The Role of TNFR family members GITR and CD30 on CD8 T cell responses

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

> Department of Immunology University of Toronto

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Abstract

GITR and CD30 are T cell costimulatory members of the TNFR superfamily known to regulate T cell responses. Elucidating the mechanisms whereby these receptors modulate T cell responses is crucial for maximizing their potential for immunotherapy. In this thesis, I examine the role of GITR and CD30 on CD8 T cell responses to influenza virus. I show that CD8 T cell intrinsic GITR is required for both maximal primary and secondary CD8 T cell expansion to influenza, while in contrast, CD30 is dispensable for anti-influenza CD8 T cell responses. GITR does not impact on CD8 T cell proliferation or homing, however, it mediates CD8 T cell survival signaling. GITR induces TRAF2/TRAF5 dependent, but TRAF1 independent, NF- κ B activation, resulting in the upregulation of the pro-survival molecule Bcl- x_L . Furthermore, I show that GITR on CD8 T cells can augment viral clearance and confer protection from death upon severe influenza infection, although the cells responsible for this effect remain to be elucidated.

In this thesis, I also show that in unimmunized mice GITR expression is upregulated to higher than basal levels on a population of CD8 memory phenotype cells in the bone marrow. In contrast, CD8 memory phenotype T cells in the spleen and LN have GITR levels similar to that on naïve T cells. The upregulation of GITR in the bone marrow is IL-15 dependent and therefore, GITR serves as a marker for cells that have recently received an IL-15 signal. Furthermore, GITR is required for the persistence, but not for the homeostatic proliferation of CD8 memory phenotype T cells in the bone marrow. Therefore, GITR plays a key role for CD8 T cell intrinsic responses to influenza, as well as for the persistence of CD8 memory phenotype T cells.

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Table of Contents

ABSTRACT	II
ACKNOWLEDGMENTS	IV
TABLE OF CONTENTS	V
LIST OF FIGURES	IX
LIST OF PUBLICATIONS	XII
ABBREVIATIONS	XIII

CHA	TER 1 INTRODUCTION1
1.1	Overview2
1.2	THE TNFR FAMILY
1.3	TNFR SIGNALING
	ראדR associated proteins (TRAFs)
	NF-κB transcription factorsθ
	Activation of the classical NF-κB pathway7
	Activation of the alternative NF-κB pathway
1.4	GITR
	Discovery and Expression of receptor10
	Signaling
	Г Cell Costimulatory Effect of GITR
	GITR and Regulatory T cells
	GITR on non-T cells
	Viral Responses
	GITR and tumor models
1.5	CD3024
	Discovery and Expression of the receptor24
	n vitro costimulatory role and signaling25
	Role of CD30 in maintaining CD4 T cell memory26
	Role of CD30 in CD4 Th1 responses28

R	ole of CD30 in CD4 Th17 responses	29
Re	ole of CD30 in CD8 T cell responses	29
1.6	T CELL ACTIVATION AND THE GENERATION OF MEMORY	
1.7	MAINTENANCE OF T CELL MEMORY	35
IL	7	35
IL	15	
Τı	rans-presentation	
CI	D4 memory T cell maintenance	
М	emory Phenotype Cells	
1.8	BONE MARROW – NICHES	
1.9	Influenza Virus	
1.10	THE IMMUNE RESPONSE TO INFLUENZA INFECTION	
In	nate Responses	
Pı	riming of Adaptive Responses	46
CI	D8 T cell responses	
CI	D4 T cell responses	
A	ntibodies	
Ge	eneration of anti-influenza T cell memory	
М	emory Responses upon influenza challenge	50
Ра	athology of immune responses to influenza	51
Со	ostimulation and the CD8 T cell response to influenza	52
1.11	THESIS OUTLINE	53

CHAPTER 2 CD8 T CELL INTRINSIC GITR IS REQUIRED FOR T CELL CLONAL EXPANSION AND

MOUSE	SURVIVAL FOLLOWING SEVERE INFLUENZA INFECTION	56
2.1	Abstract	57
2.2	INTRODUCTION	58
2.3	MATERIALS AND METHODS	60
2.4	RESULTS	64
GI	TR on the T cells is essential for maximal CD8 T cell responses to influenza virus	64
GI	TR regulates CD8 T cell numbers but not effector function	69
GI	TR on the T cells is essential for their secondary expansion in response to influenza virus	69

A	Agonistic anti-GITR antibody enhances effector T cell expansion through direct effects on t	the
C	CD8 T cells	72
F	Role of GITR in the survival of CD8 T cells responding to influenza infection	74
C	GITR enables survival signaling via enhanced Bcl-x $_{ m L}$ expression downstream of NF- κ B	77
Т	ΓRAF2 and TRAF5 but not TRAF1 enhance GITR-induced NF-κB survival signaling	79
C	GITR protects against death following severe influenza infection	81
2.5	DISCUSSION	84

CHAPTER 3 GITR CONTRIBUTES TO THE ACCUMULATION OF MEMORY PHENOTYPE CD8 T CELLS IN THE BONE MARROW WHERE LOCAL UPREGULATION OF GITR MARKS RECENT

OCCUPANTS OF AN IL-15 RICH NICHE91
3.1 Abstract
3.2 INTRODUCTION
3.3 MATERIALS AND METHODS95
3.4 RESULTS
Elevated expression of GITR on a population of CD8 memory T cells in the bone marrow98
GITR ^{hi} cells resemble cytokine-activated T cells100
Effect of IL-15 on GITR expression in vitro
The GITR ^{hi} population in the bone marrow is IL-15 dependent
IL-15 induces GITR expression on CD8 memory T cells on entrance into the bone marrow 107
GITR is not required for homeostatic proliferation of memory phenotype CD8 T cells
GITR enhances the accumulation of memory phenotype CD8 T cells in the bone marrow 109
3.5 DISCUSSION

CHAPTER 4 THE ROLE OF CD30 IN THE RESPONSE TO INFLUENZA INFECTION	117
4.1 Abstract	118
4.2 INTRODUCTION	119
4.3 MATERIALS AND METHODS	122
4.4 Results	125
CD30 is dispensable for primary influenza-specific CD8 T cell responses	125
CD30 is not required for the generation of CD8 memory T cells to influenza	125
CD30 is not required for the generation of CD8 effector function	127

	CD30 is dispensable for IgM and IgG antibody responses	129
	CD30 is dispensable for CD8 T cell recall responses to influenza virus	129
	CD30 conveys protection against death following infection with a lethal influenza	131
4.	5 DISCUSSION	135

CHAPTER 5 SUMMARY AND FUTURE DIRECTIONS	141
CHAPTER 6 REFERENCES	

List of Figures

Chapter 1

Figure 1.1: The T cell costimulatory molecules of the TNFR superfamily	4
Figure 1.2: Activation of the classical and alternative NF-kB pathways	8
Figure 1.3: GITR signaling	13
Figure 1.4: CD8 T cell activation and the generation of CD8 T cell activation	32
Figure 1.5: Structure of influenza virus	45

Chapter 2

Figure 2.1: CD8 T cell responses to influenza virus in GITR-deficient mice
Figure 2.2: Analysis of CD8 T cell intrinsic effects of GITR during influenza infection67
Figure 2.3: Role of CD8 T cell intrinsic effects of GITR in a non-competitive influenza model 68
Figure 2.4 The role of GITR on CD8 T cell effector function during influenza infection70
Figure 2.5: Role of CD8 T cell intrinsic effects of GITR in recall responses of OT-I memory T cells in vivo
Figure 2.6: GITR is required on CD8 T cells for enhancement of CD8 T cell expansion by anti- GITR in vivo
Figure 2.7: GITR on CD8 T cells is dispensable for proliferation, but required for CD8 T cell survival
Figure 2.8: GITR induced Bcl-x _L by a NF-κB-dependent signaling pathway involving TRAF2 and TRAF5
Figure 2.9: TRAF1 is dispensable for GITR induced NF-κB signaling and for the enhancement of CD8 T cell expansion to anti-GITR in vivo
Figure 2.10: GITR on CD8 T cells augments viral clearance and protects against mortality during severe respiratory influenza infection

Chapter 3

Figure 3.1: GITR is selectively upregulated on a population of CD8 memory phenotype T cells in the bone marrow
Figure 3.2: GITR ^{hi} cells have a phenotype consistent with cytokine activation102
Figure 3.3: CD8 T cells from the bone marrow upregulate GITR when cultured in vitro with IL- 15
Figure 3.4: The bone marrow GITR ^{hi} population is IL-15-dependent and splenic GITR ^{basal} memory cells convert to GITR ^{hi} cells upon entry into the bone marrow
Figure 3.5: GITR is dispensable for CD8 memory T cell homeostatic proliferation but contributes to the persistence of CD8 memory phenotype T cells in the bone marrow110

Chapter 4

Figure 4.1: CD30 is dispensable for antigen-specific primary CD8 T cell responses to influenza virus and conversion to memory
Figure 4.2: CD30 is not needed for recall antigen specific CD8 T cell responses to influenza128
Figure 4.3: CD30 is dispensable for the generation of CD8 T cell effector function to influenza virus
Figure 4.4: CD30 does not play a role in the generation of both primary and memory anti- influenza antibody responses
Figure 4.5: CD30 is required for protection from death against severe respiratory influenza infection

Chapter 5

Figure 5.1: Model of the CD8 T cell intrinsic role of GITR during influenza infection	143
Figure 5.2: The role of TNFRs in CD8 and CD4 memory T cell maintenance	149

List of Publications

<u>Snell LM</u>, McPherson AJ, Lin GH, Sakaguchi S, Pandolfi PP, Riccardi C, Watts TH. 2010.
 CD8 T cell-intrinsic GITR is required for T cell clonal expansion and mouse survival following severe influenza infection. *J Immunol* 185: 7223-34

2. <u>Snell LM</u>, Lin GH, Watts TH. Local GITR contributes to the accumulation of memory phenotype CD8 T cells in the bone marrow where local upregulation of GITR marks recent occupants of an IL-15 riche niche.

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<u>Snell LM</u>, Lin GH, McPherson A, Moraes, T, Watts TH. T cell intrinsic effects of GITR and
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Lin GH, <u>Snell LM</u>, Wortzman M, Vidric M, Pandolfi P, Riccardi C, Watts TH. Defining a niche for the maintenance of CD8 memory T cells in the bone marrow.
 Manuscript in preparation.

5. Sabbagh L, <u>Snell LM</u>, Watts TH. 2007. TNF family ligands define niches for T cell memory. *Trends Immunol* 28: 333-9

6. Lin GH, Sedgmen BJ, Moraes TJ, <u>Snell LM</u>, Topham DJ, Watts TH. 2009. Endogenous 4-1BB ligand plays a critical role in protection from influenza-induced disease. *J Immunol* 182: 934-47

Sabbagh L, Srokowski CC, Pulle G, <u>Snell LM</u>, Sedgmen BJ, Liu Y, Tsitsikov EN, Watts TH.
 A critical role for TNF receptor-associated factor 1 and Bim down-regulation in CD8 memory T cell survival. *Proc Natl Acad Sci U S A* 103: 18703-8

Abbreviations

Ad	adenovirus
AITR	activation inducible TNFR family member
APC	antigen presenting cell
BAFF	B cell-activating factor of the tumor necrosis factor family
BAL	broncheoalveolar lavage
BCG	bacillus Calmette-Guerin
Bcl	B cell lymphoma
BM	bone marrow
BrdU	bromodeoxyuridine
cIAP	cellular inhibitor of apoptosis protein
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD30L	CD30 ligand
CD	cluster of differentiation
CFSE	carboxyfluoroscein succinimidyl ester
cIAP	cellular inhibitor of apoptosis
CMV	cytomegalovirus
Cr	chromium

CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte antigen-4
DC	dendritic cell
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EOMES	eomesodermin
ERK	extracellular signal-regulated kinase
foxp3	forkhead box p3
GITR	glucocorticoid induced tumor necrosis factor receptor related protein
GITRL	GITR ligand
НА	hemagglutinin
HAU	hemagglutinin units
HIV	human immunodeficiency virus
HOIL-1L	longer isoform of hemoxidized iron-regulatory protein 2 ubiquitin ligase-1
HOIP	HOIL-1L interacting protein
HRP	horse radish peroxidase
IFN	interferon
Ig	immunogloubulin
ΙκΒα	inhibitor of NF-κB
IKK	IkB kinase

iiEL	intestinal intraepithelial lymphocytes
IL	interleukin
i.n.	intranasal
i.p.	intraperitoneal
JNK	c-Jun N-terminal kinase
KLRG1	killer cell lectin like receptor 1
L	ligand
LCMV	lymphocytic choriomeningitis virus
LFA-1	leukocyte function antigen-1
LN	lymph node
LTβR	lymphotoxin-beta-receptor
LTi	lymphoid tissue inducer
LUBAC	linear ubiquitin chain assembly complex
МАРК	mitogen activated protein kinase
MCMV	murine cytomegalovirus
MDCK	Madin-Darby canine kidney
MEF	mouse embryonic fibroblast
MFI	mean fluorescent intensity
МНС	major histocompatibility complex
MLN	mediastinal lymph node

MMP	matrix metallopeptidase
MPEC	memory-precursor effector cell
NA	neuraminidase
NEMO	NF- κ B essential modulatory (IKK γ)
NF-κB	nuclear factor KB
NIK	NF-KB-inducing kinase
NK	natural killer
NKT	natural killer T
NLS	nuclear localization sequence
NP	nucleoprotein
OX40L	ligand for OX40
OVA	ovalbumin
PAMP	pattern associated molecular patterns
pDC	plasmacytoid DC
PDTC	1-pyrrolidinecarbodithioic acid
PI	propidium iodide
Poly:IC	polyinosine:cytosine
PSGL-1	P selectin glycoprotein ligand-1
R	receptor
RAG	recombination activating gene

RANK	receptor activator of NF-kB
RING	really interesting new gene
RDC	respiratory dendritic cell
RIP1	receptor interacting protein 1
RNA	ribonucleic acid
sGITR	soluble GITR
SHARPIN	SHANK-associated RH domain-interacting protein
siRNA	small inhibitory RNAs
STAT	signal transducer and activator of transcription
S1P	sphingosine-1-phosphate
SLEC	short-lived effector cells
TCID50	tissue culture infectious dose 50
TCR	T cell receptor
T _{CM}	T central memory
T_{EM}	T Effector memory
Th	T helper
THD	TNF homology domain
TLR	toll like receptor
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor

TRAF	tumor necrosis factor receptor associated protein
Treg	regulatory T cell
UV	ultraviolet
VCAM	vascular cell adhesion molecule
VLA	very late antigen
VSV	vesicular stomatitis virus
WT	wildtype

Chapter 1

Introduction

Parts of this chapter are in press at Immunological Reviews:

<u>Snell LM</u>, Lin GH, McPherson A, Moraes, T, Watts TH. T cell intrinsic effects of GITR and 4-1BB during viral infection and cancer immunotherapy.

1.1 Overview

Immune responses are highly regulated and strive to create a balance between quickly and efficiently eliminating foreign pathogens, while at the same time limiting immunopathology. The tumor necrosis factor receptor (TNFR) superfamily encompasses multiple receptors that act as costimulatory molecules on T cells, and thereby modulate T cell responses. In this introduction, I first discuss the overall signaling of TNFRs leading to cell proliferation, differentiation and survival. I then hone in on the 2 TNFR costimulatory receptors upon which my thesis is focused, glucocorticoid induced tumor necrosis factor receptor related protein (GITR) and CD30. I describe the expression and signaling of these receptors and elaborate upon their roles, particularly on T cells, with special emphasis on in vivo studies that have been conducted to date. In addition, I describe the immunotherapeutic potential for GITR, which is currently being tested as a humanized monoclonal antibody in a phase I trial for melanoma.

My thesis details the role of GITR and CD30, primarily on CD8 T cells, and therefore I discuss the overall activation of CD8 T cells, including the conversion of these effector cells to CD8 memory T cells. In addition, I examine the mechanisms of antigen-independent CD8 memory T cell maintenance, the cellular niche whereby this occurs, and the roles of the homeostatic cytokines IL-7 and IL-15. Lastly, I end by discussing influenza, the model of infectious disease in which I test the role of GITR and CD30. I detail the immune response to the virus, and examine how costimulation can modulate the anti-influenza CD8 T cell response.

1.2 The TNFR family

The TNFR superfamily consists of an extensive group of receptors and ligands that function to regulate multiple cellular activities including immune responses, hematopoiesis, morphogenesis, bone remodeling, thermoregulation, as well as various processes within the nervous system (1-4). The role of TNFR family members is diverse even within the immune system. The family is involved in lymphoid organization, innate responses, APC activation, as well as in T cell activation and costimulation (5). However, while TNFR family members can augment immune responses leading to beneficial effects, they have also been implicated in immune pathogenesis, and are involved in such processes as septic shock, transplant rejection, autoimmune disease, and tumorigenesis (4). Therefore understanding the mechanisms whereby TNFR family members can regulate immune responses is of paramount importance.

TNF receptors are type I transmembrane proteins containing extracellular conserved cysteine rich domains that form 3 disulfide bridges (6, 7). The ligands for TNFRs are type II transmembrane proteins that express a TNF homology domain (THD) and can either be cell-bound or cleaved to form a soluble secreted form (6, 7). The ligands form trimers to interact with their respective TNFRs, which are thought to trimerize as well (6-8). The TNFR family can be broadly grouped into 3 categories: death domain containing receptors, decoy receptors and receptors that directly recruit adaptor molecules TNFR associated proteins (TRAFs) to mediate activation signals (4, 6, 8, 9). This last group includes T cell costimulatory molecules necessary for fine-tuning the T cell response (Figure 1.1).





1.3 TNFR Signaling

TNFR associated proteins (TRAFs)

Upon engagement with their respective ligands the T cell costimulatory molecules of the TNFR superfamily mediate signals that lead to proliferation, differentiation or cell survival (4). Since TNFRs do not possess intrinsic enzymatic activity, they recruit signaling adaptors to mediate these downstream effects. The direct recruitment of TNFR associated proteins (TRAFs) to the cytoplasmic tails of costimulatory TNFR family members provide links to the extracellular signal-regulated kinase (ERK), p38, and the c-Jun N-terminal kinase (JNK) mitogen activated protein kinase (MAPK) pathways, as well as the nuclear factor κ B (NF- κ B) pathway (8, 10). There are 6 known mammalian TRAFs; TRAF1-6 (9). While TRAF2, 3 and 6 are fairly ubiquitous (11-16), TRAF1 is expressed in the spleen and lung, as well as in the testis and the tonsils (11). TRAF4 has very different expression and is found in neural tissue, but mostly during embryogenesis (17). TRAF5 is expressed in the thymus, the spleen and the lung (18, 19).

All of the TRAF proteins share a highly conserved TRAF domain at their C terminus, which can be further divided into the TRAF-N and TRAF-C domains. The TRAF-N domain is crucial for oligomerization with other TRAF molecules and forms a coiled coil structure (20, 21). The TRAF-C domain is a 7-8 stranded anti-parallel β -sandwich structure allowing for the interaction of the TRAF molecules with the cytoplasmic tails of TNFR receptors (21). At the N termini of the TRAFs, with the exception of TRAF1, is a RING finger domain followed by a variable number of zinc finger domains. The RING fingers of TRAF2 and TRAF6 have E3 ubiquitin ligase activity that mediates the K63-linked polyubiquitination of target proteins, contributing to the assembly of signaling platforms (22, 23). It should be noted however, that

recently the crystal structure of TRAF2 has raised the question of whether the TRAF2 RING can adopt an appropriate conformation for E3 ligase activity (24).

TRAF2 is the prototypical TRAF molecule, and when overexpressed leads to activation of NF-κB signaling (25). TRAF5 overexpression also leads to constitutive NF-κB activation (18, 19), and TRAF2 and TRAF5 are generally thought to be redundant for NF-κB activation (26-28). Interestingly, as the 2 pathways of NF-κB (discussed below) are elucidated further, it appears that TRAF2 has dual effects. In the canonical pathway TRAF2 serves as a positive regulator of NF-κB signaling, whereas in the alternative pathway it is a negative regulator (29-31). TRAF3 is primarily involved in the activation of the alternative NF-κB pathway (29, 30), as described below, and is known to be an indirect negative regulator of the canonical pathway (32). TRAF1 is recruited to both GITR and CD30 and is known to modulate T cell survival (33, 34). TRAF4 is relatively unstudied, although it has been shown to have a role downstream of GITR signaling in overexpression studies (35). TRAF6 is primarily involved in NF-κB activation downstream of TLRs and IL-1R (32), which is beyond the scope of this thesis.

NF-KB transcription factors

The NF- κ B family of transcription factors includes: NF- κ B1 (p50 cleaved from its precursor p105), NF- κ B2 (p52 cleaved from its precursor p100) RelA, RelB and c-Rel. These transcription factors form homo or heterodimers and use their N-terminal Rel homology domain to enter the nucleus (32). There are 2 pathways of NF- κ B signaling: the classical or canonical pathway and the alternative or non-canonical NF- κ B pathway (36). The classical NF- κ B pathway

mainly forms RelA:p50 or c-Rel:p50 heterodimers, while in the alternative pathway RelB predominantly forms heterodimers with p100 or its cleaved form p52 (32).

Activation of the classical NF-KB pathway

The activation of the classical NF- κ B pathway by TRAF2 binding TNFRs involves the recruitment of the cellular inhibitor of apoptosis proteins 1 and 2 (cIAP1/2) to the cIAP interacting motif on TRAF2. This is followed by the recruitment of receptor interacting protein 1 (RIP1) and its K63-linked polyubiquitination, which in turn leads to recruitment and activation of the IKK complex. The IKK complex is composed of IKK α and IKK β catalytic subunits, as well as IKK γ , otherwise known as NEMO, which is a regulatory subunit. The activated IKK complex phosphorylates I κ B α leading to its K48-linked polyubiquitination and proteasome-dependent degradation, thereby releasing the NF- κ B dimers from I κ B α -mediated retention in the cytosol, allowing them to traffic to the nucleus (32) (Figure 1.2). The cIAP1/2 proteins also have E3 ligase activity and may act as the key modifiers of RIP1(24, 37), with the main role of TRAF2 being to recruit cIAP1/2 to K63-polyubiquitinate RIP1. (38)

Recently, linear ubiquitin chains have also been shown to play a role in the activation of the canonical NF- κ B pathway (39). The E3 ligase LUBAC (linear ubiquitin chain assembly complex) is composed of proteins HOIL-1L (longer isoform of hemoxidized iron-regulatory protein 2 ubiquitin ligase-1), HOIP (HOIL-1L interacting protein), and SHARPIN (SHANK-associated RH domain-interacting protein) (40-42), and is recruited to the TNF and CD40L signaling complexes where it linearly ubiquitinates RIP1 (42). NEMO has been shown to bind



Figure 1.2 Activation of the classical and alternative NF-κB pathways. Downstream of TNF receptors the classical NF-κB pathway is activated when activated IKK complex phosphorylates IκBα, leading to its K48-linked polyubiquitination and proteasome-dependent degradation. This then releases the NF-κB dimers (p50:p65) from IκBα-mediated retention in the cytosol, allowing them to traffic to the nucleus. Alternative NF-κB pathway signaling is normally suppressed via the constitutive degradation of NIK. Upon engagement of a TNF receptor which activates the alternative pathway, TRAF2 and TRAF3 are degraded, thereby allowing for the accumulation of NIK. NIK activates IKKα which then phosphorylates p100, allowing for its polyubiquitination and partial processing to p52. RelB:p52 heterodimers can then translocate into the nucleus.

both K63 and linear ubiquitin chains (43) and NEMO too undergoes LUBAC mediated linear ubiquitination, which is necessary for maximal canonical NF-κB activation (40-42).

Activation of the alternative NF-KB pathway

The alternative NF- κ B pathway is initiated downstream of several TNFRs such as CD40, lymphotoxin beta receptor (LT β R), B cell-activating factor of the tumor necrosis factor family receptor (BAFF-R) and receptor activator of NF- κ B (RANK) (44-46). However, it is becoming increasingly apparent that the alternative NF- κ B pathway is utilized by more TNFR family members than previously thought (47). The non-canonical pathway is regulated by the turnover of NF- κ B-inducing kinase (NIK), which has been shown to activate IKK α (48-50). IKK α phosphorylates p100, allowing for its polyubiquitination and partial processing to p52 (48, 50, 51). RelB:p52 heterodimers can then translocate into the nucleus (50) (Figure 1.2).

Basal non-canonical or alternative NF-κB pathway signaling is normally suppressed via the constitutive degradation of NIK (Figure 1.2). This occurs via a constitutive TRAF2:TRAF3 heterotrimer whereby TRAF3 recruits NIK and TRAF2 recruits cIAP1/2. This complex puts NIK in proximity to cIAP1/2, which proceeds to add K48-linked polyubiquitin to NIK, targeting it for proteasomal degradation (29, 30). Both TRAF2^{-/-} and TRAF3^{-/-} mice develop postnatal lethality, attributed to the constitutive activation of the alternative NF-κB pathway (31, 52). However, overexpressing TRAF2 in TRAF3^{-/-} MEFs and TRAF3 in TRAF2^{-/-} MEFs could not overcome the constitutive alternative NF-κB activation, demonstrating that the roles of TRAF2 and TRAF3 are non redundant (29). Indeed, it was found that TRAF2 cannot bind NIK directly under endogenous conditions, but relies on TRAF3 to recruit NIK into the complex (29). Similarly, TRAF2 is needed to recruit cIAP1/2 to the TRAF3-NIK complex, as it appears that under endogenous conditions TRAF3 cannot directly bind the cIAP proteins (29). Therefore, in the absence of either TRAF2 or TRAF3, the cIAP proteins cannot K48 polyubiquinate NIK, allowing the protein to accumulate, leading to constitutive processing of p100 to p52 (52).

Upon engagement of CD40 or BAFF-R, TRAF2 and TRAF3 are degraded, thereby allowing for the accumulation of NIK (29, 30) (Figure 1.2). This occurs whereby TRAF2 acts as an E3 ubiquitin ligase for cIAP1/2, activating the proteins via K63 ubiquitination (30). cIAP2 then K48 polyubiquitinates TRAF3 and TRAF2, allowing the proteins to be targeted for proteasomal degradation (30). NIK accumulates and is autophosphorylated, resulting in the activation of the alternative pathway (29, 30). Interestingly, it has been reported that the activation of the alternative pathway, and accumulation of NIK can feed back and enhance the canonical NF-κB pathway (53). Similarly, the canonical pathway can also feed back and enhance the alternative pathway by augmenting the expression of NF-κB2 (32).

1.4 GITR

Discovery and Expression of receptor

Glucocorticoid induced tumor necrosis factor receptor related protein (GITR) is a T cell costimulatory member of the TNFR superfamily. Murine GITR was originally cloned in 1997 using a differential display strategy which identified transcripts from a T-cell hybridoma that were upregulated after activation of the TCR in the presence of the glucocorticoid dexamethasone (54). GITR was classified as a member of the TNFR family due to the presence

of the family's characteristic extracellular domain cysteine-rich motifs, as well as its high intracellular domain homology to 2 other family members, 4-1BB and CD27 (54). Two years later, a human orthologue of GITR, activation inducible TNFR family member (AITR), was also identified (55, 56). Despite GITR's name, glucocorticoids have since been shown to be unnecessary for GITR upregulation in both mice and humans (56, 57).

GITR is a type I transmembrane protein found widely in the immune system. Perhaps most studied because of its high expression on regulatory T cells (Tregs) (58, 59), GITR is also expressed on naïve T cells and NK cells, and is further upregulated upon cell activation (60). In addition, low to intermediate levels of GITR can be found on, but not limited to, cell types such as eosinophils, basophils, mast cells, macrophages and B cells (60). On almost all immune cell types studied, activation leads to the upregulation of GITR expression, and in some cases, such as on dendritic cells, can lead to GITR expression (60).

The ligand for GITR (GITRL) is a type II transmembrane protein whose human form, AITRL, was cloned in 1999 (55, 56), followed a few years later by the cloning of its murine form (61-64). GITRL is widely expressed in the immune system and can be detected at basal levels on antigen presenting cells such as dendritic cells, B cells, monocytes and macrophages, with high expression being induced following cellular activation (60). GITR is also found on endothelial cells and is highly expressed upon activated plasmacytoid dendritic cells (60). In addition, GITRL has recently been detected on activated T cells (61, 62, 65, 66).

GITR^{-/-} mice are viable, show no gross abnormalities, and have approximately equal proportions of splenic and lymph node T and B cells as GITR^{+/+} control mice (67). To date, GITRL^{-/-} mice have not been characterized.

Signaling

GITR ligation on T cells activates NF-κB signaling (68, 69), as well as the ERK, JNK and p38 MAPK pathways (69, 70) (Figure 1.3). This signaling is mediated via the recruitment of TRAF molecules to the GITR receptor. Yeast-2-hybrid and co-immunoprecipitation studies have shown that GITR binds TRAFs 1, 2, and 3 (55, 71).

Although TRAF2 is known to be crucial for the initiation of the canonical NF- κ B pathway, the role of TRAF2 downstream of GITR has been controversial. Initial overexpression studies showed that TRAF2 was a positive regulator of GITR-induced NF- κ B activation (55, 56), however this came into question later when a report using similar methodology showed overexpressed TRAF2 downregulated NF- κ B activation downstream of GITR (71). In addition, GITR has recently been shown to activate the alternative NF- κ B pathway, (47) and indeed, overexpression studies using dominant negative NIK constructs negatively impacted NF- κ B signaling downstream of GITR (55, 56). As described above, TRAF2 is a positive regulator of canonical NF- κ B signaling but a negative regulator of the alternative NF- κ B pathway (32), which further complicated the matter as these initial experiments could not differentiate between the two pathways. In this thesis, I investigate the role of TRAF2 downstream of GITR signaling on primary CD8 T cells specifically in the canonical NF- κ B pathway.

As described above, TRAF3 is crucial for the inhibition of the alternative NF- κ B pathway, and indirectly inhibits the canonical NF- κ B pathway as well (32). Indeed, TRAF3 has been shown to be a negative regulator of NF- κ B signaling downstream of the human isoform of GITR, AITR (55). The same study also showed a weakly negative role of TRAF1 on AITR-mediated NF- κ B activation (55).



Figure 1.3 GITR signaling. GITR recruits TRAF1, 2 and 3 to its cytoplasmic tail by yeast-2hybrid analysis. TRAF5 is also utilized for downstream GITR signaling, and TRAF4 has been implicated in the augmentation of GITR-induced signaling in an overexpression model. GITR signaling induces JNK, ERK and p38 MAPK pathways, as well as the NF- κ B pathway, which leads to the upregulation of Bcl- x_L .

Although not originally identified in the yeast-2-hybrid screen as binding to GITR, TRAF4, in an in vitro overexpression model, was also found to be a positive modulator of GITRmediated NF- κ B signaling (35). In addition, it has been convincingly shown in studies using TRAF5^{-/-} CD4 T cells that TRAF5 is essential for GITR induced NF- κ B, p38, and ERK signaling (72). In this thesis, I further examine the role of both TRAF5 and TRAF1 in GITR induced canonical NF- κ B signaling in primary CD8 T cells.

GITR has been shown to be a pro-survival molecule, and stimulation through the receptor induces proliferation, cytokine secretion and cell survival. It has previously been described that GITR is required for CD28 mediated Bcl-x_L upregulation (66). In this thesis, I show that Bcl-x_L expression is enhanced directly downstream of GITR-mediated NF-κB signaling. Interestingly, in certain instances GITR has also been reported to induce apoptosis, and as described below GITR^{-/-} T cells hyperproliferate to anti-CD3 (67). It should be noted that GITR can bind Siva, a cytoplasmic molecule containing a death domain (73). Siva, which also binds CD27, induces apoptosis in T cells via a mitochondrial pathway, dependent upon both effector and initiatior caspases (74). In addition, Siva can bind directly to Bcl-x_L and inhibit its anti-apoptotic rescue in response to UV radiation (75). Interestingly, Siva has been mapped to bind to domain 2 of GITR, which also binds TRAF2 (73). Therefore, it is possible that differential binding can lead to different outcomes, depending on the circumstances (60). However, as will be discussed further, most studies show that GITR signaling promotes T cell expansion rather than apoptosis.

T Cell Costimulatory Effect of GITR

In vitro studies first identified GITR as a modulator of T cell responses. GITR^{-/-} T cells hyperproliferate to anti-CD3 stimulation which initially caused confusion and had GITR labeled as a negative regulator (67). However, many studies have since shown that GITR is unquestionably a T cell costimulatory molecule. Triggering of GITR on T cells, either by agonistic antibody, soluble GITRL or cells transfected with GITRL, enhances both CD4 and CD8 T cell proliferation to suboptimal anti-CD3 stimulation (58, 61, 62, 64, 68, 70) (69) (76). In addition, anti-GITR stimulation enhances anti-CD3 upregulation of activation markers CD69 and CD25 (IL-2R α) and augments the production of IFN- γ , IL-2, IL-10 and IL-4 to anti-CD3 stimulation (68, 70, 76). GITR has also been shown to rescue T cells from anti-CD3 induced apoptosis (70).

Anti-GITR agonistic antibody can enhance anti-CD3 mediated CD28^{-/-} T cell proliferation (58, 66) and similarly, GITR augments T cell activation to antigen in the presence of CTLA-4-Ig, which blocks CD28:CD80/86 binding (69). Indeed, when a constant dose of agonistic anti-CD28 is used to stimulate CD4 T cells, anti-GITR further augments the response, suggesting the 2 costimulatory molecules signal through independent pathways (68). However, whereas GITR can costimulate CD8 T cells in the absence of CD28, CD28 mediated CD8 T cell costimulation is defective in the absence of GITR (66). Although GITR is not required for CD28 mediated ERK or JNK activation, it is crucial for NF- κ B activation and Bcl- x_L upregulation downstream of CD28 (66). T cells can upregulate GITRL in response to anti-CD3/CD28. Thus it appears that the induction of GITRL by anti-CD3/CD28 can result in GITRL binding to GITR on T cells leading to GITR-dependent NF- κ B induction (66). Agonistic anti-GITR can also augment CD28 upregulation in response to suboptimal anti-CD3 concentrations (66). Thus the presence of GITR on the resting T cells allows it to play an important role in augmenting anti-CD3/CD28 induced survival signals on the T cells and in this regard GITR appears to play a role that is similar to that of CD27 during primary T cell activation (77).

In vivo, the administration of anti-GITR agonistic antibody augments T cell responses in various viral and tumor models (reviewed below). While this is consistent with GITR's in vitro T cell costimulatory role, the targets of the antibody are unknown and the T cell enhancement could be due to indirect effects. Indeed, much remains to be elucidated as to GITR's physiological role in vivo. My thesis will examine the intrinsic role of GITR on CD8 T cells during influenza infection in vivo.

GITR and Regulatory T cells

In 2002 it was discovered that GITR is highly expressed on regulatory T cells (58, 59). This suggested that perhaps GITR could be a long sought after marker to identify $CD4^+CD25^+$ regulatory T cells. However, like CD25, GITR expression is upregulated to high levels upon the activation of effector T cells and therefore cannot be used to discriminate between effector and regulatory T cells during an immune response (58, 59, 65). More importantly, however, was the discovery that when agonistic anti-GITR antibody was administered to in vitro cultures of effector and regulatory T cells, Tregs cells could no longer suppress the effector T cells (58, 59). Indeed, when agonistic GITR antibody was administered to 2 week old Balb/c mice once a week for 3 weeks, the mice developed autoimmune gastritis (58). Examination showed that the antibody bound $CD4^+CD25^+$ T cells, but did not cause their deletion, thereby inferring that the suppressive effect of the regulatory T cells was being diminished (58). Further studies, however,

suggested that it was GITR ligation on the effector T cells and not the regulatory T cells that was necessary for this costimulatory effect (62). Although this appears to be the predominating view, some evidence remains that GITR signaling may be able to directly inhibit the suppressive activity of Tregs, and thus some of the costimulatory effects of anti-GITR may be due to contributions on both effector and regulatory T cells (60, 65).

GITR^{-/-} mice have approximately 33% fewer regulatory T cells in spleen and lymph nodes, but equal numbers in the thymus, when compared to WT control mice (62). This suggests that GITR is not necessary for the development of Tregs, but may play a role in their peripheral maintenance. Furthermore, the ability of GITR knockout cells to suppress effector T cell responses does not differ from that of WT regulatory cells, suggesting that GITR is not required for functional suppression (62, 70).

GITR not only costimulates effector T cells, but can also costimulate regulatory T cells. CD4⁺CD25⁺ T cells treated with suboptimal anti-CD3 and either anti-GITR or GITRL-P815 cells and IL-2 break their anergic state and undergo cell proliferation in vitro (62, 68, 70, 76). In addition, stimulation of CD4⁺CD25⁺ regulatory T cells with GITRL-P815 enhances the production of various cytokines, most notably IL-10 (76). Interestingly, the proliferating Tregs did not lose suppressive function but instead seemed to enhance their function on a per cell basis (65, 76). This may be due to the enhanced secretion of the immunosuppressive cytokine IL-10 (76). Therefore, the proliferation of regulatory T cells may ultimately increase their numbers and allow for more suppression (78).

It is somewhat perplexing that GITR can costimulate both effector and regulatory T cells, which serve opposite roles in an immune response. GITR is rapidly and transiently upregulated on antigen activated effector T cells and the expression of GITRL also increases upon activation of APC. Shevach and Stephens suggest a model where the interaction between effector T cells and APCs might predominate at the beginning of the immune response, but as the response progresses and GITR on effector T cells returns to baseline, the GITR on regulatory T cells would then predominate. The interaction of GITR on the Tregs with GITRL on APCs, together with exogenous IL-2 would then allow expansion and local accumulation of the Tregs to prevent excess inflammation and pathology (42).

The dual costimulation of both effector and regulatory T cells must be taken into consideration when examining the potential use of GITR for immunotherapy, as the overall effect may simply be a balance between positive and negative regulation and may be dependent on precursor numbers of each type of cell (65, 76). It may prove useful to first deplete regulatory T cells before administration of agonistic GITR antibody (65). However, although many studies in mice have shown that GITR agonistic antibody enhances T cell responses and can exacerbate autoimmunity, the cell types targeted by the antibody in these studies are largely untested and need to be understood in order to fully harness the power of GITR for immunotherapy.

GITR on non-T cells

The role of GITR on DCs is somewhat controversial. An initial report showed that DCs from GITR^{-/-} mice more effectively stimulated IL-2 and IFN- γ production from CD4 T cells than did GITR^{+/+} DCs. (79) However, recently, GITR on bone marrow derived dendritic cells was reported to be necessary for optimal T cell proliferation in vitro (80). In addition, when activated GITR^{-/-} loaded dendritic cells were injected into mice, the local lymph nodes were visibly smaller than those in mice that received GITR^{+/+} DCs (80).

Despite GITR's positive role on the activation of multiple types of immune cells, curiously this is not always the case. Several reports have implicated GITR as having a negative role for human NK cell activation (81-83) as well as for invariant NKT cell activation (84). Interestingly, human tumor cells have been discovered to secrete a soluble form of GITRL (sGITRL) that diminishes NK cell cytotoxicity and IFN- γ production. Neutralization of sGITRL augmented anti-tumor responses (82).

Viral Responses

Very little is known about the role of GITR in the context of infectious disease. In vitro, GITR triggering by agonistic antibody increases the proportion of cytokine-producing HIV specific CD4 T cells (85). This was attributed to the decreased levels of activated caspase 3 in TNF- α producing HIV-specific CD4 T cells, thereby inferring that GITR may protect these cells from apoptosis (85).

In vivo, studies administering an agonistic anti-GITR antibody to mice infected with herpes simplex virus (HSV), showed increased numbers of activated CD4 and CD8 T cells, as well as enhanced cytokine responses in comparison to mice which were treated with a Rat IgG control antibody (86, 87). Interestingly, in a model of ocular HSV, anti-GITR treatment actually reduced the number of corneal lesions, through effects on MMP9 expression, thereby decreasing angiogenesis and T cell entry into the cornea. Thus although costimulatory, this treatment protects from pathology (86). Treatment with an anti-GITR agonistic antibody during persistent retroviral infection of mice led to a decrease in splenic viral load which correlated with higher antigen-specific CD8 T cell responses, increased memory CD8 T cell formation, and a higher
proportion of CD8 T cells producing IFN- γ (88). Whereas anti-GITR clearly augments virusspecific T cell responses, none of these studies could differentiate whether GITR was working primarily on the regulatory T cells or on the effector T cells, or perhaps on some other cell type to indirectly stimulate the T cell response. As discussed in a recent review, the role of GITR on CD8 T cells in viral responses remains to be elucidated (89), and this question will be addressed in my thesis using an influenza infection model.

GITR and tumor models

Although my thesis does not focus on tumor models, GITR has in fact been studied more in tumor models than in infectious disease models and I will briefly review these studies here. Like the study of GITR in viral responses, many of the studies examining GITR in tumor models use agonistic GITR antibody. In a model of B16 melanoma, several groups have shown that the administration of agonistic GITR antibody, DTA-1, can lead to reduced tumor growth, and in some cases rejection of tumors altogether (90-93). DTA-1 stimulated mice show augmented CD8 and CD4 T cell activation, and depletion studies demonstrated that T cells, as well as NK and NKT cells were necessary for GITR-induced primary tumor rejection (91). The mechanism of rejection was found to be dependent upon IFN- γ and FasL (91). Interestingly, DTA-1 treatment also led to T cell responses against several self melanoma differentiation antigens and induced mild autoimmunity in a small percentage of mice (91). Therefore, although DTA-1 induced antitumor responses, the antibody also overcame tolerance/ignorance to self melanoma antigens (91). Although DTA-1 induces regulatory T cell proliferation and enhances Treg numbers in various tumor models (91, 94), it has also been found to specifically inhibit regulatory T cell infiltration into tumors (93, 95), thereby increasing the CD8 T cell to regulatory T cell ratio (93). This was shown to be a direct effect of GITR ligation on regulatory T cells, as DTA-1 treated GITR^{-/-} Tregs did not traffic differently than control treated Tregs (93). Interestingly, in one report, the regulatory T cells in tumors of mice treated with DTA-1 also downregulated the transcription factor foxP3 (93). In this particular study, delay of tumor growth was evidenced only when GITR was present on both regulatory and effector T cells (93). Most studies, however, show that GITR's anti-tumor role on effector T cells is crucial while its role on regulatory T cells is minimal (91, 92).

While administration of DTA-1 has been shown to have the ability to clear primary tumors in a B16 melanoma model as described above (91, 93), other studies using this model demonstrate instead that DTA-1 elicits concomitant tumor immunity (90, 92); the failure to clear a primary tumor but the ability to control a secondary tumor challenge at a distal site. The differences in outcome between studies can most likely be attributed to differences in the establishment of tumors, as well as variations in the number and kinetics of antibody injections, as well as the dose of antibody administered (92, 93, 96). Similar to the other melanoma studies, depletion experiments showed that CD8 and CD4 T cells were the predominant cell type crucial for this concomitant immunity, with NK and NKT cells playing a small role (91, 92). Using reconstitution of RAG^{-/-} mice with splenocytes containing CD8 T cells from either WT or GITR^{-/-} mice (92), Cote et al. demonstrated a requirement for DTA-1 to act directly on the CD8 T cells for this concomitant immunity (92). Moreover, anti-GITR was shown to lead to higher avidity CD8 T cell responses towards tumor-specific antigens. In contrast, reconstitution of RAG^{-/-} mice

with splenocytes containing Tregs from either WT or GITR^{-/-} mice showed no difference in concomitant immunity (92).

Other tumor models have been found to have similar results as those seen using melanoma. In a model of murine fibrosarcoma CMS5, DTA-1 led to enhanced tumor rejection by tumor-specific adoptively transferred CD8 T cells (95). GITR-stimulation of transgenic cells enhanced proliferation, cytotoxicity, and infiltration into the tumor. In addition, not only did DTA-1-treated transgenic cells make more IFN- γ , TNF- α and express higher levels of CD107a on a per cell basis, but a higher proportion of them were also multifunctional as compared to control treated cells. In a fibrosarcoma model, Meth A, DTA-1 treatment enhanced the infiltration of T cells into the tumor, including that of regulatory T cells. Anti-GITR synergized with a non-depleting anti-CTLA-4 antibody to provoke tumor immunity in the absence of overt autoimmunity (96). Moreover, in a colorectal carcinoma CT26 tumor, not only did DTA-1 cause the expansion and activation of NK and T cells in the tumor draining lymph nodes, but the antibody also induced B cell expansion (94). Interestingly, in this model depletion of CD4 T cells led to complete abrogation of anti-tumor DTA-1 effects, whereas depletion of NK or CD8 T cells separately could only partially abrogate the effects (94). Depletion of CD4 T cells caused decreased DTA-1 activation and cytolytic function of NK and CD8 T cells (94).

In addition to the GITR agonistic antibody being used in tumor studies, some studies have transfected GITRL into tumors (97). Injection of an adenovirus expressing either soluble or full length GITRL into palpable melanomas increased CD4 and CD8 T cell infiltrates compared to the control group (97). Interestingly, however, tumors which received soluble rather than full length GITRL expressed by the adenovirus vector were more efficient at inhibiting B16

melanoma growth (97). The mechanism of this effect is unclear, however, as the two forms of GITRL had similar *in vitro* activities on CD4 and CD8 T cells (97).

In another study, injection of GITRL-transfected tumor cell lines caused tumor regression spontaneously, in contrast to the progressive growth of the parental tumor cell lines (98). A blocking GITRL antibody reversed the tumor regression in GITRL transfected tumors, where it had little effects in the parental lines (98). It was found that in the parental lines, where GITRL was lacking, regulatory T cells generally induced tolerance to the tumors. When GITRL was highly expressed on tumors, however, the regulatory T cell effect could be overcome by CD8 T cells, which were found to be required for tumor specific immunity (98).

Taken together, there is extensive evidence that the action of GITR directly on effector T cells can enhance anti-tumor immunity. Additionally, effects on Tregs have been identified in some but not all models. GITR, therefore, remains a promising therapeutic candidate and in 2007, an NCI workshop rated GITR 12th in a list of potential agents for cancer immunotherapy. (http://web.ncifcrf.gov/research/brb/workshops/NCI%20Immunotherapy%20Workshop%207-12-07.pdf). As of June 2011, a phase I trial of anti-GITR for malignant melanoma is in the recruiting phase in the United States. GITRL is also currently being used for modification of dendritic cells in a metastatic melanoma trial (http://clinicaltrials.gov/; search for key word: GITR).

23

1.5 CD30

Discovery and Expression of the receptor

CD30 is another member of the TNFR family with T cell costimulatory function. Originally discovered as a marker on Reed Sternberg cells in Hodgkin's lymphoma (99), CD30 is overexpressed in multiple cancers, but particularly lymphomas such as Hodgkin's and non-Hodgkin's lymphoma, anaplastic large cell lymphoma, multiple-myeloma and skin-associated lymphomas (100). In addition, a soluble form of CD30 can frequently be detected in the serum of patients with various autoimmune diseases, chronic viral infections and neoplasms, and increased levels of soluble CD30 correlate with poor outcome (101).

While overexpression of CD30 can lead to aberrant signaling and pathology, (102) CD30 is expressed on multiple different cell types in the immune system and is important for the generation of normal immune responses (5). CD30 is not expressed on naïve T cells, but is upregulated upon T cell activation in a CD28 or IL-4 dependent manner (101, 103). Originally thought to be preferentially expressed on Th2 CD4 cells (104, 105), CD30 has since been shown to be expressed on Th0 and Th1 cells as well (106). In addition, CD30 is also expressed on NK cells, macrophages, eosinophils, as well as naïve B cells, which when activated upregulate CD30 even further (5, 101).

CD30^{-/-} mice show no gross abnormalities and have normal subsets and numbers of lymphocytes in their secondary lymphoid organs (107). Although CD30^{-/-} mice were originally thought to have a defect in negative selection (107), this has been disputed (108). In addition, it was later observed that CD30^{-/-} mice have significantly reduced splenic T zones and defective splenic B/T zone segregation (109). Interestingly, this defect was not intrinsic to the

lymphocytes, which segregated normally in a RAG^{-/-} host, and could not be attributed to differential chemokine expression (109). Rather, it was associated with a lack of podoplanin, a transmembrane mucin-type protein involved in tube formation and cell adhesion, on the T zone stroma of CD30^{-/-} spleens (109). It appears that CD30 signaling, possibly through interaction with CD4⁺CD3⁻CD11c⁻ cells (discussed below), regulates the expression of podoplanin post-transcriptionally (109). However this is spleen specific, as levels of podoplanin were normal in CD30^{-/-} lymph nodes and thymus (109).

The ligand for CD30 (CD30L) or CD153 is a 40 kDa type II transmembrane protein. CD30L is expressed on dendritic cells, macrophages, resting B cells, neutrophils, eosinophils, the medulla of the thymus and activated T cells (5, 101). As well, CD30L and OX40L, have also been shown to be expressed on a specialized CD4⁺CD3⁻CD11C⁻ accessory cell, believed to be the adult equivalent of a LTi (lymphoid tissue inducer) cell, which plays a role in the development of lymph nodes and Peyer's Patches (110). The function of CD30L on this specialized cell will be discussed below.

In vitro costimulatory role and signaling

In vitro stimulation of T cells with either anti-CD3 antibody or antigen-primed dendritic cells and agonistic anti-CD30 antibody or recombinant CD30L enhances proliferation, as well as the production of various cytokines, including IFN- γ , IL-5 and IL-4 (111-114). CD30 signaling has been suggested to more efficiently costimulate CD4 Th2 cells, and it has been reported that CD30 stimulation of peripheral blood mononuclear cells causes a skewing towards the development of Th2 responses (112). However, it should be noted that CD30 is also expressed

on Th1 and CD8 T cells and, as will be discussed below, has recently been found to play a role in various Th1 in vivo models (115, 116). Interestingly, recent data has also indicated that CD30 plays a role in the polarization of Th17 cells (117).

CD30 has been shown to mediate downstream signaling via the recruitment of TRAFs 1, 2, 3 and 5 (118-121). Like many of the other T cell costimulatory molecules of the family, CD30 signaling activates JNK and p38 (100), as well as the NF- κ B pathway (122, 123). NF- κ B signaling downstream of CD30 has been shown to be positively regulated by TRAF1, TRAF2 and TRAF5, but negatively regulated by TRAF3 (120, 121, 124, 125). CD30 overexpression in states of disease is often associated with ligand independent constitutive NF- κ B signaling, which correlates with pathology (102, 126).

Role of CD30 in maintaining CD4 T cell memory

The function of CD30 has been well examined on CD4 T cells in vivo. Peter Lane's group has done extensive work implicating CD30 in the survival of CD4 Th2 cells (110, 116, 127, 128). They have shown that CD30 knockout mice have defective memory antibody responses and cannot sustain follicular germinal center responses following immunization with a T dependent antigen (128). This is attributed to defective CD4 T cell help for antibody responses (128). In addition, Lane's group demonstrated that CD30 works synergistically with OX40, another T cell costimulatory molecule, to sustain CD4 T cell memory help for secondary antibody responses (128). Strikingly, in CD30^{-/-}OX40^{-/-} mice there is a complete ablation of the memory antibody response due to a severe defect in the ability of CD4 memory populations to survive and generate help (128). As discussed above, CD30L and OX40L are expressed on a

CD4⁺CD3⁻CD11c⁻ accessory cell localized at the T/B cell interface (110). Interestingly, CD4 memory T cells have been visualized to interact with these accessory cells in vivo, and upon coculture in vitro, CD4⁺CD3⁻CD11c⁻ cells augmented the survival of Th2, but not Th1 polarized CD4 T cells (110). Moreover OX40^{-/-}CD30^{-/-} Th2 cells had significant survival defects compared to WT controls when cultured with the accessory cells in vitro (128). Therefore, a model arises where, following the priming and activation by dendritic cells, CD4 Th2 cells which have high expression of CD30 and OX40, migrate into B cell follicles and interact with CD30L and OX40L expressing CD4⁺CD3⁻CD11c⁻ cells at the B:T area interface to receive survival signals (110, 127, 128). It is of interest to note that IL-7, which has been implicated in the maintenance of CD4 memory T cells, can upregulate OX40 and CD30L expression on CD4 T cells independent of TCR signaling (128). Therefore, when antigen is absent or limiting, IL-7 may induce OX40 on CD4 memory T cells so that these cells can receive survival signals from CD4⁺CD3⁻ accessory cells (128).

While the above studies focused on a role for CD30 and OX40 in the maintenance of CD4 memory T cells in secondary lymphoid organs, further work demonstrated that OX40 and CD30 are also crucial for the survival of CD4 memory T cells in the lamina propria of the small intestine (129). While CD30^{-/-} and OX40^{-/-} mice individually have CD4 memory T cell defects in the lamina propria, the lack of the two molecules caused an almost entire ablation of this population (129). The cell type providing the survival signals through CD30L and OX40L is yet unknown, and though a population of CD4⁺CD3⁻ accessory cells also exists in the lamina propria, these cells seem to have a distinct phenotype from those found in the spleen (129). While it is possible that these lamina propria CD4⁺CD3⁻ cells may be the source of the survival signals, it is also feasible that other antigen presenting cells in the lamina propria play that role (129).

Role of CD30 in CD4 Th1 responses

While much work has focused on CD30 in the maintenance of CD4 Th2 responses, recently a role for CD30 and CD30L in CD4 Th1 infectious disease has emerged. Indeed, studies using CD30^{-/-}, CD30L^{-/-} mice or a neutralizing antibody against CD30L, show that CD30 and CD30L play a role in controlling *M. bovis* infection (115, 130). Indeed, it has been nicely demonstrated that following *M. bovis* bacillus Calmette-Guerin (BCG) infection, interactions between CD30 and CD30L on CD4 T cells are required for maximal numbers of CD4⁺CD44^{hi} IFN- γ^+ cells and for optimal bacterial control (115). Interestingly, when the mechanism was dissected further, it was CD30 signaling on the CD4 T cell that was required, as the IFN- γ production of CD30L^{-/-} CD4 effector T cells could be rescued by providing exogenous CD30L or by stimulating with an agonistic anti-CD30 antibody (115). Accordingly, following BCG infection, CD30L was primarily expressed on activated CD4 T cells, and could not be detected on APCs, suggesting a T-T cell interaction for CD30 signaling (115).

A role for CD30 in the immune response to Salmonella typhimurium has also been reported. Following *S. typhimurium* infection the proportion of CD4⁺ CD62L¹⁰ T cells could not be maintained in OX40^{-/-}CD30^{-/-} mice compared to WT controls (116). It was previously demonstrated that clearance of *S. typhimurium* is antibody independent but CD4 Th1 dependent, and double knockout mice failed to control the bacteria as effectively as WT controls (116). Similarly, transferred splenocytes from infected double knockout mice could not control infection in RAG^{-/-} mice (116). Interestingly, the single knockouts had no difference in bacterial control or proportions of CD4 T cells, indicating both CD30 and OX40 play a role, and can compensate for the loss of the other (116).

Role of CD30 in CD4 Th17 responses

A recent report implicates T-T CD30/CD30L signaling in the polarization of CD4 T cells to Th17 conditions (117). Indeed, CD30L^{-/-} mice were more resistant to the onset of both acute and chronic models of colitis, whose pathology is known to be IL-17A dependent (131). The administration of a CD30-Ig fusion protein to mice with colitis also ameliorated the disease (131).

Role of CD30 in CD8 T cell responses

Several studies have suggested that CD30 and CD30L play a role in the CD8 memory T cell response, although these results may be model dependent. A preliminary report demonstrated that OT-I cells had defective expansion, failed to clonally contract and could not generate a secondary response when adoptively transferred into CD30L^{-/-} hosts following immunization with tumor antigen EG7 fused to the fusion protein gp96Ig, which directs the complex to dendritic cells to be presented (100). In addition, a more recent study using a model of listeria infection demonstrated that CD30L was required for the formation of long lived antigen-specific CD8 central memory (see below) (132). CD30L was also required for protective responses to bacterial rechallenge (132). Indeed, stimulation of CD8⁺CD44^{bi} CD62L^{lo} effector cells with an agonistic anti-CD30 antibody in vitro caused the upregulation of CCR7 mRNA, suggesting that CD30/CD30L signaling might play a role in the generation of central memory by upregulating homing receptors to lymph nodes (100, 132). Further adding to the evidence of a role for CD30 in regulating CD8 T cell responses, when a 1:1 ratio of OT-I:CD30^{-/-}OX40^{-/-}OT-I cells was adoptively transferred into mice which were then immunized with OVA peptide, the

double knockout cells failed to expand as well as the WT cells early, and had poor recall responses when challenged (133).

In contrast to the above studies, however, while $OX40^{-/-}CD30^{-/-}$ mice infected with murine CMV (MCMV) had reduced antigen-specific CD8 T cell responses 7 days post-infection, surprisingly they had unimpaired generation and maintenance of CD8 T cell memory as compared to WT mice (133). Similarly, CD30 was dispensable for the generation of in vitro memory CTL responses to vesicular stomatitis virus (VSV) (107). Despite these findings, it is unlikely that all viral responses are CD30 independent. Interestingly, cowpox and mousepox virus encode a soluble homologue of CD30 that is secreted and specifically binds human CD30L, blocking binding with the cellular receptor (134, 135). This viral homologue inhibited type I cytokine responses in vivo, and in vitro almost completely blocked the production of IFN- γ -producing T cells in a mixed lymphocyte reaction (134). Poxviruses most likely evolved this homologue as an immune evasion mechanism, suggesting that CD30 is important for the immune response to these viruses (134, 135).

1.6 T cell activation and the generation of memory

A naïve T cell typically migrates through the lymphoid system, surveying the environment by random contact with antigen presenting cells, which under non pathogenic or non inflammatory conditions are immature and present self antigen. However, in the event of a TCR interaction with its respective peptide presented in the context of MHC on an APC, in the presence of inflammation and secondary signals such as costimulation, a T cell becomes activated (Figure 1.4). Over the next few days extensive proliferation ensues, when antigenspecific T cells can expand ten thousand fold (136). In addition, the T cells upregulate homing receptors to allow them to migrate to sites of infection, and differentiate effector functions to combat the foreign pathogen once they arrive. Broadly speaking, CD8 T cells develop the ability to directly kill infected cells and tissues via the release of granzymes and perforin, as well as through the ligation of Fas (137). In addition, T cells also produce cytokines such as IFN- γ and TNF, and CD4 T cells generate help for the activation of antigen-specific B cells to promote antibody responses. Furthermore, CD4 T cells are necessary for CD8 memory T cell responses (138). The expansion of antigen-specific CD8 T cells upon secondary encounter with antigen has been shown to be dependent upon the presence of CD4 T cells upon priming (139-141). Although there has been some debate as to the exact nature of the CD4 T cell help, it was recently demonstrated that CD4 T cells are required for the CD40:CD40L dependent "licensing" of dendritic cells, which then can activate CD8 T cells to produce autocrine IL-2 (142). Moreover, in response to vaccinia virus, CD4 mediated CD40L signaling induces the production of IL-12p70 by dendritic cells which acts to upregulate CD25 (IL-2Ra) on CD8 T cells, enhancing the sensitivity of these cells to IL-2 (143). It has been postulated that the degree of



an APC, in an inflammatory milieu with costimulatory signals, it is activated. The primed CD8 T cell proliferates and acquires effector functions to fight the antigen insult. Effector cells are often characterized as either SLECs (short lived effector cells) or MPECs (memory precursor effector cells), and these appear to be lineages driven by distinct transcription factors. Upon contraction, most of the T cells undergo apoptosis, however 5-10% remain as CD8 memory T cells and are maintained by homeostatic cytokines IL-15 and IL-7. In the event that the cells are re-exposed to their respective peptide, they proliferate and undergo a recall response, which has been shown to be dependent upon CD4 T cell help upon original priming. This memory response is both quantitatively and qualitatively superior to the primary response. help required by CD8 T cells from CD4 T cells may depend on the degree of inflammation present (144).

Upon resolution of infection, 90-95% of effector T cells contract and only a small proportion of T cells survive as memory cells. This cell fate decision has been the subject of many studies, and it has recently been shown that naïve T cells are not programmed pre-priming to become either effector or memory T cells, but rather this differentiation occurs only following T cell activation (145). Multiple markers have been identified to characterize populations of effector T cells and determine their potential for memory, and a wide spectrum of phenotypes exist as T cells undergo activation and differentiation (136). Following activation the majority of CD8 T cells downregulate CD127 (IL-7R α), which is constitutively expressed on naïve cells (146-148). There is an inverse correlation between the expression of CD127 on T cells and the expression of killer cell lectin like receptor 1 (KLRG1) (149). These 2 cell surface molecules are widely used to characterize 2 distinct populations of effector cells following acute infection: KLRG1^{hi}CD127^{lo} short-lived effector cells (SLECs) and KLRG1^{lo}CD127^{hi} memory precursor effector cells (MPECs) (147, 149). SLECs are terminally differentiated cells with high effector potential. MPECS, on the other hand, tend to be longer lived and preferentially survive contraction, converting to memory cells (136, 149).

Memory T cells have been broadly characterized by the markers CD62L and CCR7, with CD62L^{hi}CCR7^{hi} cells termed central memory and CD62L^{lo}CCR7^{lo} cells termed effector memory (150). These two cell populations preferentially localize to different areas of the body. Central memory T cells have a resting phenotype and can traffic to lymphoid organs, due to their expression of homing molecules. Effector memory T cells are typically found in non-lymphoid sites and are more primed to be activated to exogenous antigen. This preferential localization of

the 2 memory subsets is not absolutely fixed, however, and both effector and central memory can be found in most organs to a certain extent (136). Whether central and effector memory T cells derive from distinct lineages, or can interconvert remains somewhat controversial. Although not yet completely elucidated, the current predominating view seems to be that these memory T cell subsets are derived from separate lineages (151-153).

The transcription factors T-bet and eomesodermin (EOMES) have also been shown to play prominent roles in the differentiation of effector and memory T cells, and control expression of CD122 (IL-15R β chain) on CD8 memory T cells (154). T-bet is known to promote Th1 responses in CD4 T cells (155, 156), and in CD8 T cells is crucial for the formation of effector cells, having higher expression levels in SLECs as opposed to MPECs (149). Consistent with this, T-bet deficient mice have enhanced central memory (157). Interestingly, in contrast, a study using EOMES knockout virus-specific CD8 T cells showed defects in longterm survival and the formation of central memory (158). Despite the distinct roles of T-bet versus EOMES in the promotion of terminal differentiation versus long-lived central memory (158), the 2 transcription factors are both needed to generate effector functions in CD8 T cells (154). Following LCMV infection in double deficient (*Tbx21^{-/-} Eomes^{-/-}*) knockout mice, CTLs are not generated and rather, CD8 T cells produce IL-17, leading to a lethal inflammatory disease (159).

Similarly the transcription factor Blimp-1 has also been implicated for the terminal differentiation of CD8 T cells (160, 161). Indeed, Blimp-1 knockout CD8 T cells differentiate into MPECs and have augmented memory cell populations upon viral infection (160, 161). It has also been noted that CD8 T cells lacking Blimp-1 express higher levels of Bcl-6 than do WT cells (160, 161). Bcl-6 expression is upregulated in memory CD8 T cells and the overexpression of Bcl-6 in CD8 T cells leads to enhanced central memory formation (162). In contrast, Blimp-1

is expressed only at low levels in central memory CD8 T cells, but at high levels in effector cells (160, 161). Therefore, it has been suggested that Blimp-1 and Bcl-6 reciprocally regulate the differentiation of CD8 T cells (163).

1.7 Maintenance of T cell Memory

After a pathogen is cleared and infection resolved, the majority of the resultant effector T cells undergo apoptosis. However, approximately 5-10 % of these cells survive as memory T cells and persist to generate a fast and efficient response in the event of re-exposure to that particular pathogen (164, 165). Memory T cells undergo homeostatic proliferation, dividing approximately once every 3-6 weeks. Previously, it was thought that T cell memory was maintained due to encounter with persistent antigen. However it has been demonstrated that homeostatic proliferation is both antigen and MHC independent (166-168) and rather is driven by the cytokines IL-7 and IL-15 (169). The regulation and maintenance of CD4 and CD8 T cell memory. It is thought that generally in steady state conditions IL-7 is crucial for the survival of antigen-specific CD8 T cells, while IL-15 is more important for the homeostatic proliferation of CD8 memory T cells. (169, 170).

IL-7

IL-7 is a pleiotropic cytokine produced constitutively by stromal cells (171). The receptor for IL-7 is composed of the IL-7R α chain, (CD127) which is paired with a common γ chain (CD132) also used by IL-2, IL-4, IL-9, IL-15 and IL-21 (169). As described above, IL-7R α is expressed on naïve T cells and is downregulated on the majority of T cells upon activation (146). In various acute infection models, however, a small subset of cells remains IL-7R α high and preferentially converts to memory T cells (147, 148). Because CD127 expression is high on those T cells that survive and are destined to become memory, it was thought that repression of the IL-7R might be necessary for contraction and apoptosis, and therefore IL-7 might provide a selection signal for effector CD8 T cells to become memory (136, 172). However in 2 infection models that prevented the downregulation of CD127 on effector CD8 T cells, the LCMV or listeria-specific CD8 memory T cell response was not enhanced (173, 174).

Consistent with the expression of its receptor, IL-7 is crucial for the survival of both naïve and memory T cells in vivo (170). In models where IL-7R signaling is abolished selectively on CD8 T cells, activation and development into memory was not impaired, but IL-7R knockout or mutant antigen-specific CD8 T cells could not survive longterm and gradually diminished over time (146, 175-177). In addition, when exogenous IL-7 was administered as an adjuvant, it enhanced the survival of activated and memory CD8 T cell populations (172, 178, 179).

Interestingly, in vitro, II-7 can also mediate CD8 T cell proliferation, but in vivo it seems that IL-15 predominates for the homeostatic proliferation of CD8 memory T cells and that IL-7 is either at too low a concentration for use by memory CD8 T cells (180), or perhaps it is being competed out by naïve T cells. However, the overexpression of IL-7 can compensate and augment memory phenotype CD8 T cell numbers when IL-15 is lacking (181). It should also be noted that IL-7 not only promotes T cell survival, but also is important for both T and B cell development. The details of these functions, however, are beyond the scope of this thesis.

Although the IL-15 protein has been difficult to detect in vivo, IL-15 can be synthesized by various cell types including bone marrow derived dendritic cells and monocytes (182). The receptor for IL-15 is composed of three subunits: IL-15R α , β (CD122) and the common γ chain (CD132). The β chain is common to the IL-2 receptor, and the common γ chain is also used by IL-2, IL-4, IL-7, IL-9 and IL-21. IL-15R α is widely expressed on most cell types in the body (183) and it is this chain which confers specificity for IL-15 (182).

The role of IL-15 has been studied using knockout mice. $IL-15^{-/-}$ and $IL-15Ra^{-/-}$ mice have diminished numbers of CD8 memory phenotype T cells, a lack of NK cells, and defects in NKT cell and intestinal intraepithelial lymphocyte (iIELs) populations (184, 185). Furthermore, overexpression of IL-15 increases numbers of memory phenotype CD8 T cells (186). IL-15 and IL-15Ra are dispensable for the generation and function of antigen-specific CD8 T cells to virus, however are necessary for the maintenance of these CD8 memory T cells (187, 188). Indeed, homeostatic proliferation of antigen-specific CD8 memory T cells is severely compromised in IL-15^{-/-} mice, and WT CD8 memory T cells fail to proliferate when adoptively transferred into IL-15^{-/-} recipients, whereas IL-15^{-/-} memory CD8 T cells divide if transferred into a WT recipient. (188)

Trans-presentation

IL-15 is presented via a unique mechanism, termed trans-presentation. Originally proposed by Dubois et al. (189), trans-presentation involves the binding of IL-15 to the IL-15R α subunit intracellularly and the subsequent transportation of the complex to the surface of the cell,

where it is presented to the IL-15R β and γ chains of an adjacent cell (182). Indeed, earlier studies by Averil Ma's group had curiously demonstrated that IL-15R α was not required on T cells for their IL-15 dependent poly-i.c. mediated proliferation (190). Furthermore, adoptive transfer experiments and mixed bone marrow chimeras with IL-15R α deficient or sufficient CD8 T cells demonstrated that the expression of the IL-15R α chain on antigen-specific CD8 T cells was not necessary for their maintenance in vivo, as long as IL-15R α was present in the mouse (191, 192). Of interest, trans-presentation has also been shown to be the mechanism of NK and NKT cell maintenance (193-196).

As described above, the cell that produces IL-15 is also the trans-presenting cell (197, 198), as the IL-15:IL-15R α complex is assembled intracellularly and then shuttled to the cell surface. Using bone marrow chimeras, it was demonstrated that the cells which trans-present IL-15 to CD8 memory T cells are of a haematopoietic origin (192, 193). Interestingly, the development of both invariant NKT cells, as well as CD8aa iIELs is mediated by IL-15 transpresentation by parenchymal cells (193). NK cells and naïve CD8 T cells can be stimulated via both haematopoietic or parenchymal cells (192, 193). Recently, Averil Ma's group elegantly dissected the cell types mediating trans-presentation to NK and CD8 T cells by generating conditional knockout mice lacking IL-15R α on either dendritic cells, macrophages, both dendritic cells and macrophages or intestinal epithelial cells (199). Interestingly, they demonstrated that IL-15Ra on macrophages was necessary for the transition from effector to memory CD8 T cells, as well as for supporting the maintenance of both effector and central CD8 memory T cells (199). The trans-presentation of IL-15 via dendritic cells was crucial for supporting only central memory CD8 T cells (199). Similarly, studies using transgenic mice where IL-15R α is expressed only under the CD11c promoter, and therefore, only on dendritic cells, showed partial recovery of NK cell numbers, as well as enhanced numbers of memory

phenotype CD8 T cells (200). In addition, the dendritic cell derived IL-15 signals supported the homeostatic proliferation of differentiated memory CD8 T cells (200). Therefore, IL-15 transpresentation is diverse, yet highly regulated. The specificity of the IL-15 transpresentation may also be due in part to the localization of the different cell types, and therefore may reflect the nature of the niches where IL-15 signaling can occur (182, 199).

Interestingly, because IL-15 is not produced by CD8 T cells, and the IL-15R α is not necessary on these cells for CD8 T cell maintenance, the question arises as to why the IL-15R α chain is present on CD8 T cells at all? There has been *in vitro* data suggesting that CD8 T cells respond to IL-15 more efficiently when the cells express the IL-15R α chain (201). Indeed, it has been speculated that there might be cases when IL-15 could be presented in cis, and this may be driven by the availability of the cytokine (182). For instance, upon initiation of an immune response IL-15 is produced in abundance and it is possible that this excess cytokine could be secreted and be available for pickup and cis presentation (182). In contrast, the lower basal levels of IL-15 present without infection most likely predisposes to trans-presentation (182). Despite the possibility for cis-presentation, however, trans-presentation remains the dominant mechanism of IL-15 presentation for CD8 T cell maintenance in vivo (182).

CD4 memory T cell maintenance

The regulation of CD4 T cell memory is much different from that of CD8 T cell memory. Interestingly, antigen-specific CD4 memory T cells are less stable and diminish much more quickly (202). Because IL-15^{-/-} mice show no defect in CD4 memory T cell numbers (185), IL-15 was originally thought to be dispensable for CD4 T cell maintenance. However, IL-15 has since been shown to play a small role in the homeostatic proliferation of antigen-specific CD4 T cell memory (203, 204). CD4 memory T cells are highly dependent on IL-7 for their survival (205, 206), and IL-7 can drive homeostatic proliferation of these cells as well (203). The difference in the requirements for these cytokines may be due to the high expression of CD127 and the lower levels of CD122 found on CD4 memory T cells (180).

Memory Phenotype Cells

Memory phenotype T cells are defined as cells in unimmunized or naïve mice that express surface markers of antigen exposure, i.e. CD44^{hi}CD122^{hi}Ly6C^{hi}. While the antigenspecificity of these memory phenotype cells is unknown, it was originally thought that they were generated from exposure to environmental antigens or commensal flora (207). However, it was discovered that germ-free and antigen-free mice also have populations of these cells (208-210). Therefore, it is possible that memory phenotype cells arise from homeostatic proliferation of naïve T cells driven by encounter with MHC and self peptide (207). Indeed, CD44^{lo} cells can convert to CD44^{hi} cells in lymphopenic environments in response to self peptide MHC stimulation (211, 212), and it was recently shown that in unimmunized mice the antigen-specific CD8 T cell repertoire includes cells which had homeostatically proliferated to become memory phenotype cells (213). To date, CD8 memory phenotype T cells have served as a good surrogate for antigen-specific CD8 memory T cells in many studies. Similar to antigen-specific responses, memory phenotype cells can induce rapid proliferation upon T cell activation and elicit immune protection (213, 214). In addition, the maintenance of CD8 memory phenotype cells is very similar to that of antigen specific CD8 memory T cells, with IL-15 and IL-7 playing key roles (215, 216). It has been noted, however, that memory phenotype cells are slightly less dependent on IL-7 for T cell survival, as IL-15 can also promote survival (217). It has been speculated that this may be due to differences in the priming of these cells. Memory phenotype cells may be primed in the context of weak TCR signals to self antigens, in the presence of γ chain cytokines (207).

1.8 Bone marrow – niches

Bone marrow is a rich source of memory T cells (218-220). Although in unimmunized mice CD8 T cells compose only approximately 2.5% of mononuclear cells in the bone marrow, two thirds of these cells have a memory phenotype (219). Similarly, approximately 40% of $CD3^+CD4^+$ T cells identified in the bone marrow are of a memory phenotype, which is in stark contrast to the naïve phenotype of the majority of T cells in blood, spleen and lymph nodes (218, 219). Consistent with the data from the mouse, human bone marrow has also been shown to be a reservoir for memory T cells (219, 221-223) (224).

Following an immune response, antigen-specific memory T cells can be found in the bone marrow (218, 219, 225-228). Indeed, both naïve and memory T cells can home to the bone marrow, however memory T cells were found to home more efficiently to the bone marrow than naïve T cells (219, 229). Furthermore, central memory CD8 T cells homed more effectively to the bone marrow than effector memory T cells (219). To gain access to the bone marrow from the blood, central memory CD8 T cells first roll on the bone marrow endothelium via a mechanism dependent on P, E and L-selectin expression on the bone marrow microvasculature (219). Rolling cells are then arrested via a mechanism involving the adhesion molecule VCAM-1, which is expressed on stromal cells and bone marrow microvasculature, binding to the α 4

integrin, VLA-4, expressed on central memory T cells (219). In addition, the chemokine CXCL12, which binds to its ligand CXCR4 on central memory T cells is also involved in T cell arrest (219). CXCL12 is not required for diapedesis, however, and this is mediated via pertussis toxin sensitive Gai proteins (219). Furthermore, CD4 memory T cell homing to the bone marrow has been shown to require the integrin $\alpha 2$, which interestingly, is expressed on the majority of CD4 memory T cells found in the bone marrow, but not those in the spleen (225). $\alpha 2$ integrin binds collagen I, which is mainly found in the stroma of the bone marrow (230). Egress of T cells from the bone marrow has also been studied, and has been shown to be S1P1 dependent, as S1P1 deficiency, as well as the use of the S1P receptor inhibitor FTY720 sequestered and caused accumulation of T cells in bone marrow (231, 232).

Though naïve and memory T cells can be activated in the bone marrow to initiate immune responses (233-235), it appears that the bone marrow plays a more important role for the maintenance of memory T cells. CD8 memory T cells preferentially homeostatically proliferate in the bone marrow (236, 237), and it is thought that the organ contains CD8 memory T cell niche has been more clearly elucidated. After infection, antigen-specific CD4 resting memory T cells accumulate in the bone marrow where they interact with IL-7 producing VCAM1⁺ stromal cells (225). This association presumably allows CD4 memory T cells to receive IL-7 derived maintenance signals (225), as IL-7 can drive both survival and homeostatic proliferation of CD4 memory T cells. Indeed, it has been found that T cells in the bone marrow have a more activated phenotype (228, 238), and this may be due to exposure to IL-7 and IL-15. CD8 memory T cells from mouse bone marrow have lower CD127 levels and increased phosphorylation of STAT5 and p38 MAPK levels than those memory cells from the spleen (238). In addition, bone marrow memory cells have a higher proportion of larger blasting cells

despite a lack of antigen, and when challenged in vitro they reactivate more quickly than their counterparts from the spleen and lymph nodes (228). Memory T cells in human bone marrow are also in a heightened state of activation (223, 224). When compared to their peripheral blood effector memory counterparts, effector memory T cells from the bone marrow express elevated levels of CD69 and other activation markers, and upon in vitro stimulation are more cytotoxic and have a higher proportion of IFN- γ -producing memory cells (223, 224). In addition, a higher proportion of bone marrow CD4 and CD8 memory T cells are polyfunctional, expressing IL-2, IFN- γ and TNF- α (223). Interestingly, a recent study also showed that in humans both CD4 and CD8 T cells in the bone marrow are found in close proximity to IL-15 producing cells (223).

1.9 Influenza Virus

Influenza viruses are negative-sense single stranded RNA viruses belonging to the *Orthomyxoviridae* family. Type A influenza viruses infect humans and have the potential to cause pandemics. This group of viruses can be further classified into subtypes by their envelope glycoproteins hemagglutinin (HA) and neuraminidase (NA), which are the major targets of the anti-influenza antibody response (Figure 1.5). Currently there are 16 viral subtypes based on HA and 8 on NA (239). In this thesis, I make use of 2 strains of influenza virus: A/HK-X31 (X31), which is a H3N2 virus, and A/HK-PR8 (PR8), a H1N1 virus. X31 is a recombinant virus made up of the HA and NA genes derived from the 1968 Hong Kong strain of virus and the 6 RNA segments encoding the internal viral proteins from the A/PR8 virus. X31 elicits a mild infection that is typically cleared within 8 days (240). In contrast, PR8 elicits a more severe influenza infection, which at certain viral doses can mediate the death of mice (241). Although both strains

of influenza differ significantly in their virulence, they share common CD8 T cell epitopes (240), allowing for the study of secondary T cell responses to influenza upon rechallenge of mice. Indeed, infection of mice with X31 elicits protection from a subsequent challenge with the more lethal PR8 strain (240, 242).

Influenza viruses evolve and continually mutate to evade the immune response. This occurs by 2 specific mechanisms, antigenic drift and antigenic shift. Antigenic drift occurs continually and is mediated by mutations caused by the error prone polymerase. As the immune response exerts pressue on the neutralizing epitopes in HA, viruses tend to be selected that have mutations in and around the sialic acid binding site on HA, to prevent the binding of neutralizing antibodies. As the virus is always mutating, the need for seasonal vaccines against the pertinent strains becomes important to elicit immunity. Antigenic shift is less frequent and occurs when a host is infected with 2 separate viruses, allowing the RNA segments of the two viruses to reassort to form a new subtype containing a novel HA or NA not typically found in humans. In this case, often the general population has little or no immunity to this resultant virus, which can spark pandemics.



Figure 1.5. The structure of an influenza virus. Influenza viruses are negative-sense single stranded RNA viruses. This group of viruses can be further classified into subtypes by their envelope glycoproteins hemagglutinin (HA) and neuraminidase (NA), which are the major targets of the anti-influenza antibody response. Each of the single stranded RNA segments is associated with an RNA polymerase as well as the intracellular nucleoprotein (NP).

1.10 The Immune Response to Influenza Infection

Innate Responses

Respiratory viruses, such as influenza, infect and replicate in the lung epithelium (243). Infection of respiratory epithelial cells by influenza initiates the activation of the innate immune response via various mechanisms, including the detection of pattern associated molecular patterns (PAMPs), such as viral proteins or single stranded RNA by toll like receptors (TLRs) (244). This results in the generation of an inflammatory reaction, where cells such as alveolar macrophages and plasmacytoid DCs (pDCs) produce type I interferons, in addition to other cytokines and chemokines, leading to the recruitment first of innate cells, such as neutrophils and NK cells, and then of adaptive immune cells into the lung. (243, 245)

Priming of Adaptive Responses

In addition to epithelial cells, influenza can infect resident dendritic cells located in the upper respiratory tract (246, 247). Respiratory dendritic cells (RDCs) are diverse and include conventional RDCs and pDCs (248). Conventional RDCs can be further subsetted into 2 major subsets: CD103⁻CD11b^{hi} cells which are found in the submucosa or interstitium, and CD103⁺CD11b⁺ cells which are localized to the intraepithelium (248). Virally infected DCs in the local proinflammatory milieu, are then activated to a mature state, expressing high levels of MHC, costimulatory molecules and adhesion molecules in order to present viral antigen. (244) Within the first 48 hours post-infection, the DCs migrate out to the cervical and mediastinal draining lymph nodes of the lung, to present viral antigen to naïve T cells and generate an

adaptive immune response. The chemokine receptor CCR7 is required for DC trafficking to the draining lymph node in steady state non-inflammatory conditions (249). Interestingly, influenza infection augments CCR7 expression on DCs even further, which enhances migration (250). Although the mechanism is not completely elucidated, the upregulation of CCR7 appears to be dependent on the chemokine receptor CCR5 and its ligand CCL5 (250, 251).

Different subsets of dendritic cells can activate influenza-specific T cells differentially, with the most potent activator being the CD103⁺ dendritic cell subset (252). Initial priming is mediated primarily by this CD103⁺ subset, but after this, CD11b^{hi} dendritic cells continue to present antigen in the lymph node throughout the infection. (252). The first few days following infection, the T cells specific for the viral antigens presented are primed, activated, and undergo expansion in the draining lymph nodes. They then home into the lung 5-7 days post-infection where they lyse virus-infected epithelial cells and produce anti-viral cytokines such as IFN- γ and TNF- α , to combat the influenza virus (253). T cell homing to the lung has been found to be dependent upon the adhesion molecules, LFA-1 and PSGL-1 (254, 255). Interestingly, LFA-1 is downregulated on T cells upon entry into the lung, though the functional significance of this remains unknown.

CD8 T cell responses

CD8 T cells play a pivotal role in combating virus and protecting from severe infection and death. Virally infected cells in the lungs are killed by primed cytotoxic CD8 T cells in a Fas/FasL or a perforin/granzyme dependent mechanism (256). In addition these activated effector CD8 T cells also can also produce proinflammatory cytokines such as TNF- α or IFN- γ which function to recruit other innate cells to the lung to further fight the virus (248). Recently it was shown that the effector mechanisms elicited by CD8 T cells in the lungs are dependent on the target cell type encountered (257). CD45⁻ influenza infected respiratory epithelial cells triggered CTL cytotoxicity, while CD45⁺ antigen-bearing dendritic cells triggered both target cell lysis, as well as the production of inflammatory cytokines (257). Interestingly, optimal anti-viral responses have also necessitated the interaction of recruited CD8 T cells with inflammatory dendritic cells in the lung (258). These dendritic cells enhance CD8 T cell survival by transpresenting IL-15 (259). It should also be noted that IL-15 has been implicated in the recruitment of CD8 T cells to the lungs (260).

CD4 T cell responses

Anti-influenza CD4 T cell responses are largely known to be important for B cell activation to promote the production of anti-influenza antibodies (248). CD4 T cells also produce anti-viral cytokines such as IFN- γ , TNF- α and IL-2, and play a role in the formation of CD8 T cell responses, as mice deficient for CD4 T cells have normal primary, but defective memory anti-influenza CD8 T cell responses, resulting in a delay of viral clearance (261). Interestingly, however, the number of CD8 T cells in the BAL is not defective, implying that high levels of inflammation can compensate for a lack of CD4 T cell help (261).

Not only can CD8 T cells mediate protection against lethal strains of influenza, but primed or memory CD4 T cells have also been shown to clear virus and protect mice from death (262-264). Protection by primed CD4 T cells can be mediated via 2 separate mechanisms: by the CD4-driven enhancement of anti-influenza antibody titers, and by the perforin-dependent killing of infected cells, independent of any CD4-mediated effects on B cells (264). In addition, transferred antigen-specific memory CD4 T cell protection was dependent on IFN- γ (262). Moreover, these memory CD4 T cells could elicit protection in CD8 depleted, B cell deficient mice (262).

Antibodies

Virus-specific antibodies are also very important in the clearance of influenza, particularly upon infection with highly pathogenic viruses (265). Mice that are B cell deficient fail to clear virus and succumb to infection (266, 267), with early IgM antibodies playing a large role in mediating protection (268, 269). B-1 B cells also generate T-independent neutralizing IgM antibody, which can help neutralize the virus (270). However, as discussed above, CD4 T cell help is needed for most, though not all antibody responses (263, 271). Upon the resolution of influenza infection, long lived plasma cells traffic to the bone marrow where they continue to secrete antibody (271). Resting memory B cells are distributed widely but have high frequencies in the lung (271).

Generation of anti-influenza T cell memory

Following the resolution of influenza infection, a minimal amount of T cell proliferation can still be induced for up to 60 days post-infection (271). Although somewhat controversial, it is thought that this is due to remaining antigen pools that are presented in the draining lymph nodes (272, 273). Memory T cell numbers in the lung airways begin to wane upon loss of antigen and T cell numbers decline for approximately 6 months and then stabilize, so that a small population of influenza-specific memory T cells remain in the lung (227, 274). At this point of time, the levels of memory T cells are maintained via basal recruitment from the circulation, which has been suggested to be driven by homeostatic cytokines or low levels of inflammation from environmental antigens (244, 275). At first, effector memory T cells (T_{EM}) predominate in non-lymphoid organs (271). These cells have an activated phenotype, and express markers such as CD69 and CD25, while downregulating LFA-1 upon entry into the lungs (244, 276, 277). However with time, the bulk of the anti-influenza memory population shifts to central memory (T_{CM}) and therefore is localized in lymphoid tissue, such as lymph nodes, spleen and bone marrow (271). This is not thought to be due to a direct conversion between T_{EM} to T_{CM} , but rather a competitive outgrowth of T_{CM} cells (271).

Memory Responses upon influenza challenge

Upon secondary challenge with influenza, memory T cells residing in the lungs are the first wave of defense to counter the virus (278). Though these airway resident cells lack cytolytic function (279), they produce cytokines and recruit other immune cells to control the virus until the dendritic cells can activate T cells in the draining lymph nodes (280). The second wave of the response involves the recruitment of non-dividing CD8 memory T cells into the lung airways from the circulation (277). Recruitment of these memory T cells to the lung airways is CCR5 dependent, but antigen-independent, and aids in decreasing viral burden (281). This may occur via cross-reactivity, or perhaps the inflammatory conditions of the lung can non-specifically activate these cells to secrete cytokines (244). These cells do not proliferate and cannot sustain the response, but function to decrease viral burden until the antigen-primed CD8 T cells can

come into the lung (277). The last stage of the memory response is the arrival of the DC primed antigen-specific T cells from the draining lymph nodes, which occurs approximately 4 days post-infection (271). These cells can sustain a longer immune response. Though similar to the primary response, the higher precursor frequency, the reduced dependency on costimulation, and the enhanced kinetics of T cell activation and the acquisition of effector functions, leads to a more qualitatitive, as well as quantitative anti-influenza secondary response (271).

Pathology of immune responses to influenza

It should be noted that while a potent immune response to influenza is necessary for the clearance of virus, in certain cases it may also be detrimental to the host, resulting in immunopathology. Patients who developed severe disease when infected with pandemic H1N1 had high levels of pro-inflammatory cytokines such as TNF- α and IL-6 in their serum (282). In addition, during the 1918 influenza pandemic, the number of healthy 15-34 year olds who succumbed to the virus was unusually high, and this, as well as mortality in H5N1 cases was associated with haemophagocytic syndrome (283, 284), which has been shown to be driven by cytokines (285). These young people were found to die from a phenomenon termed "cytokine storm", which involves the excessive production of inflammatory cytokines, leading to pulmonary edema, pneumonias, and alveolar haemorrhage (286). Indeed, while such cytokines are crucial for the immune response to influenza, elevated cytokine levels have also been associated with viral burden and disease severity (286). Furthermore, while CD8 T cells are crucial for the clearance of the virus, an exaggerated response can also be pathogenic. In transgenic models CD8 T cells are protective both at low viral dose, and in cases when fewer T cells are transferred to mice, while higher viral doses, and the transfer of more T cells resulted in

immunopathology and death (287, 288). Activated T cells in the lung can recruit other inflammatory cells, and produce pro-inflammatory cytokines such as IFN- γ , TNF- α and IL-17, which cause pulmonary inflammation in the lung (248). However, the role of these T cell derived cytokines can be complex. For instance, there is conflicting data as to whether CD8 mediated IFN- γ is pathogenic or not (288, 289). Interestingly, as discussed above, T cells can also produce IL-10, which helps to dampen the immune response to influenza (290), although it should be noted that IL-10^{-/-} mice are more resistant to lethal influenza infection (291, 292). In addition, regulatory T cells also play a role in the suppression of both innate and adaptive immune responses to influenza (293, 294). Clearly the regulation of the immune response is very finely regulated and the balance can be tipped either way.

Costimulation and the CD8 T cell response to influenza

T cell costimulation provides another mechanism for fine-tuning the immune response to influenza infection. Although this thesis focuses on TNFR family members, the prototypical costimulatory molecule CD28 has been well studied in this regard, and CD28/B7 signaling is essential for maximal CD8 T cell responses to influenza (295-300). CD28^{-/-} mice have substantially decreased primary CD8 T cell expansion to influenza infection, and upon rechallenge, have very poor recall anti-influenza CD8 T cell responses (300). In contrast, the ligand for another inducible TNFR family member expressed on T cells, 4-1BB, is dispensable for primary antigen-dependent CD8 T cell responses to mild influenza infection, however is needed for the maintenance of CD8 T cell memory and for secondary recall responses to the virus (300). Interestingly, upon infection of 4-1BBL^{-/-} mice with a more pathogenic strain of influenza that causes severe respiratory infection, 4-1BBL^{-/-} mice show defective recruitment of

CD8 T cells to the lung, which correlates with decreased viral clearance and lower lung function (287). 4-1BBL is clearly protective, as the knockout mice succumb to death far more readily, and the protection appeared to be CD8 T cell mediated, as transferred CD8 T cells could protect B6 but not 4-1BBL^{-/-} hosts from death (287). To be discussed in this thesis, both GITR and CD30 also augment mouse survival from severe influenza infection. Although generally beneficial to the anti-influenza immune response, the augmentation of T cell responses through costimulation can also, in certain instances, be detrimental to the host. This is the case when OX40/OX40L interactions are blocked with a soluble form of OX40, OX40Ig, in mice infected with influenza (301). The OX40Ig decreased T cell proliferation at the peak of the response and ameliorated T cell mediated pathology in the lung (301).

1.11 Thesis Outline

When I entered the laboratory we were investigating the role of TRAF1 in CD8 T cell activation and memory and discovered that TRAF1 was crucial for the CD8 T cell response to influenza and for the maintenance of CD8 memory (34). We further identified 4-1BB as a TNFR receptor through which TRAF1 mediates survival effects (33, 302). However, as defects in TRAF1^{-/-} mice appeared to occur throughout the immune response (34), whereas 4-1BBL^{-/-} mice had defects in the CD8 T cell response to influenza only at late time points (300), we reasoned that other TRAF1 binding TNFRs might play a role. The prototypical TRAF1 binding TNFR is TNFR2, however, others had suggested that TNF-deficient mice did not have defects in the response to influenza (303). Therefore, I decided to focus my efforts on two members of the

family that were reported to recruit TRAF1 and yet had been understudied to date, GITR and CD30.

In Chapter 2, I investigate the role of GITR on CD8 T cell responses to influenza virus. GITR, one of the newest members of the TNFR superfamily, had not been well characterized and there is little data on GITR's role in infectious disease. In this chapter, I show that CD8 intrinsic GITR is crucial for maximal CD8 T cell responses to influenza. I show that GITR contributes to the survival of the CD8 T cells and medisates TRAF2/TRAF5 dependent NF- κ B signaling leading to the upregulation of Bcl- x_L . In addition, I demonstrate that GITR on CD8 T cells can decrease viral burden in the lungs and protect mice from death.

In Chapter 3, I further investigate the role of GITR on CD8 memory T cells in unimmunized mice. I show that the bone marrow contains a population of CD8 memory phenotype T cells that express high levels of GITR which is absent in the spleen and lymph nodes. Furthermore, this GITR^{hi} population is IL-15 dependent and adoptive transfer experiments show that GITR becomes actively upregulated on a subset of CD8 memory T cells by local IL-15 upon entrance into the bone marrow. GITR is not necessary for the homeostatic proliferation of these CD8 memory phenotype T cells, however GITR is required for their persistence in the bone marrow. Therefore, elevated expression of GITR on CD8 memory T cells in the bone marrow marks a population of memory T cells which have recently received an IL-15 signal. Furthermore, GITR expression is needed for the maintenance of CD8 memory phenotype T cell populations in the bone marrow.

In Chapter 4 I show my earliest work on the role of CD30 in the response to influenza virus. With the use of CD30^{-/-} mice I examine both the primary and secondary antigen-specific CD8 T cell response to the virus at multiple time points. At all times, however, CD30 is

dispensable for this response. In addition, I show that CD30 does not play a role for the generation of anti-influenza antibody responses. Interestingly, when I switched and infected with a much more severe and potentially lethal strain of influenza, A/PR8, CD30 did confer some protection from death. This project is being completed by another graduate student in our laboratory, Michael Wortzman, who is investigating the nature of CD30's protective role.

In Chapter 5, I summarize my findings and compare and contrast the role of GITR, CD30 and 4-1BB on CD8 T cells in the influenza model. In addition, I examine the signaling of the 3 molecules, and compare the role of TRAF1 downstream of each receptor. Furthermore, I discuss the new found role of GITR in the persistence of CD8 memory T cells, and how this parallels to other TNFR family members in maintaining T cell memory in the absence of antigen. Finally, I discuss my data on CD30, the questions it raises, and suggest future directions for this project.
Chapter 2

CD8 T cell intrinsic GITR is required for T cell clonal expansion and mouse survival following severe influenza infection

The results in this chapter (Figures 2.1-2.10) were published in the Journal of Immunology:

<u>Snell, L. M.</u>, A. J. McPherson, G. H. Lin, S. Sakaguchi, P. P. Pandolfi, C. Riccardi, and T. H. Watts. CD8 T cell-intrinsic GITR is required for T cell clonal expansion and mouse survival following severe influenza infection. *J Immunol* 185:7223-7234.

Figure 2.9b is unpublished data.

A. McPherson and G. Lin contributed to this study by assisting with several experiments and gave technical advice.

S. Sakaguchi contributed to this study by allowing the use of the DTA-1 antibody.

P. Pandolfi and C. Riccardi contributed to this study by providing the GITR^{-/-} mice.

2.1 Abstract

The regulation of T cell expansion by TNFR family members plays an important role in determining the magnitude of the immune response to pathogens. As several members of the TNFR family, including GITR, are found on both regulatory and effector T cells there is much interest in understanding how their effects on these opposing arms of the immune system affect disease outcome. While much work has focused on the role of GITR on regulatory T cells, little is known about its intrinsic role on effector T cells in an infectious disease context. Here we demonstrate that GITR signaling on CD8 T cells leads to TRAF2/5 dependent, TRAF1 independent NF-kB induction resulting in increased Bcl-xL. In vivo, GITR on CD8 T cells has a profound effect on CD8 T cell expansion, via effects on T cell survival. Moreover, GITR is required on CD8 T cells for enhancement of influenza-specific CD8 T cell expansion upon administration of agonistic anti-GITR antibody, DTA-1. Remarkably, CD8 T cell intrinsic GITR is essential for mouse survival during severe but dispensable during mild respiratory influenza infection. These studies highlight the importance of GITR as a CD8 T cell costimulator during acute viral infection and argue that despite the similarity among several TNFR family members in inducing T lymphoctye survival, they clearly have non-redundant functions in protection from severe infection.

2.2 Introduction

Glucocorticoid induced tumor necrosis factor receptor associated protein (GITR) is a member of the tumor necrosis factor receptor (TNFR) superfamily that modulates immune responses. Perhaps most extensively studied for its high basal expression on CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs) (58, 59), GITR is also expressed on many other immune cell types, including CD4 and CD8 T cells, natural killer (NK) cells, B cells and macrophages (60). The ligand for GITR, GITRL, can be expressed on dendritic cells, macrophages, B cells, endothelial cells, and activated T cells (60).

The role of GITR in immune responses is complex and may be cell-type specific. Stimulation of macrophages with an anti-GITR antibody induces a proinflammatory response (304), however recent reports have shown that GITR can play an inhibitory role for human NK cell activation (81, 82). GITR has also been extensively studied in the context of Tregs and generated much excitement when it was observed that agonistic anti-GITR antibody in co-cultures of CD4⁺CD25⁻ effector T cells and CD4⁺CD25⁺ Tregs caused the abrogation of suppression by Tregs (58, 59). Interestingly, it was shown that GITR signaling on the effector T cells was necessary to render the cells less refractory to Treg-mediated suppression (62). A recent report has shown that this phenomenon is not unique to CD4 T cells, and GITR signaling can induce resistance to CD4⁺CD25⁺ Treg suppression in CD8 T cells as well (305).

In addition to enhancing T cell responses by raising the threshold for Treg suppression, GITR has also been shown to costimulate CD4 and CD8 T cells *in vitro* by enhancing proliferation, survival and cytokine secretion (61, 70). GITR acts early in the immune response to lower the threshold for CD28 signaling in both CD4 and CD8 T cells (66). Whereas GITR's

costimulatory role for T cells is well documented *in vitro*, much remains to be elucidated as to its function *in vivo*, particularly on CD8 T cells. Notably lacking from the literature is any thorough assessment of GITR's physiological role on CD8 T cells in an infectious disease context. Although a few studies have shown that agonistic anti-GITR antibody can enhance anti-viral CD8 T cell responses *in vivo* (86, 87, 306), the mechanism whereby this occurs remains to be determined, as GITR is broadly expressed and its signaling on other immune cells may influence the CD8 T cell response indirectly. Clearly, more defined models are needed to study the intrinsic role of GITR on CD8 T cells in response to pathogens *in vivo*.

In this study, we sought to address the role of GITR on CD8 T cells during viral infection. By using an adoptive transfer model in which GITR is only absent on the responding CD8 T cells, we show that GITR is intrinsically required on CD8 T cells for maximal primary and secondary CD8 T cell responses to influenza. Moreover, we demonstrate that GITR-specific agonistic antibodies enhance the response to influenza virus through direct effects of GITR on CD8 T cells. Although GITR does not influence cell division, it augments CD8 T cell survival by upregulating the pro-survival molecule $Bcl-x_L$ in an NF- κ B dependent manner. We found that GITR-induced NF- κ B activation is positively regulated by both TNF receptor associated factor (TRAF) 2 and 5, whereas TRAF1 is dispensable for this survival signaling. Although the absence of GITR on T cells did not affect disease outcome during mild influenza virus infection, during a severe and potentially lethal model of influenza infection we show that GITR on CD8 T cells augmented viral clearance and protected mice from death. These results highlight the importance of GITR as a CD8 T cell intrinsic costimulatory receptor.

2.3 Materials and Methods

Mice. C57BL/6 mice were obtained from Charles River Laboratories. CD45.1 and Thy1.1 congenic mice were crossed with OT-I mice (Jackson Laboratories). GITR^{-/-} mice have been previously described (67). GITR^{-/-} mice were backcrossed on to the C57BL/6 background for at least 8 generations and then further crossed to generate CD45.1 GITR^{-/-} OT-I mice. TRAF1^{-/-} mice (Tsitsikov et al. 2001) were originally provided by E. Tsitisikov (Center for Blood Research, Boston, MS), and crossed to OT-I TCR transgenic mice, as previously reported (34). Mice were maintained under specific pathogen-free conditions in sterile microisolator cages. Animal studies were approved by the University of Toronto animal care committee in accordance with the regulations of the Canadian Council on Animal Care.

Influenza Virus Infection. Six to ten week old C57BL/6 and GITR^{-/-} mice were infected i.n. with 5 hemagglutinin units (HAU) of influenza A/HKx31 (H3N2). Thirty days after infection, some mice were challenged with 5 HAU of influenza A/PR8 (H1N1). For survival experiments using influenza A/PR8, mice were infected i.n. with 10⁵ TCID50/mouse. For immunization after adoptive transfer of WT/GITR^{-/-} OT-I T cells, mice were infected with 5 HAU or 6118 TCID50/mouse of A/HKx31-OVA (X31-OVA) or 10⁶ TCID50/mouse of A/PR8-OVA (PR8-OVA) (307) both generously provided by P. Doherty and P. Thomas (St. Jude Children's Research Hospital, Memphis, TN). After transfer of OT-I or TRAF1^{-/-} OT-I cells, mice were infected with 64 HAU/mouse of influenza A/WSN-OVA. At the indicated times after X31-OVA infection, spleen, MLN, bone marrow and lungs were harvested. Lungs were perfused with PBS, and lymphocytes were enriched by isolation over an 80/40% Percoll gradient. Single-cell suspensions were prepared from all organs and were subjected to flow cytometry staining.

Following PR8-OVA or PR8 infection, mice were sacrificed when they had lost 30% body weight or were moribund.

Flow Cytometry. MHC class I tetramers were obtained from the National Institute for Allergy and Infectious Diseases tetramer facility (Emory University, Atlanta, GA). Influenza NP₃₆₆₋₇₄specific CD8 T cells were surface stained with anti-CD8a, anti-CD62L (eBioscience) and D^{b}/NP_{366-74} tetramers. For intracellular IFN- γ staining, splenocytes were restimulated with 1 μ M of NP₃₆₆₋₇₄ for 6 hours with Golgi Stop (BD Biosciences) at 37⁰C. Cells were surfaced stained as above, fixed and intracellularly stained for IFN-y (BD Biosciences). Adoptively transferred OT-I cells were detected using anti-Thy1.1 and anti-CD45.1 (eBioscience) in conjunction with anti-CD8 α . For intracellular IFN- γ staining, splenocytes and lymphocytes isolated from lung were restimulated at 37^oC with 1 µM OVA₂₅₇₋₆₄ using Golgi Stop for 6 and 4 hours respectively. Cells were then surface stained for CD8, CD45.1 and/or Thy1.1, fixed and intracellularly stained for IFN-y (BD Biosciences). For detection of degranulation 5 µg/mL of anti-CD107a (BD Biosciences) was added at the beginning of the restimulation culture. Fluorescent minus one controls or unstimulated samples (no peptide) were used as negative controls. Certain experiments also required staining with anti-GITR, anti-CD69, anti-CD44, anti-CD4 and anti-CD25 (eBioscience). Foxp3 staining was performed using a mouse regulatory T cell staining kit (eBioscience). Staining for apoptosis was carried out by adding annexin V (BD Biosciences) to surface stained cells. Samples were analyzed using a FACScalibur (BD Biosciences) and FlowJo (TreeStar Inc.) software.

T cell isolation, adoptive transfers and agonistic antibody studies. WT and GITR^{-/-} OT-I T cells were purified from lymph nodes and spleens of naïve mice using a negative selection mouse CD8 T cell enrichment kit (Stem Cell Technologies). For memory experiments, memory-

like WT and GITR^{-/-} OT-I cells were generated as previously described (308). In certain experiments T cells were stained with 1 μ M CFSE for 10 minutes at 37^oC. T cells were injected intravenously at 10⁴ cells/mouse (for ratio experiments 5x10³ of each cell type/mouse) with the exception of CFSE experiments which used 10⁶ cells/mouse and PR8-OVA experiments which used 10³ cells/mouse. A day later the mice were infected with X31-OVA or PR8-OVA as described above. For experiments using agonistic antibodies, X31-OVA or WSN-OVA was administered i.p. at a dose of 100 HAU/mouse or 64 HAU/mouse respectively with either 200 µg of anti-GITR (DTA-1) hybridoma (58) or purified rat IgG (Sigma-Aldrich).

Cell Viability Assay. WT and GITR^{-/-} OT-I CD8 T cells were isolated from naïve mice and cultured *in vitro* with irradiated splenocytes from C57BL/6 mice at a ratio of 1:4. Cultures were stimulated with a suboptimal dose of SIINFEKL (10⁻¹⁰ M) and in some cases T cells were CFSE labeled as described above. CFSE analysis was performed on day 2 and annexin V staining of cultures was performed on days 0, 2 and 3.

Signaling studies, siRNA knockdowns. OT-I T cells and in some experiments TRAF1^{-/-} OT-I cells were stimulated *in vitro* at 0.1 µg/mL SIINFEKL. Cells were subjected to lympholyte (Cedarlane) on day 3 of the culture, rested and then stimulated with either 10 µg/mL of DTA-1 or Rat IgG. In some experiments cells were preincubated with either 20 µg/mL of NF- κ B inhibitor 1-Pyrrolidinecarbodithioic Acid, Ammonium Salt (PDTC) (Calbiochem) or vehicle for 1 hour prior to DTA-1 or Rat IgG stimulation. For siRNA knockdown experiments, cells were cultured with 20 ng/mL of IL-15 (R&D Systems) for 72 hours after lympholyting. 9x10⁶ OT-I cells were then transfected with 1 µM siRNA targeting TRAF2, TRAF5, both TRAF2 and TRAF5 or a control scrambled duplex RNA (IDT Technologies). Transfections were performed

using the Amaxa mouse T cell Nucleofector kit (Lonza). Cells were rested for 20 hours and then stimulated with 10 µg/mL of either DTA-1 or control Rat IgG.

Western blots. Cells were lysed in 1% NP40 with complete protease inhibitor mix (Roche). Lysates were quantified using a BCA protein assay kit (Pierce Protein). Lysates were subjected to SDS/PAGE and then transferred to PVDF membranes. Membranes were probed with antibodies specific for I κ B α , Bcl- x_L , TRAF2 (Cell Signaling Technology), TRAF5, (Santa Cruz Biotechnology) or β -actin (Sigma Aldrich) and incubated with horseradish peroxidase-conjugated anti-rabbit or anti-goat IgG (Sigma Aldrich). Signals were revealed by chemiluminescence (GE Healthcare) and visualized by autoradiography. Where indicated, quantitation was performed using Quantity One software (Bio-Rad).

Viral Clearance. Lungs were excised from mice at various time points post X31-OVA and PR8-OVA infection and then homogenized in RPMI medium (1 g of lung tissue/10 mL). Supernatant was obtained and stored at -70° C. Viral loads were determined by the MDCK assay with the Reed and Muench technique as previously described (309).

Statistical Analysis. Where indicated, p values were obtained using the Student's t-test, unpaired, or paired for ratio experiments (two tailed, 95% confidence interval). The log rank test was used to determine significance for mouse survival experiments. Statistically significant differences are indicated as *, p < 0.05, **, p < 0.01 or ***, p < 0.001.

2.4 Results

GITR on the T cells is essential for maximal CD8 T cell responses to influenza virus

To examine the role of GITR on CD8 T cells during viral infection *in vivo*, we intranasally (i.n.) infected GITR^{-/-} and wildtype (WT) C57BL/6 mice with Influenza A/HKx31 (H3N2) virus. Some mice were challenged with the serologically distinct Influenza A/PR8 (H1N1) 30 days after the initial X31 infection to examine the secondary response (Figure 2.1A). Antigen-specific CD8 T cell responses to the immunodominant NP₃₆₆₋₇₄ epitope were assessed using fluorescently labeled H-2D^b/NP₃₆₆₋₇₄ tetramers. Decreased influenza-specific CD8 T cell numbers were observed in GITR^{-/-} mice in a subset of organs, but only at particular times of the immune response (Figure 2.1B-2.1D). As GITR can be expressed on many different cell types during the course of an infection (60), it was possible that the lack of GITR signaling on other cell types in the local environments, such as regulatory T cells, was indirectly affecting the CD8 T cell response.

Therefore, in order to dissect the CD8 T cell intrinsic effects of GITR during viral infection *in vivo*, we crossed GITR^{-/-} mice with CD45.1 OT-I mice, whose CD8 T cells have transgenic T cell receptors (TCR) specific for the H-2K^b/OVA₂₅₇₋₆₄ epitope. GITR^{-/-} mice had been backcrossed at least 8 times before a further backcross to generate the GITR^{-/-} OT-I transgenic mice. Purified CD8 T cells from CD45.1 GITR^{-/-} OT-I and Thy1.1 WT OT-I mice were mixed at a ratio of 1:1 and adoptively transferred into WT CD45.2 recipients (Figure 2.2A and 2.2B). A day later, mice were infected with Influenza A/HKx31-OVA (X31-OVA), which carries the OVA₂₅₇₋₆₄ epitope in its neuraminidase stalk (307). The ratio of WT to GITR^{-/-} OT-I T cells at the peak of the response was greater than one in all organs assayed, indicating significant



Figure 2.1. CD8 T cell responses to influenza virus in GITR-deficient mice. A. WT and GITR^{-/-} mice were infected i.n. with Influenza A/HKx31 and sacrificed at day 10. 30 days after X31 infection, some mice were challenged with 5 HAU of influenza A/PR8 and sacrificed 8 days later to assess the secondary response. B. Representative FACS plot for data shown in C and D. Plots are gated on live CD8 T cells. C&D. Total number of D^b/NP₃₆₆₋₇₄ CD8 T cells at day 10 post X31 infection (C) and day 8 post PR8 infection (D). Data for each organ were pooled from 2 independent experiments with 5-6 mice per group.

defects in the recovery of GITR^{-/-} OT-I T cells in spleen, lung, mediastinal lymph node (MLN), and bone marrow (Figure. 2.2C and 2.2D). Differences ranged from two to four-fold depending on the particular organ. A similar defect in GITR^{-/-} T cell recovery was observed at days 7, 9 and 12 post-infection (Figure. 2.2E). Thus, the defect in recovery of GITR-deficient CD8 T cells could not be attributed to a difference in the kinetics of expansion between WT and GITR^{-/-} OT-I T cells. Therefore, these results demonstrate that GITR is required on the CD8 T cells for their maximal expansion during the primary response to influenza virus.

Competitive models can sometimes exacerbate differences between two cell populations, as each population is forced to compete for the same pool of resources. As the previous experiments were performed in a competitive fashion, we also investigated whether GITR^{-/-} OT-I cells would show a similar impairment when they did not have to compete with OT-I cells for survival factors. When CD45.1 OT-I or CD45.1 GITR^{-/-} OT-I T cells were injected into separate mice and infected with X31-OVA, similar defects were observed, although slightly delayed, particularly in the lung (Figure 2.3A-2.3D).

Although the GITR^{-/-} OT-I mice had been extensively backcrossed (n=9), to rule out any differences in cell recovery due to rejection, we injected a 1:1 mix of in vitro expanded (see methods and below) memory-like WT and GITR^{-/-} OT-I T cells (3 million cells total) into mice and monitored their persistence without infection. At day 21 post injection both cell types could still be detected and the GITR^{-/-} T cell numbers were similar and if anything slightly higher in number than their WT counterparts (data not shown), indicating that the defects observed at day 7-12 of the primary response could not be attributed to rejection of the knockout cells.



Figure 2.2. Analysis of CD8 T cell intrinsic effects of GITR during influenza infection. A. 5X10³ each of Thy1.1 OT-I and CD45.1 GITR^{-/-} OT-I CD8 T cells were injected as a 1:1 mixture into WT mice. A day later the mice were infected i.n. with X31-OVA. On days 7, 9 and 12 the mice were sacrificed and the ratio of WT:GITR^{-/-} OT-I T cells was analyzed. Data are representative of 2 independent experiments, each using 4-5 mice. B. Representative FACS plot of original 1:1 Thy1.1 OT-I:CD45.1 GITR^{-/-} OT-I ratio upon injection. Plot gated on CD8 T cells. C. Ratio of WT:GITR^{-/-} OT-I T cells in various organs at day 9. D. Representative FACS plots of Thy1.1 and CD45.1 gating for ratio determination. E. Kinetics of WT and GITR^{-/-} OT-I primary response. Graphs show % of transferred cells of total CD8 T cells at day 7, 9, and 12. At each time point 5 mice were used.





Figure 2.3 Role of CD8 T cell intrinsic effects of GITR in a non-competitive influenza model. A. CD45.1 OT-I or CD45.1 GITR^{-/-} OT-I T cells were transferred into WT mice. A day later the mice were infected i.n. with X31-OVA. **B, C & D.** # CD45.1 CD8 T cells in spleen, mediastinal lymph node (MLN) and lung at days 7, 10 and 12. Each data point represents an individual mouse.

GITR regulates CD8 T cell numbers but not effector function

Multiple costimulatory molecules in the TNFR superfamily have been implicated in enhancing not only the magnitude of the T cell response, but also its quality (5, 310). In order to assess whether GITR was also regulating effector function of the responding CD8 T cells, we examined IFN- γ production and the expression of CD107a, a marker of degranulation, on WT and GITR^{-/-} OT-I cells at the peak of the primary response (Figure 2.4A & 2.4B). Despite the fact that there were fewer GITR^{-/-} OT-I T cells overall, the proportion of GITR^{-/-} cells that was producing IFN- γ and expressing CD107a was similar to the proportion of WT OT-I cells showing this phenotype. Thus, whereas GITR is critical for maximal CD8 T cell expansion in the primary response to influenza, it does not influence effector function per T cell.

GITR on the T cells is essential for their secondary expansion in response to influenza virus

Under some circumstances, memory T cells are less dependent on costimulation than their naïve counterparts (311). Therefore, we next assessed whether GITR would play a role in the recall CD8 T cell response to influenza virus. As there were already significant defects in CD8 T cell numbers after the primary response, to avoid the complications of these defects and focus on the effect of GITR during the secondary response, we generated memory-like cells *in vitro*, by culturing WT and GITR^{-/-} OT-I splenocytes with antigen followed by IL-15 treatment (308). At the end of the cultures, the WT and GITR^{-/-} OT-I cells showed a similar memory phenotype: CD44^{hi}, CD69^{lo}, with GITR expression detected on the WT cells (Figure 2.5B). A 1:1 mixture of Thy1.1 OT-I and CD45.1 GITR^{-/-} OT-I memory-like cells was adoptively transferred into WT recipient mice and a day later the mice were challenged with X31-OVA



Figure 2.4 The role of GITR on CD8 T cell effector function during influenza infection. $5X10^3$ each of Thy1.1 OT-I and CD45.1 GITR^{-/-} OT-I CD8 T cells were injected as a 1:1 mixture into WT mice. A day later the mice were infected i.n. with X31-OVA as in Figure 4.1. A & B. % of IFN- γ positive (A) and % of CD107a positive (B) cells of transferred Thy1.1 OT-I or CD45.1 GITR^{-/-} OT-I T cells at day 9 post-infection. Representative FACS plot of data in F and G are shown on the left. Plots are gated on CD8 T cells and then either Thy1.1 or CD45.1 cells. No ag refers to no antigen (peptide) controls.



Figure 2.5 Role of CD8 T cell intrinsic effects of GITR in recall responses of OT-I memory T cells *in vivo.* **A.** Thy1.1 OT-I and CD45.1 GITR^{-/-} OT-I memory-like T cells were generated *in vitro* and were injected as a 1:1 mixture into WT mice as in Figure 1. A day later the mice were infected i.n. with X31-OVA. On days 6, 8, and 10 the mice were sacrificed and the ratio of WT:GITR^{-/-} OT-I T cells was analyzed. Data are representative of 2 independent experiments, each using 4-5 mice. **B**. GITR, CD69 and CD44 expression on naive, activated and memory T cells was analyzed at days 0, 2 and 9 respectively of *in vitro* memory cell culture. **C**. Ratio of WT:GITR^{-/-} OT-I memory-like T cells in various organs at day 8. **D**. Kinetics of WT and GITR^{-/-} OT-I secondary response. Graphs show % of transferred cells of total CD8 T cells at day 6, 8 and 10 in various organs. At each time point 4 mice were used. **E**. % of IFN-γ positive cells of transferred Thy1.1 OT-I or CD45.1 GITR^{-/-} OT-I T cells in spleen and lung. **F** % of CD107a positive cells of transferred Thy1.1 OT-I or CD45.1 GITR^{-/-} OT-I T cells in spleen.

(Fig. 2.5A). At several time points throughout the response, the number of WT OT-I cells consistently outnumbered the number of $\text{GITR}^{-/-}$ OT-I cells in all organs examined (Figure 2.5C and 2.5D). As in the primary response, the proportion of WT and $\text{GITR}^{-/-}$ cells producing IFN- γ and expressing CD107a after *ex vivo* stimulation was approximately equal (Figure 2.5E and 2.5F). GITR, therefore, is important for the maximal expansion of CD8 T cells in both the primary and recall response to influenza virus, and this effect is intrinsic to the CD8 cells.

Agonistic anti-GITR antibody enhances effector T cell expansion through direct effects on the CD8 T cells

The agonistic anti-GITR antibody DTA-1 enhances effector T cell responses *in vivo* (60). DTA-1 was originally thought to mediate its effects by directly inhibiting Treg suppression (58, 59), however this has recently come under question (62, 65), as GITR can also costimulate T cells and make them refractory to Treg suppression (62, 70). To test the CD8 T cell intrinsic role of GITR in response to the anti-GITR agonist, we compared the response of WT and GITR^{-/-} OT-I T cells to X31-OVA in the presence of DTA-1 or control Rat IgG antibody (Figure 2.6A). For this experiment, we used the i.p. route of infection, which results in minimal virus replication. At the dose of virus chosen, with this route of infection, the response of the OT-I T cells to the virus is very weak and almost completely dependent on inclusion of the DTA-1 agonist, thereby focusing the analysis on the DTA-1-dependent effects. At the peak of the primary response there was a 3-fold increase in the proportion of OT-I T cells recovered after the administration of DTA-1 as compared to Rat IgG (Figure 2.6B). Strikingly, this effect was lost if the T cells lacked



Figure 2.6. GITR is required on the CD8 T cells for enhancement of CD8 T cell expansion by anti-GITR *in vivo.* **A.** 10⁴ CD45.1 OT-I or CD45.1 GITR^{-/-} OT-I T cells were transferred into WT mice. A day later the mice were infected i.p. with 100 HAU/mouse of X31-OVA and 200 µg/mouse of DTA-1 or Rat IgG. Mice were sacrificed at day 7. Data were pooled from 2 independent experiments. **B**. % (middle panel) and # (right column) of CD45.1 cells of total CD8 T cells in spleen. Representative FACS plot of data in B is shown on the left. Plots are gated on CD8 T cells. **C.** % (middle column) and # (right column) of CD44^{hi} cells of CD4 T cells in spleen. Representative FACS plot of data in C is shown on the left. Plots are gated on CD4 T cells. **D**. % (middle column) and # (right column) of Foxp3⁺CD25⁺ of CD4 T cells in spleen. Representative FACS plot of data in D is shown at left. Plots are gated on CD4 T cells.

GITR (Fig. 2.6B). Thus, DTA-1 enhances CD8 T cell expansion largely through direct effects on the CD8 T cells. Although our model did not allow us to specifically examine CD4 T cell intrinsic effects of DTA-1, we did observe a modest enhancement in the number of activated CD4 T cells, defined as having a CD44^{hi} phenotype, in the mice that had been administered DTA-1 versus Rat IgG (Figure 2.6C). In addition, it has been reported that DTA-1 can also costimulate Tregs, causing them to proliferate (59, 65, 68, 70). We surveyed the number of CD4⁺CD25⁺Foxp3⁺ cells in the mice and found that there was a trend toward slightly higher numbers of Tregs with DTA-1 treatment (Figure 2.6D). Whereas DTA-1 can costimulate both CD4 effectors and Tregs in our model, the increase in CD8 T cell expansion observed with DTA-1 treatment was found to be almost exclusively through CD8 T cell intrinsic effects.

Role of GITR in the survival of CD8 T cells responding to influenza infection

Having demonstrated that GITR is essential for maximal CD8 T cell responses to influenza infection, we next sought to dissect the mechanism by which GITR was regulating cell recovery. The defect in numbers of GITR^{-/-} OT-I cells in both the primary and recall responses of the i.n. influenza model could be attributable to differences in homing or a defect in either proliferation or survival. To study homing, the seeding of the cells was examined prior to influenza infection, 24 hours after a 1:1 mixture of WT:GITR^{-/-} OT-I cells was injected into mice. The ratio in all organs examined was close to 1 (data not shown), implying that the cells homed in an equal manner, at least before they encountered antigen. Moreover, the finding that defects in cell recovery of GITR^{-/-} CD8 T cells were observed in all organs examined, and that

kinetic studies in the lung showed equal numbers of both WT and GITR^{-/-} OT-I T cells early, with defects accumulating only later, argues against a trafficking defect.

To investigate whether the defect in recovery of GITR^{-/-} OT-I T cells was due to a proliferative difference, WT mice were injected with CFSE labeled WT or GITR^{-/-} OT-I cells and infected with X31-OVA. We observed that both the number of divisions and the proportion of WT and GITR^{-/-} OT-I cells that had undergone cell division were very similar (Figure 2.7A). These results suggest that GITR is dispensable for antigen-dependent CD8 T cell division *in vivo*.

It is difficult to examine cell death in vivo because apoptotic cells are cleared very quickly by macrophages (312). Therefore, to test whether GITR^{-/-} OT-I cells had a survival defect, we moved to an *in vitro* model. Equal numbers of either WT or GITR^{-/-} OT-I CD8 T cells were cultured with irradiated B6 splenocytes and a suboptimal dose of peptide (10⁻¹⁰ M). At the beginning of the culture, the percentage of annexin V and propidium iodide (PI) positive WT and GITR^{-/-} OT-I cells was approximately equal (data not shown). By day 2, the cells had begun to proliferate and CFSE labeling indicated that approximately equal numbers of WT and GITR^{-/-} OT-I cells had undergone division (Figure 2.7B), consistent with the in vivo data. Whereas the proliferation of the two populations was similar, there was a marked difference in the survival of WT versus GITR^{-/-} OT-I cells. On day 2, a higher proportion of GITR^{-/-} OT-I cells compared to OT-I cells were annexin V positive and primed to undergo apoptosis (Figure 2.7C and 2.7D). This trend became statistically significant by day 3, as the GITR^{-/-} OT-I cells became increasingly annexin V positive. Interestingly, at a peptide dose which was too low to induce proliferation (10⁻¹² M), both WT and GITR^{-/-} OT-I cells had the same percentage of annexin V positive cells (data not shown). This result implies that the difference in survival in WT and



Figure 2.7. GITR on CD8 T cells is dispensable for proliferation but required for CD8 T cell survival. A. 10⁶ CFSE labeled CD45.1 OT-I or CD45.1 GITR^{-/-} OT-I T cells were transferred into WT mice. A day later mice were infected with X31-OVA and 3 days after infection were sacrificed and CFSE analyzed via flow cytometry. Shown are representative FACS plots of CD45.1 gating and CFSE staining of transferred cells. Four to five mice were used per group in 3 independent experiments. **B, C & D**. Purified CFSE labeled WT and GITR^{-/-} OT-I T cells were cultured with irradiated splenocytes and 10⁻¹⁰ M SIINFEKL. **B**. CFSE analysis of WT and GITR^{-/-} OT-I cells at day 2 of culture. **C.** Annexin V analysis on days 2 and 3. Plots are gated on CD8 CD45.1 T cells. **D.** % of Annexin V positive cells of CD45.1 cells on days 2 and 3. Data in all panels are representative of 2-4 independent experiments.

GITR^{-/-} cells is TCR signal dependent and not due to some intrinsic homeostatic difference between the WT and GITR^{-/-} OT-I cells. Based on these data we infer that GITR signaling on CD8 T cells is required for their *in vivo* survival.

GITR enables survival signaling via enhanced Bcl-x_L expression downstream of NF-κB

Activation of the NF- κ B signaling pathway is essential for cell survival. It was previously reported that GITR can initiate NF- κ B signaling in primary T cells (66, 68, 69). To confirm this in our model, OT-I CD8 T cells were activated with peptide and subsequently stimulated with anti-GITR (DTA-1) or Rat IgG. Activating the cells first and then stimulating with DTA-1 allows GITR to be upregulated above basal levels before its triggering, mimicking the situation that would occur during an antigen-specific immune response *in vivo*. Stimulating activated CD8 T cells with DTA-1 led to NF- κ B activation as observed by I κ B α degradation (Figure 2.8A). In contrast, stimulation with control Rat IgG did not lead to significant I κ B α degradation.

To further dissect the mechanism by which GITR enables survival signaling, we examined the expression of Bcl- x_L , a pro-survival molecule downstream of NF- κ B (313). When activated OT-I cells were stimulated with DTA-1, Bcl- x_L expression was significantly enhanced compared to Rat IgG stimulation, as measured at 3 hours and as late as 20 hours post stimulation (Figure 2.8B). In contrast, when the OT-I cells were preincubated with an NF- κ B inhibitor, PDTC, DTA-1 stimulation could no longer induce Bcl- x_L to levels greater than in the Rat IgG control, as measured at 3 hours post-stimulation (Fig. 2.8C). Similar results were seen at 20 hours (data not shown), however by this time the cells treated with the NF- κ B inhibitor were beginning to die, presumably from the inhibition of this critical pathway. These results suggest



Figure 2.8. GITR induces Bcl-x_L by an NF-κB dependent signaling pathway involving TRAF2 and TRAF5. A. Activated OT-I T cells were stimulated with anti-GITR (DTA-1) or Rat IgG and analyzed for IκBα degradation (A) or Bcl-x_L expression (B). C. Activated OT-I cells were preincubated with 20 mg of NF-κB inhibitor, PDTC, or vehicle for 1 hour. Cells were then stimulated with DTA-1 or Rat IgG and analyzed for Bcl-x_L expression 3 hours post stimulation. D. WT OT-I T cells were activated in vitro, cultured with IL-15 and transfected with siRNA targeting TRAF2 (T2), TRAF5 (T5), both or a scrambled (Scr) control. Representative blots of TRAF2 and TRAF5 siRNA knockdowns (KD). E. siRNA treated cells were stimulated with either DTA-1 or Rat IgG and analyzed for IκBα degradation. Representative IκBα blot at 30 minutes post stimulation. F. Graphs show DTA-1:Rat IgG IκBα ratio at 15 and 30 minutes. Data are pooled from 2 independent experiments.

that GITR regulates CD8 T cell survival, at least in part, by upregulating the expression of the pro-survival molecule $Bcl-x_L$ downstream of NF- κB signaling.

TRAF2 and TRAF5 but not TRAF1 enhance GITR-induced NF-KB survival signaling

Signaling downstream of TNFR family members is known to be mediated by the recruitment of TNFR associated factors (TRAFs) to the activated receptor (314). To further understand how GITR induces survival signaling, we investigated the role of several TRAF family members in mediating activation of NF-kB downstream of GITR. GITR has been shown to bind TRAFs 1, 2, and 3 via yeast-2-hybrid and overexpression studies (55, 71). In addition, TRAF5 has been shown to enhance NF- κ B signaling downstream of GITR (72). The role of TRAF2 in GITR mediated NF-κB signaling remains controversial (55, 71), and TRAF1, which is known to modulate TRAF2 signaling (302, 315-317), has not been well investigated downstream of GITR. We sought, therefore, to determine the role of TRAFs 1, 2 and 5 in the activation of NF-kB downstream of GITR in CD8 T cells. To do this, we made use of siRNA to knockdown TRAF2 and TRAF5, as well as use of TRAF1^{-/-} OT-I mice. OT-I cells were activated in vitro and transfected with siRNA targeting TRAF2, 5, both TRAF2 and 5 or a scrambled siRNA control (Fig. 2.8D). NF-kB activation was diminished in individual TRAF2 and TRAF5 knockdown cells treated with DTA-1, as observed by decreased degradation of IkBa compared to the scrambled control (Fig. 2.8E and 2.8F). In cells where both TRAF2 and 5 were knocked down, an additive effect was observed, with a higher level of intact IkBa persisting compared to the individual TRAF knockdowns (Fig. 2.8F). In contrast, when preactivated TRAF1^{-/-} OT-I T cells were stimulated with DTA-1, the lack of TRAF1 did not significantly affect GITR-induced NF-



Figure 2.9. TRAF1 is dispensable for GITR induced NF- κ B signaling and for the enhancement of CD8 T cell expansion to anti-GITR in vivo. A. In vitro activated WT and TRAF1^{-/-} OT-I cells were stimulated with anti-GITR (DTA-1) or Rat IgG and analyzed for I κ B α degradation. Graph shows DTA-1:Rat IgG I κ B α ratio 5, 15, and 30 min post stimulation. Data are pooled from 3 independent experiments. B. 10⁴ CD45.1 OT-I or CD45.1 TRAF1^{-/-} OT-I T cells were transferred into WT mice. A day later the mice were infected i.p. with 64 HAU/mouse of A/WSN-OVA and 200 µg/mouse of DTA-1 or Rat IgG. Mice were sacrificed at day 7. % of CD45.1 positive cells of CD8 T cells in spleen (left) and bone marrow (right). This experiment was performed once with 4 mice per group.

 κ B signaling (Figure 2.9A). Consistent with a lack of a role for TRAF1 downstream of GITR signaling, TRAF1^{-/-} OT-I cell expansion was similar to that of WT OT-I cells when mice were infected with influenza and treated with DTA-1. (Figure 2.9B) Thus, TRAF1 is dispensable for GITR-induced NF-κB signaling, whereas TRAF2 and TRAF5 act as positive regulators to enhance NF-κB signaling downstream of GITR.

GITR protects against death following severe influenza infection

Although GITR was required for maximal T cell expansion during mild influenza infection (Figure 2.2), it had no impact on mouse survival (data not shown). Therefore, to examine the potential role of GITR on influenza disease outcome, we turned to a more severe respiratory infection model using Influenza A/PR8. Following intranasal infection with Influenza A/PR8 (Figure 2.10A), only 25% of the GITR^{-/-} mice survived the infection under conditions where 62% of the WT mice fully recovered (p<0.05). These results indicate that GITR can protect mice from death in a lethal influenza model.

To further investigate whether GITR's protective function was due to its intrinsic role on CD8 T cells, we transferred WT or GITR-deficient naïve OT-I T cells into mice and then challenged them with X31-OVA or Influenza A/PR8-OVA (PR8-OVA) (307). X31-OVA induces a mild respiratory disease whereby mice lose approximately 5-10% of their body weight, but clear the virus by day 8 and completely recover. Under these circumstances the absence of GITR on the T cells had no effect on initial weight loss or viral clearance and 100% of mice survived (Fig. 2.10B and 2.10C). In contrast, infection with PR8-OVA causes up to 30% weight loss and can be lethal in mice. Previous results from our laboratory had shown that transfer of



Figure 2.10. GITR on CD8 T cells augments viral clearance and protects against mortality during severe respiratory influenza infection. **A.** Mouse survival following influenza A/PR8 infection: WT and GITR^{-/-} mice were infected with Influenza A/PR8. Mice were monitored post-infection and sacrificed when moribund. Data are pooled from 2 independent experiments, each with 6-7 mice per group. **B-F:** One thousand CD45.1 OT-I or CD45.1 GITR^{-/-} OT-I cells were transferred into WT mice. A day later mice were infected with X31-OVA (**B**, **C**) or PR8-OVA (**D,E,F**). Mice were monitored post PR8-OVA infection and sacrificed when moribund. **B, E.** Body weight was monitored daily post infection. Body weights were not read after day 7 for PR8-OVA infected mice as several were moribund and had to be sacrificed. Data in B are representative of 3 independent experiments, each having 5-7 mice per group. **C, F.** Lung homogenates were analyzed for viral load at days 3, 6 post-infection and for X31-OVA on day 3, 6 and 8. At each time point a minimum of 5 mice per group was used.

just 1000 naïve OT-I T cells to mice prior to infection with PR8-OVA can protect WT mice from death, whereas transfer of high numbers of T cells abrogates the protective effect due to immune pathology (287). Using this optimized T cell dose, we observed that 100% of the mice that received 1000 GITR^{-/-} OT-I cells or no transferred cells succumbed to the infection. In contrast, 40% of the mice that received WT OT-I cells recovered and survived (Fig. 2.10D). Thus the absence of GITR on CD8 T cells recapitulates the mouse survival defect seen when GITR is absent on all cells. Although the absence of GITR on the CD8 T cells had no effect on the initial

weight loss (Fig. 2.10E) induced in response to the early innate response to the virus, the mice which received GITR^{-/-} OT-I cells had a 5.6 fold (0.75 log) higher viral load by day 6 (Fig. 2.10F) and were visibly sicker at day 7-8 post infection as compared to those that had received 1000 WT OT-I cells. Thus CD8 T cell intrinsic GITR can protect mice from death during severe respiratory influenza infection but is dispensable during mild respiratory influenza infection.

2.5 Discussion

GITR is emerging as an important molecule for regulating CD8 T cell responses. Moreover, the use of agonistic anti-GITR antibodies to augment immune responses *in vivo* is of interest as a therapeutic tool. In administering GITR agonists in a therapeutic context, it will be important to understand GITR's mechanisms of action and its cellular targets. Until now, the intrinsic role of GITR on CD8 T cells *in vivo* has been unclear. In this study we make use of transgenic GITR^{-/-} OT-I T cells to dissect the intrinsic role of GITR on CD8 T cells during influenza infection. We demonstrate that GITR is required on the CD8 T cells for maximal CD8 T cell expansion. Moreover, GITR on the CD8 T cells is also required for the enhancement of their responses following systemic administration of agonistic anti-GITR antibody. While GITR did not enhance CD8 T cell proliferation, it played a key role in CD8 T cell survival. Under conditions of severe respiratory influenza infection, the presence of GITR on CD8 T cells led to augmented viral clearance and protection of the mice from death.

We first examined the NP₃₆₆₋₇₄ specific CD8 T cell response when GITR^{-/-} or WT C57BL/6 mice were infected with a mild strain of influenza. GITR^{-/-} mice, however, showed defects only in certain organs at certain times. The lack of concordance between the GITR^{-/-} OT-I adoptive transfer model, in which defects were seen in all organs examined, and the complete knockout, which showed only a subset of these defects, is most likely due to indirect effects of the lack of GITR on other cell types. Whereas GITR is present on naïve T cells with increased expression upon infection, GITR can also be expressed on dendritic cells, macrophages, NK, NKT and B cells upon infection. Furthermore, it is well established that GITR is highly expressed on CD4⁺CD25⁺ regulatory T cells and aids in their costimulation. It has been noted

that immune responses are highly dependent on the ratio of effector T cells to regulatory T cells in the local environment. Therefore, since GITR works to costimulate both effector and regulatory T cells, it is possible that the defects seen in one organ and not the others are due in part to differences in the ratios of effector T cells to regulatory T cells in the various organs. In addition, it has been shown that GITR can negatively regulate DC function. DCs from GITR^{-/-} mice have been shown to more effectively stimulate IL-2 and IFN- γ production from CD4 T cells than GITR^{+/+} DCs. (79) It is difficult, therefore, to interpret the role of GITR on CD8 T cells in a model where GITR may be interacting on multiple cell types to influence the CD8 T cell response indirectly.

Using the GITR^{-/-} OT-I adoptive transfer model we showed profound recovery defects of GITR^{-/-} CD8 T cells in both the primary and recall response. Having ruled out rejection, kinetic, proliferative and homing differences between WT and GITR^{-/-} OT-I T cells, we showed that GITR is essential for CD8 T cell survival. Consistent with this we demonstrated that GITR signaling induces NF- κ B, which in turn leads to induction of the prosurvival molecule Bcl- x_L . Riccardi et al. previously showed that GITR is required for CD28 mediated upregulation of Bcl- x_L expression (66). Here we have observed that Bcl- x_L expression is enhanced directly downstream of GITR-mediated NF- κ B signaling, as early as 3 hours after GITR ligation.

It is well established that GITR can activate NF-κB signaling (60). Recently it was shown that several TNFR family members, including GITR, are capable of activating both the canonical and non-canonical NF-κB pathways (36, 47). In the present study we show that the canonical NF-κB pathway is rapidly activated downstream of GITR signaling, requiring both TRAF2 and TRAF5. Our data are consistent with previous studies showing that TRAF5 is essential for CD8 T cell survival (318) and for maximal NF-κB activation downstream of GITR in CD4 T cells (72). There has been controversy, however, as to the role of TRAF2 in activating NF- κ B downstream of GITR signaling. Kwon et al. and Gurney et al. report TRAF2 to be a positive regulator of GITR-dependent NF- κ B signaling (55, 56), in agreement with our data and consistent with studies showing that TRAF2 and TRAF5 play redundant roles downstream of TNF signaling (27). Esparza et al., however, have reported that GITR uses TRAF2 in a novel fashion as an inhibitor of NF- κ B activation (71). All 3 studies overexpressed TRAF2 and performed an NF- κ B luciferase assay 24 hours after GITR stimulation. This assay does not distinguish between activation of the canonical and noncanonical NF- κ B pathways. As TRAF2 has been shown to be positive for activation of the canonical NF- κ B luciferase assay readout would be a balance between these two roles and may depend on the levels of TRAF2 expression. Our study specifically tested the role of TRAF2 and TRAF5 in the canonical pathway of NF- κ B activation downstream of GITR in primary T cells and showed that they have additive and positive effects.

TRAF1 has been shown to bind to GITR in a yeast-2-hybrid assay (71) and in overexpression studies (55), however its role in GITR signaling in primary T cells has not been examined. We observed that TRAF1 was dispensable for the DTA-1 induced expansion of OT-I cells to influenza virus. In addition, TRAF1^{-/-} OT-I CD8 T cells could initiate NF- κ B signaling in a manner similar to WT cells, indicating that TRAF1 is dispensable for GITR induced NF- κ B activation. However, it is possible that TRAF1 has a redundant role in NF- κ B signaling, which in its absence can be compensated for by TRAF2 and TRAF5.

Several studies have shown that the agonistic anti-GITR antibody, DTA-1, enhances both CD8 and CD4 T cell responses to viruses and tumors *in vivo* (60). It is not known, however,

whether this is a direct consequence of GITR ligation on the T cells or whether it occurs indirectly through effects on other cell types. Earlier studies attributed the DTA-1-induced effector T cell expansion to an indirect mechanism whereby the antibody was targeting Tregs and attenuating their function (58, 59). Indeed, it has been shown that DTA-1 can cause the proliferation of Tregs, though whether this enhances or abrogates their suppression remains controversial (60, 65). DTA-1 can also costimulate effector T cells (61, 70), and cause them to become more refractory to Treg suppression (62). A few groups have attempted to use depletion studies to rule out the role of DTA-1 on various cellular subsets, such as Tregs, thereby suggesting a direct role for DTA-1 in T cell costimulation (91, 319). To our knowledge, however, no study has been able to directly address the question of whether GITR is required on CD8 T cells for DTA-1 induced expansion. By using GITR^{-/-} OT-I T cells we show that the CD8 T cells lacking GITR failed to expand in response to DTA-1, with responses similar to those of WT OT-I cells treated with Rat IgG. Therefore, we conclusively show that DTA-1 ligation of GITR on CD8 T cells is essential for maximal CD8 T cell responses to influenza and that in this model DTA-1 ligation on other cell types, such as Tregs, does not play a major role.

It is of interest to note that shortly after our report was published, another study emerged examining DTA-1's target cells in a model of concomitant immunity to a B16 melanoma tumor. Turk's group elegantly reconstituted RAG^{-/-} mice with splenocytes containing CD8 T cells from either WT or GITR^{-/-} mice before inducing tumors and treating with DTA-1. (92) The proportion of mice that cleared the secondary tumor was significantly reduced in those mice that lacked GITR on their CD8 T cells, thereby demonstrating the need for DTA-1 to act directly on the CD8 T cells to induce concomitant immunity. (92) In addition, because DTA-1 has been reported to work on regulatory T cells, the same group also reconstituted RAG^{-/-} mice. Indeed, there was no difference in

concomitant immunity when the Tregs lacked GITR, therefore demonstrating that DTA-1 stimulation directly on CD8 T cells, and not CD4⁺CD25⁺ regulatory T cells, is required to induce the clearance of challenge tumors in this melanoma model (92). This study agreed nicely with our data demonstrating that GITR was required on CD8 T cells for DTA-1 induced expansion to influenza virus.

In apparent contrast to the present study, however, a recent paper demonstrated that transgenic expression of GITRL on B cells expands CD4 T cells but not CD8 T cells in mice (320). This might reflect the failure of CD8 T cells and B cells to efficiently interact *in vivo*.

We demonstrate that GITR is essential for the survival of influenza-specific CD8 T cells, with little effect on proliferation or effector function per cell. In contrast, other studies have shown that administration of agonistic GITR antibodies in viral or cancer models can enhance both the effector function and proliferation of CD8 T cells (60). It is likely that the administration of the anti-GITR antibody gives a supraphysiological signal, which is much stronger than that of the endogenous GITRL in the OT-I model. Therefore, it is possible that the signal from the agonistic antibody would be sufficient to drive enhancement of proliferation and effector function, whereas the endogenous signal is not. It should be noted that in our studies, the addition of agonistic antibodies to *in vitro* cultures with suboptimal peptide did not substantially enhance proliferation (data not shown) and therefore the effects of antibody may depend on the dosage and administration schedule used. Physiologically, however, our study shows that GITR's main role on CD8 T cells *in vivo* is in promoting their survival.

A striking difference was observed in the requirement for GITR on CD8 T cells for disease outcome in mild versus severe influenza infection. Although expansion of CD8 T cells during infection with X31-OVA is impaired when the transferred T cells lack GITR, the mice

still readily handle this infection and fully recover with complete viral clearance that is indistinguishable from mice receiving WT T cells. This is likely because in the milder influenza infection model, the combination of endogenous CD4, CD8 and antibody responses are able to readily control the virus (253). In contrast, in a more severe infection model with PR8-OVA, transfer of 1000 WT T cells protected 40% of the mice from death, whereas 100% of mice that received either no cells or 1000 GITR-deficient T cells succumbed to the infection. This decreased survival of mice that received GITR-deficient T cells as compared to WT T cells was associated with a 6-fold higher viral load at day 6 of the infection. As the only difference between the two groups is the presence of GITR on the transferred T cells, this argues that GITR on the CD8 T cells contributes to viral clearance and mouse survival. It is of interest to note that the mouse survival defect observed in the adoptive transfer model with GITR^{-/-} OT-I cells and PR8-OVA infection, recapitulates the defect seen when GITR^{-/-} mice are infected with unmodified PR8. This suggests that while GITR on other immune cell types may aid in the protection of mice from lethal influenza, GITR on CD8 T cells most likely plays a major protective role.

Our laboratory has previously shown that the TNF family ligand 4-1BBL is also required for survival to severe influenza virus, but dispensable during mild flu infection (287). In that study, however, it was not determined whether this was a T cell intrinsic effect. It has been suggested that the large number of TNF family receptors and ligands may have arisen by gene duplication followed by diversification (321). The finding the GITR and 4-1BB costimulatory pathways are critical for mouse survival during severe influenza infection leads us to speculate that survival against severe viral infections may have contributed to the accumulation of TNF / TNFR costimulators during evolution. On the other hand, there may also be circumstances where such increased responses are pathological (301). In summary, this study extensively dissects the role of GITR on CD8 T cells during viral infection. We show that GITR is essential for maximal primary and secondary CD8 T cell responses to influenza. GITR is required for the survival of CD8 T cells and GITR signaling induces TRAF2 and TRAF5 mediated NF- κ B activation leading to enhanced Bcl- x_L protein expression. Moreover, during severe influenza infection, GITR on CD8 T cells can play a critical role in augmenting viral clearance and decreasing mouse mortality. In addition, we demonstrate that GITR is required on CD8 T cells for agonistic anti-GITR antibody mediated CD8 T cell expansion in response to influenza virus, thus providing a mechanism for the potential immunotherapeutic use of anti-GITR agonistic antibody.

Chapter 3

GITR contributes to the accumulation of memory phenotype CD8 T cells in the bone marrow where local upregulation of GITR marks recent occupants of an IL-15 rich niche

This chapter was submitted to Cutting Edge, Journal of Immunology and was invited for resubmission as a full paper.

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G. Lin contributed Figure 3.1D and Figure 3.5D and assisted with several experiments.

Figure 3.5D is taken from Gloria Lin's thesis 2011.
3.1 Abstract

CD8 memory T cells are enriched in the bone marrow, a site where these cells are thought to receive homeostatic signals. In this report, we demonstrate that the bone marrow contains a population of CD8 memory phenotype T cells with elevated expression of the TNFR family member GITR. In contrast, CD8 memory phenotype T cells in the spleen and LN have GITR levels similar to that on naïve T cells. The bone marrow GITR^{hi} memory T cells have a cytokine-activated phenotype and are almost completely ablated in the absence of IL-15. Moreover, splenic CD8 memory phenotype T cells upregulate GITR in response to IL-15 upon entry into the bone marrow. Whereas GITR does not play a role for CD8 memory T cell homeostatic proliferation to IL-15, GITR contributes to the accumulation of CD8 memory phenotype T cells in the bone marrow. The quantification of the IL-15-dependent GITR^{hi} population in the bone marrow reveals that 30% of the CD8 memory phenotype T cells are recent occupants of the IL-15-rich niche and demonstrates the strong presence of this niche in the bone marrow relative to spleen.

3.2 Introduction

Upon the resolution of an infection, approximately 90 - 95% of effector T cells undergo apoptosis. The remaining antigen-specific T cells are retained as memory cells and provide rapid protection upon re-exposure to the pathogen. The homeostatic maintenance of these memory T cells is independent of antigen, and relies instead on cytokines, most notably IL-7 and IL-15 (169, 170). Within lymphoid organs, it is thought that areas rich in these cytokines characterize niches for memory T cell maintenance. Indeed, it was recently shown that CD4 memory T cells interact with IL-7 expressing stromal cells in the bone marrow to receive survival signals (225). The niche that maintains CD8 memory T cells, however, remains poorly characterized.

The bone marrow contains a reservoir of CD8 memory T cells and is thought to be their preferred location for undergoing homeostatic proliferation (236, 237). IL-15 is required for the homeostatic proliferation and maintenance of CD8 memory T cell populations (187, 188, 216), however the cytokine has been difficult to detect and quantify *in vivo* (322). Although the enrichment of CD8 memory T cells that show evidence of homeostatic division in the bone marrow implies that they have received their cytokine signals in that organ, the site in which IL-15 is provided to memory T cells has not been definitively identified.

Memory phenotype CD8 T cells are defined as cells in unimmunized or naïve mice that express surface markers of antigen exposure, i.e. CD44^{hi} CD122⁺. While the antigen-specificity of these cells is unknown, it is thought that memory phenotype cells can arise from the homeostatic proliferation of naïve T cells driven by encounter with MHC and self peptide (207). Although CD8 memory phenotype cells and antigen-specific CD8 T cells are formed under different conditions, CD8 memory phenotype T cells have served as a good surrogate for antigen-specific CD8 memory T cells in many studies. Similar to antigen-specific responses, memory phenotype cells can induce rapid proliferation upon T cell activation and elicit immune protection (213, 214). The maintenance of both cell types is also dependent on IL-15 and IL-7 (215, 216), although memory phenotype cells are slightly less dependent on IL-7 for T cell survival, as IL-15 can also promote their survival (217).

GITR (glucocorticoid induced TNFR-related protein) is a TNFR family member that is of interest for its role as a T cell costimulatory molecule both on effector and regulatory T cells (61, 70, 323). GITR is expressed on resting naïve T cells and shows increased levels of expression with activation. GITR can also be further upregulated by IL-15 signaling in the absence of antigen in vitro. (324). This led us in the present study to examine whether the expression of GITR on memory CD8 T cells in vivo could also be regulated by cytokines, such as IL-15, and whether this might have a functional impact on their homeostasis.

In this report, we show that GITR upregulation identifies a population of CD8 memory phenotype T cells in the bone marrow that has recently received an IL-15 signal. This population is virtually absent from spleen, but adoptive transfer studies demonstrated that IL-15 could actively upregulate GITR on splenic CD8 memory T cells once they enter the bone marrow. The expression of GITR was found to be dispensable for IL-15 induced homeostatic proliferation of CD8 memory phenotype T cells in vivo, but aids in the maintenance of this population in the bone marrow. Thus, the upregulation of GITR identifies a subpopulation of CD8 memory T cells that has recently occupied an IL-15 rich niche that is enriched in bone marrow relative to spleen.

3.3 Materials and Methods

Mice. C57BL/6 (B6) mice were obtained from Charles River Laboratories (Wilmington, MA). BALB/c, Sv129 and CD45.1 mice were obtained from the Jackson Laboratory (Bar Harbour, ME). IL-15^{-/-} mice were obtained from Taconic (Germantown, NY) and crossed with B6 mice to obtain IL-15^{+/-} mice, and then further crossed to obtain IL-15^{+/+} littermate controls. GITR^{-/-} mice were a generous gift from Carlo Riccardi and Pier Paolo Pandolfi and have been previously described (67). Mice were maintained in sterile microisolator cages under specific pathogen-free conditions. Animal studies were approved by the University of Toronto animal care committee in accordance with the regulations of the Canadian Council on Animal Care.

Tissue Preparation and Influenza Infection. Six to eight week old unimmunized wildtype (WT) B6 mice were sacrificed and spleen, bone marrow and lymph nodes (brachial, axial and inguinal) were harvested. Single cell suspensions were prepared from all organs. Bone marrow was obtained by flushing the femur and tibia bones. Cells were then subjected to flow cytometry analysis. Three month old WT and GITR^{-/-} mice were used for BrdU experiments and experiments examining the accumulation of CD8 memory phenotype T cells. In certain experiments, mice were infected intraperitoneally with 100 HAU/mouse of influenza A/HK/x31. Twenty-one days post-infection the mice were sacrificed and bone marrow was harvested for flow cytometry analysis.

Flow Cytometry. Cells were stained with anti-CD8, anti-CD3, anti-CD44, and anti-GITR (eBioscience, San Diego, CA). In addition, certain experiments also used anti-CD45.1, anti-CD127, anti-CD122, anti-KLRG1, anti-CD69, anti-CD25, anti-CD62L and anti-mouse-4-1BB (clone 3H3) (eBioscience). For intracellular staining of Eomesodermin (EOMES) and Bcl-x_L,

cells were surfaced stained as above and permeabilized using Cytofix/Cytoperm buffer (BD Biosciences, San Jose, CA). Cells were then intracellularly stained with anti-EOMES (eBioscience) or anti-Bcl-x_L (Cell Signaling Technology, Beverly, MA). For Bcl-x_L staining a labeled anti-rabbit (Molecular Probes, Carlsbad, CA) was used as a secondary antibody. The influenza D^b/NP₃₆₆₋₇₄ MHC class I tetramer was obtained from the National Institute for Allergy and Infectious Diseases tetramer facility (Emory University, Atlanta, GA). Samples were run on a FACSCanto II (BD Biosciences) and data analyzed using FlowJo software (Treestar, Ashland, OR).

In vitro bone marrow culture with IL-15. Total bone marrow was isolated from WT or $GITR^{-/-}$ mice as described above. A single cell suspension was generated and in some experiments was labeled with 1µM CFSE (Molecular Probes) for 10 minutes at 37⁰C. Cells were then cultured at 2x10⁶ cells/mL with either 0, 5, 20 or 100 ng/mL of IL-15 (R&D Systems, Minneapolis, MN) for 72 hours. Depending on the particular experiment, the cells were stained with anti-CD8, anti-CD3, anti-CD44 and anti-GITR (eBioscience) at 0, 24, 48 and/or 72 hours of IL-15 treatment.

Cell Sorting and Adoptive Transfer of Memory Cells. For sorting experiments, CD8 T cells were isolated from the splenocytes of CD45.1 mice using a negative selection mouse CD8 T cell enrichment kit (Stem Cell Technologies, Vancouver, BC). The resulting cells were then stained with anti-CD8 and anti-CD44 (eBioscience) and sorted on a FACS Aria (BD Biosciences) for the CD8⁺CD44^{hi} population. 6x10⁵ sorted CD8⁺CD44^{hi} cells were adoptively transferred to either WT or IL-15^{-/-} mice. Spleens and bone marrow were harvested at 5 or 8 days post-injection.

BrdU Staining. WT and GITR^{-/-} mice were fed 0.8 mg/mL of bromodeoxyuridine (BrdU) (Sigma) in their drinking water for 8 consecutive days. Upon sacrifice, single cell suspensions were obtained from spleens and bone marrow and cells were stained according to the manufacturer's protocol using the BrdU Flow Kit (BD Bioscience). Cells were first surface stained with anti-CD8, anti-CD3, anti-CD44, before fixation, permeabilization and then BrdU staining.

Statistical Analysis. Where indicated, p values were obtained using the Student t test, unpaired or paired (two tailed, 95% confidence interval) for experiments where organs were taken from the same mouse. Statistically significant differences are indicated as p < 0.05, p < 0.01 or p < 0.001.

3.4 Results

Elevated expression of GITR on a population of CD8 memory T cells in the bone marrow

There is currently much interest in identifying markers that can characterize subpopulations of memory T cells, as these markers can shed light on the processes that control memory T cell survival and function (147, 325, 326). Although GITR is expressed on all CD8 T cells, the finding that it could be upregulated on CD8 T cells by IL-15 in the absence of antigen in vitro (324) led us to examine GITR expression on T cells from different organs immediately *ex vivo*. CD3⁺CD8⁺CD44^{hi} memory phenotype T cells from the spleen, LN, and bone marrow of unimmunized C57BL/6 mice were analyzed for GITR expression. In the bone marrow we observed two populations of memory phenotype CD8 T cells based on GITR staining: a population that expresses GITR at high levels (GITR^{hi}), and a population that has GITR levels which are indistinguishable from the CD44^{lo} naive CD8 T cells within that organ, (GITR^{basal}) (Figure 3.1A). Remarkably, the GITR^{hi} population is virtually undetectable in the spleen and LN (Figure 3.1A). This population makes up approximately 10% of total CD8 T cells, and 36% of CD8 memory phenotype T cells in the bone marrow, whereas it accounts for less than 1% of total splenic CD8 T cells and less than 3% of CD8 memory phenotype T cells in the spleen (Figure 3.1B) This is further reflected in the difference between the mean fluorescent intensities (MFI) of GITR on CD8 memory phenotype T cells in the different organs (Figure 3.1C). We also examined GITR expression in the bone marrow and spleens of BALB/c and Sv129 mice, and observed a similar pattern as in the C57BL/6 mice, indicating that the GITR^{hi} memory T cells observed in the bone marrow were not strain specific (Figure 3.1D). We conclude,



Figure 3.1. GITR is selectively upregulated on a population of CD8 memory phenotype T cells in the bone marrow. A. Spleen, LN and bone marrow were obtained from naïve C57BL/6 (B6) mice. Representative gating strategy (bone marrow) and FACS staining of GITR expression on CD8 T cells in the various organs. Gates are based on GITR^{-/-} controls. **B**. % of GITR^{hi} of total CD8 T cells or of CD8 CD44^{hi} cells in the bone marrow and the spleen. Representative FACS plots are gated to show the GITR^{hi} population out of the total CD8 T cell population. Data are pooled from 3 independent experiments with 3-5 mice each. **C**. MFI of GITR on CD8⁺CD3⁺CD44^{hi} cells from organs in A. FACS staining (**A & B**) and GITR MFIs (**C**) are representative of at least 3 independent experiments each with 4-5 mice. **D**. Representative FACS plots of GITR expression on CD8 memory T cells in the spleens and bone marrow of B6, Sv129 and BALB/c mice. Plots are gated on GITR^{-/-}controls. **E**. B6 mice were infected with Influenza A/HK/x31 and 21 days later were sacrificed. Representative FACS staining of D^b/NP₃₆₆₋₇₄ specific CD8 T cells (gray) in the bone marrow. Figures are representative of 2 independent experiments each having 2-4 mice.

therefore, that GITR is upregulated on a population of CD8 memory T cells in the bone marrow relative to the naïve CD44^{lo} population and that this population is greatly enriched in the bone marrow compared to the spleen and LN, where it is virtually undetectable.

Because our studies had examined memory phenotype cells from unimmunized mice, the antigen-specificity of these CD44^{hi}GITR^{hi} "natural" memory in the bone marrow was unknown. Since it has been reported that there can be differences in the the generation and regulation of memory phenotype versus antigen-specific memory T cells (327), we questioned whether antigen-specific memory T cells could also express high levels of GITR in the bone marrow. We immunized WT mice with Influenza A/HK/x31, which causes a mild infection that is completely cleared by day 8 (253). On day 21 post-infection we stained with MHC-tetramers specific for the immunodomonant D^b restricted NP₃₆₆₋₇₄ epitope, to detect the antigen-specific CD8 memory T cells. Whereas a subset of the NP-specific CD8 T cells detected were GITR^{hi}, GITR levels on these cells showed a distribution similar to the diverse GITR expression of the total CD44^{hi} population in the mouse (Figure 3.1E). The proportion of GITR^{hi} cells in the tetramer positive population ranged from 23% to 37%. As the antigen-specific CD8 memory T cells in the bone marrow also exhibit the GITR^{hi} population it is likely that the upregulated GITR expression in unimmunized mice also contains functional CD8 memory T cells, perhaps specific for unknown environmental antigens.

GITR^{hi} cells resemble cytokine-activated T cells

To further characterize the GITR^{hi} CD8 memory T cell population in the bone marrow, we examined the expression of several activation markers and cytokine receptors on this GITR^{hi}

population and compared them to the levels of these molecules on the GITR^{basal} CD8 memory T cell population. Upon antigen activation, CD127, the IL-7 receptor α chain normally expressed on naïve T cells, is downregulated, and CD122, the IL-15 receptor β chain, is upregulated to high levels of expression. Interestingly, a greater proportion of CD8 memory T cells express CD122 and have downregulated CD127 in the bone marrow as compared to the spleen (Figure 3.2A). The GITR^{hi} cells had even higher levels of CD122 and slightly lower levels of CD127 than the GITR^{basal} cells in the bone marrow (Figure 3.2B), consistent with a more activated phenotype. In agreement with this, the GITR^{hi} cells had downmodulated CD8 expression and a greater proportion of the GITR^{hi} cells expressed the activation marker CD69 as compared to the GITR^{basal} population (Figure 3.2B). The killer cell lectin like receptor 1 (KLRG1), which is a marker of terminal differentiation on CD8 T cells, was not expressed on either GITR^{hi} or GITR^{basal} cells in the unimmunized mice, and only very low and comparative levels of CD25 (IL-2 receptor α chain) were detected on both populations (Figure 3.2B). It should also be noted that whereas the GITR^{hi} and GITR^{basal} populations consisted of a mixture of both CD62L^{lo} (effector) and CD62L^{hi} (central) CD8 memory T cells, a larger proportion of the GITR^{hi} population was of the central memory phenotype (Figure 3.2B). The above data demonstrate that the GITR^{hi} T cell population represents a more activated subset of CD8 memory T cells than the GITR^{basal} population, whereas the absence of KLRG1, the low levels of CD25 and the presence of CD62L on a substantial proportion of GITR^{hi} CD8 memory T cells, argue against the majority of these cells representing a recently antigen activated /effector population.

It has been demonstrated that the transcription factor EOMES (Eomesodermin) is required for the formation of central memory T cells and is also necessary for cells to compete for the CD8 memory T cell niche (328). We reasoned, therefore, that perhaps EOMES would be more highly expressed in GITR^{hi} cells, which seemed to have proportionally more central



Figure 3.2. GITR^{hi} cells have a phenotype consistent with cytokine activation. A. Unimmunized mice were sacrificed and the the % of CD122 and CD127 positive cells of CD8 CD44^{hi} cells was analyzed in both the bone marrow and spleen. B. Representative FACS plot defining GITR^{hi} (solid line) and GITR^{basal} (dashed line) populations in the bone marrow. Plot is gated on CD8⁺CD3⁺ population. Below are histograms showing expression of CD127, CD122, CD69, CD25, KLRG1, CD62L, EOMES, Bcl-x_L and CD8 on defined GITR^{hi} versus GITR^{basal} gates. FMO controls are in gray. Plots are representative of 2-3 independent experiments, each using 3-4 mice.

memory. There was no difference, however, in the expression of EOMES between the GITR^{hi} and the GITR^{basal} cell population (Figure 3.2B). In addition, we have previously shown that antigen-dependent GITR signalling could upregulate Bcl- x_L (329) However, upon examination of Bcl- x_L expression in both populations we could not detect an appreciable difference in the levels of the pro-survival molecule (Figure 3.2B).

Effect of IL-15 on GITR expression in vitro.

IL-15 can promote an activation phenotype and drive similar gene expression as crosslinking of the TCR in CD8 memory phenotype T cells (330). In addition, CD127 can be downregulated by both IL-7 and IL-15 via a transcriptional mechanism (331) and CD69 expression is upregulated upon IL-15 stimulation (332). Thus, it was possible that the GITR^{hi} CD8 T cells in the bone marrow represent a cytokine-activated memory T cell population. We therefore examined the effect of IL-15 on bone marrow CD8 T cells *ex vivo*. Indeed, we observed that IL-15 can augment GITR expression levels on CD8 T cells from the bone marrow (Figure 3.3A). Moreover, as GITR expression was upregulated, CD8 expression was downmodulated (Figure 3.3C). This pattern was very similar to the downregulation of CD8 expression observed on GITR^{hi} cells in the bone marrow of unimmunized mice (Figure 3.3B). In addition, we noted that in culture with IL-15, GITR was upregulated on CD8 T cells very early, even before the first peak of division. Even CD8 T cells that received a low concentration of IL-15 (5 ng/mL) that induced little or no proliferation had a slight upregulation of GITR on a subset of the undivided cells (Figure 3.3C). These data indicate that the IL-15 threshold for GITR



Figure 3.3 CD8 T cells from the bone marrow upregulate GITR when cultured in vitro with **IL-15. A.** Bone marrow was harvested from WT mice and either cultured *in vitro* with 100 ng/mL of IL-15 or left untreated. At days 0, 1, 2 and 3 CD8 T cells were analyzed for GITR expression. All plots are gated on CD8⁺ cells and gates are based on FMO controls. FACS staining is representative of 3 independent experiments. **B.** Representative FACS staining of CD8 versus GITR expression from bone marrow of unimmunized WT mice *ex vivo*. Plot is representative of 2-3 independent experiments, each using 3-4 mice. **C.** Bone marrow was harvested as in A, CFSE labeled and cultured with 5 ng/mL, 20 ng/mL or 100 ng/mL of IL-15. 72 hours later cultures were harvested and analyzed for CD8, GITR expression and CFSE dilution. Bottom plots are gated on CD8 T cells. Plots are representative of 2 independent experiments.

upregulation on CD8 T cells is lower than the threshold for CD8 T cell proliferation. Thus, GITR is upregulated on CD8 memory T cells by IL-15 prior to their undergoing homeostatic proliferation and as such represents a useful marker of cells that have recently received IL-15 stimulation.

The GITR^{hi} population in the bone marrow is IL-15 dependent.

Based on the above studies, we hypothesized that IL-15 was the most likely candidate for the *in situ* upregulation of GITR on CD8 memory T cells in the bone marrow. Although IL-15^{-/-} mice have decreased numbers of CD8 memory T cells (184, 185), a population of CD8 CD44^{bi} cells remains and can be readily detected in the bone marrow. Strikingly, when IL-15 was absent from the mouse, the remaining CD8 memory T cells completely lacked the GITR^{hi} population (Figure 3.4A). Whereas the MFI of GITR on CD8 memory T cells was significantly higher in the bone marrow versus the spleen of WT mice, there was no appreciable difference between the MFI of GITR on CD8 memory T cells in the bone marrow and spleen of IL-15^{-/-} mice (Figure 3.4A and 3.4B). Moreover, we observed a gene dosage effect of IL-15 on GITR expression on the CD8 memory T cell population, with no effect on the basal expression of GITR on the naïve CD44^{lo} CD8 T cells (Figure 3.4C and 3.4D). From these results we conclude that IL-15 is necessary for the elevated levels of GITR seen on a subset of CD8 memory T cells in the bone marrow.



Figure 3.4. The bone marrow GITR^{hi} population is IL-15-dependent and splenic GITR^{basal} memory cells convert to GITR^{hi} cells upon entry into the BM. A. Representative FACS staining of GITR expression on CD8 T cells from spleen and bone marrow of WT or IL-15^{-/-} mice. All FACS plots are gated on CD8⁺CD3⁺ cells and gates are based on FMO or GITR^{-/-} controls. Histograms represent GITR expression on CD8 CD44^{hi} memory T cells of WT versus IL-15^{-/-} mice. **B**. MFI of GITR staining on CD8 CD44^{hi} memory T cells from organs in A. FACS staining (A) and GITR MFIs (B) are representative of at least 3 independent experiments each with 4-5 mice of each group. C. Representative FACS staining of GITR expression on CD8 T cells from the bone marrow of of IL-15^{+/} +, IL-15^{+/-} or IL-15^{-/-} mice. **D**. GITR MFI of CD8 CD44^{hi} cells from the bone marrow of mice shown in C. Data are representative of 2 independent experiments with 3-5 mice of each group. E. Representative FACS staining of GITR and CD27 expression on CD8 T cells from bone marrow of WT and IL-15^{-/-} mice F. Splenic CD8 or CD8 CD44^{hi} T cells were sorted from CD45.1 mice and injected into either WT or IL-15^{-/-} recipients. G. Recipients were sacrificed 8 days later and the GITR MFI of transferred CD8 memory T cells (and transferred CD8 CD44^{hi} T cells from experiments using total CD8 T cells) recovered from each organ was determined. We conducted two independent experiments with transfer of sorted CD8 T cells into 2-4 mice per group, as indicated in F. In one experiment we used sorted total CD8 T cells, equalized for number of CD44^{hi} cells and in another experiment we sorted the CD8 CD44^{hi} memory population with indistinguishable results; therefore the results shown are pooled from the two experiments.

We further sought to determine if the upregulation of GITR by IL-15 was simply a characteristic of general CD8 T cell activation and whether the expression of other TNFR receptors would also be augmented in response to this cytokine. Like GITR, the TNFR CD27 has basal expression on naïve CD8 T cells and we have previously reported that IL-15 can further upregulate CD27 on CD8 memory T cells *ex vivo* (324). Interestingly, the expression of CD27 was similar on CD8 memory T cells in both WT and IL-15^{-/-} mice (Figure 3.4E). Therefore, the upregulation of GITR by IL-15 is not simply a general characteristic of cytokine activation of the cells, but appears to be a unique mechanism of enhancing GITR expression in vivo.

IL-15 induces GITR expression on CD8 memory T cells on entrance into the bone marrow

The finding that the bone marrow contains an IL-15-dependent GITR^{hi} population of CD8 memory T cells that is virtually absent from the spleen or LN raises the question of how this apparent differential distribution of memory T cell subsets arises. It is possible that the GITR^{hi} and GITR^{basal} CD44^{hi} CD8 memory populations represent two distinct subsets of memory T cells, with the GITR^{hi} population showing preferential localization to the bone marrow. Alternatively the bone marrow may be a site of local upregulation of GITR. To distinguish between these two possibilities, we sorted CD8 CD44^{hi} memory T cells from the spleens of unimmunized CD45.1 mice. These cells, which have only basal expression of GITR, were then adoptively transferred into either WT or IL-15^{-/-} recipient mice (Figure 3.4F). Analysis of the MFI of GITR on the transferred CD8 memory phenotype T cells on day eight following adoptive transfer revealed that GITR expression was significantly higher on the transferred cells harvested from the bone marrow as compared to those taken from the spleen of the same WT mouse

(Figure 3.4G). In IL-15^{-/-} mice, however, there was little difference in the expression of GITR on transferred cells harvested from either organ and GITR levels were similar to the basal expression seen on transferred cells harvested from WT spleens (Figure 3.4G). The same pattern also held true when the mice were sacrificed on day 5 post adoptive transfer (data not shown). Therefore local IL-15 can upregulate GITR expression on CD8 memory T cells when they enter the bone marrow.

GITR is not required for homeostatic proliferation of memory phenotype CD8 T cells

IL-15 can support both the homeostatic proliferation and the survival of memory phenotype CD8 T cells (217). Taken together with IL-15's striking upregulation of GITR on CD8 memory T cells in the bone marrow we sought to examine whether GITR might have a functional role for the maintenance of memory phenotype CD8 T cells. While it is technically difficult to study the role of the upregulation of GITR in the bone marrow because this is a very small and likely transient population, we used GITR^{-/-} cells and mice to query whether the complete lack of the receptor might result in defective homeostatic proliferation or maintenance of CD8 memory phenotype cells. When we stimulated WT and GITR^{-/-} bone marrow cells with various concentrations of IL-15 in vitro, the proliferation of both CD8 T cell types was equivalent (Figure 3.5A). To follow up this observation in vivo, we fed GITR^{-/-} and WT mice bromodeoxyuridine (BrdU) in the drinking water for 8 days to examine basal homeostatic proliferation of CD8 memory phenotype T cells. Indeed, in both spleen and bone marrow the incorporation of BrdU into the DNA of CD8 T cells occurred in those cells which were CD44^{hi} or CD44^{int}, indicating that memory phenotype cells, and not naïve cells were undergoing homeostatic proliferation (Figure 3.5B). Again, the GITR^{-/-} CD8 T cells did not have impaired proliferation and surprisingly seemed to incorporate more BrdU than the WT CD8 T cell controls (Figure 3.5C).

GITR enhances the accumulation of memory phenotype CD8 T cells in the bone marrow

Despite enhanced homeostatic proliferation of GITR^{-/-} T cells in vivo, we sought to examine whether GITR might yet play a role in the maintenance of CD8 memory phenotype cells. GITR^{-/-} and WT mice were aged for approximately 3 months and the proportion of CD8 CD44^{hi} memory phenotype cells in spleen and bone marrow were determined. Interestingly, there was a small but statistically significant defect in the number of CD8 memory phenotype cells in the bone marrow, but not the spleen (Figure 3.5D). Therefore GITR plays a role in the accumulation of memory phenotype CD8 T cells in the bone marrow of unimmunized mice, but does not enhance the homeostatic proliferation of these cells.



Figure 3.5. GITR is dispensable for CD8 memory T cell homeostatic proliferation but contributes to the persistence of CD8 memory phenotype T cells in the bone marrow. A. Bone marrow was harvested from WT and GITR^{-/-} mice, CFSE labelled and cultured with either 20 or 100 ng/mL of IL-15. 72 hours later cultures were harvested and analyzed for CD8 expression and CFSE dilution. Plots are gated on CD8 T cells. B & C. WT and GITR^{-/-} mice were treated with 0.8 mg/mL in their drinking water for 8 days. Mice were sacrificed and cells were stained for BrdU incorporation. **B**. Representative FACS data of BrdU staining in the spleen. Plots are gated on CD8 T cells of CD8 T cells in spleen and bone marrow of WT and GITR^{-/-} mice. **D**. Spleen and bone marrow were harvested from unimmunized 3 month old WT and GITR^{-/-} mice. Graphs show % of CD44^{hi} of CD8 T cells in spleen and bone marrow.

3.5 Discussion

In this report we demonstrate that GITR is highly expressed on a population of CD8 memory T cells in the bone marrow of unimmunized mice. In contrast, CD8 memory T cells in the spleen and LN have GITR levels similar to that on naïve T cells. We go on to show that the GITR^{hi} population is not a unique subset of cells, but rather following entrance into the bone marrow, IL-15 acts locally to upregulate GITR expression on CD8 memory T cells. As IL-15 has been implicated in the maintenance of CD8 memory T cell populations, we sought to determine whether GITR might be required for either homeostatic proliferation or the maintenance of these CD8 memory phenotype cells. We show that GITR^{-/-} mice do not have impaired CD8 memory T cell homeostatic proliferation, but do have a small but significant defect in the proportion of CD8 memory phenotype cells in bone marrow but not the spleen. The finding that GITR is required for the accumulation of memory phenotype T cells in the bone marrow, but not the spleen, but is not required for their IL-15-dependent proliferation, could suggest a role for GITR in the survival of these cells, although an effect of GITR on trafficking to the bone marrow cannot be completely ruled out.

Memory phenotype T cells and defined antigen-specific T cell memory are known to have slightly different requirements for the maintenance of memory (327). While both cell types typically use IL-15 for homeostatic proliferation, antigen-specific cells depend on IL-7 for survival, while memory-phenotype CD8 T cells are less dependent on IL-7, as IL-15 can promote their survival as well (217). Although we focused our study on memory phenotype cells, we also showed that GITR can be upregulated on influenza-specific cells in the bone marrow. Therefore, endogenous CD8 CD44^{hi} memory T cells in unimmunized mice, as well as influenza

NP₃₆₆₋₇₄ specific CD8 CD44^{hi} cells can be divided into a GITR^{hi} and a GITR^{basal} population, with 30% of the endogenous and approximately 30% of antigen-specific CD8 memory T cells falling into the GITR^{hi} group. The observation that similar proportions of both memory phenotype and influenza specific CD8 memory T cells are GITR^{hi} (Figure 1C), suggests that similar mechanisms of GITR regulation occur in both types of CD8 T cell memory. In addition, the finding that only approximately one third of CD8 memory T cells are GITR^{hi} may reflect competition among the cells for cytokines or other resources necessary to induce the upregulation of GITR expression, as well as the transient nature of the IL-15 induced signal. The much lower proportion of GITR^{hi} memory T cell populations in the spleen and LN might, therefore, reflect limited availability or stores of IL-15 in those organs.

One key result of this study was that splenic memory T cells can migrate to and upregulate GITR in the bone marrow in response to IL-15. This finding argues against the possibility that GITR^{hi} and GITR^{lo} cells are different subsets of T cells within the spleen versus bone marrow, but rather suggests that GITR upregulation in the bone marrow reflects the local microenvironment. Indeed, we observed that CD8 memory T cells in the bone marrow appear to have a cytokine-activated phenotype, although the upregulation of GITR to IL-15 seemed to be unique among the TNF receptors tested, and not simply a readout of enhanced activation. Our data correspond nicely to studies from Francesca Di Rosa's group, which indicate that despite the lack of recent antigen exposure, bone marrow CD8 memory T cells have a more activated phenotype than their counterparts in other organs (228, 238). They have also demonstrated that although homeostatic proliferation is augmented in the bone marrow, CD8 memory T cells in this organ do not have an intrinsic ability for enhanced cytokine-mediated proliferation, suggesting that they are stimulated to proliferate locally in the bone marrow (238).

We demonstrated in this study that GITR^{-/-} mice do not have impaired CD8 memory T cell homeostatic proliferation. Consistent with this, our lab has shown that CFSE labelled adoptively transferred WT and GITR^{-/-} memory CD8 T cells do not have proliferative differences 3 weeks post transfer into recipient unimmunized mice (Lin et al., unpublished). Our observations agree with previous reports showing that homeostatic proliferation of CD8 T cells is independent of costimulation by 4-1BB and CD28 (333). Interestingly, although GITR was upregulated on IL-15 stimulated bone marrow CD8 memory T cells just prior to proliferation, the receptor was not required for division. Even at very low doses of IL-15 which elicit little if any T cell proliferation, we observed some GITR upregulation. It has been shown *in vitro* that IL-15 can protect CD8 T cells from apoptosis at doses too low to stimulate proliferation (334). Taken together with the low threshold for IL-15's upregulation of GITR, we questioned whether GITR might be required for the survival of CD8 T cells in vivo.

It has been previously reported that GITR^{-/-} mice have normal peripheral CD8 and CD4 T cell compartments (67). Indeed our own group reported that upon in vitro culture, GITR^{-/-} CD8 T cells did not appear to have basal survival defects in the absence of antigen (329). These studies, however, examined proportions of GITR^{-/-} CD8 T cells only in young mice or at short time points in vitro, and failed to examine the bone marrow (67, 329). In this study, GITR^{-/-} mice used to query the role of GITR on endogenous CD8 memory T cell survival were aged in parallel with WT controls to at least 3 months post-birth, therefore allowing small defects to become more apparent over time.

Interestingly, the role that we observed for GITR in the accumulation and potentially the survival of CD8 memory T cells is quite consistent with our studies of GITR on CD8 T cells in a

viral model of influenza. We have previously reported that GITR does not play a role in influenza-induced CD8 T cell proliferation but rather is important for the survival of these cells, with GITR signalling leading to TRAF2/TRAF5 dependent NF- κ B activation, resulting in the upregulation of the pro-survival molecule Bcl- x_L (323).

It is of interest to compare and contrast the maintenance of both CD8 and CD4 memory T cells in the bone marrow. While the niche for CD8 memory T cells remains to be fully defined, the maintenance of CD4 memory T cell populations has been more fully studied. A recent study showed that CD4 memory T cells in the bone marrow interact with VCAM-1+ expressing stromal cells in a 1:1 ratio (225). The stromal cells produce IL-7, which is essential for CD4 memory T cell proliferation and survival (225). Interestingly, Peter Lane's group has demonstrated that IL-7 upregulates the expression of OX40 on CD4 memory T cells (128). Furthermore, they have shown that the survival of CD4 Th2 memory cells is dependent on the interaction of memory cells with a CD4⁺CD3⁻CD11c⁻ accessory cell that expresses both OX40L and CD30L (110, 128). OX40^{-/-}CD30^{-/-} CD4 memory Th2 cells have severe survival defects, implicating the signalling of these TNF receptors as playing a crucial role in CD4 memory T cell homeostasis (128).

There is accumulating evidence to suggest that IL-15 may play a parallel role for the maintenance of CD8 memory T cell populations, and this may be dependent on TNF receptor family members as well. It is known that IL-15 is crucial for the maintenance of CD8 memory T cells that preferentially homeostatically proliferate and derive survival signals in the bone marrow (170, 236, 237). Recently Averil Ma's group demonstrated that macrophages were necessary for the trans-presentation of IL-15 to maintain effector and central CD8 memory T cell populations, whereas dendritic cell trans-presentation of IL-15 supported primarily the central

memory T cell population (199). In this report, we have shown that IL-15 upregulates GITR on CD8 memory T cells locally in the bone marrow, and that GITR is required for the accumulation of CD8 memory phenotype T cells in the bone marrow. It should be noted that while we have shown that IL-15 causes the upregulation of GITR expression in the bone marrow, IL-15^{-/-} mice had basal levels of GITR. Due to limitations in our model and the difficulty with sorting such small populations, we were unable to assess whether it was the upregulation of GITR in the bone marrow that was important for the survival of the cells, or whether GITR at basal levels may suffice. Therefore, it is possible that the IL-15 upregulation of GITR in the bone marrow is not functionally necessary for the survival of CD8 memory T cells, but may simply be a marker of an IL-15 stimulated cell.

It has been previously reported that CD8 T cells in the bone marrow show evidence of homeostatic proliferation (236, 237). Moreover, IL-15 deficient mice show less of these dividing cells in bone marrow (although there are effects on spleen and LN as well) (187, 188, 216, 335). These studies have been used to infer that the bone marrow is the site of IL-15 dependent homeostatic proliferation, although it cannot be ruled out that the cytokine signal is obtained elsewhere and that the cells then home to the bone marrow to divide. We now take this one step further and provide a marker, elevated expression of GITR, for the identification of activated cells that have recently seen an IL-15 stimulus. The finding that IL-15 can directly upregulate GITR on CD8 memory T cells *ex vivo* under conditions of high cell viability, argues for a direct effect of IL-15 on GITR expression, rather than selective survival of GITR^{hi} cells. Our results show that GITR upregulation marks cells within the bone marrow that have just received their IL-15 signal; these cells may be within the IL-15-rich niche or may have recently exited this niche. While GITR expression is not required for homeostatic proliferation of CD8 memory T cells, the receptor is required for the maintenance of these CD8 memory phenotype T cells,

although it is unclear whether basal expression of GITR suffices or whether upregulation in the bone marrow is required for this survival effect. The results presented here suggest that at a snapshot in time, about 30% of CD8 memory T cells in the bone marrow versus 3% in the spleen have recently occupied the IL-15 niche, arguing that the IL-15 niche is strongly associated with bone marrow.

Chapter 4

The Role of CD30 in the response to influenza infection

The results in this chapter are unpublished.

Brad Sedgmen assisted with several experiments.

A graduate student in our laboratory Michael Wortzman is continuing this project.

4.1 Abstract

CD30 is a member of the TNFR superfamily that is upregulated on T cells following antigen activation. While CD30 has recently been implicated in the formation of long-term CD8 T cell memory to bacterial infection, its role in viral responses remains unclear. Here, we examine the role of CD30 in the immune response to influenza A/HKx31. WT and CD30^{-/-} mice have similar influenza-specific CD8 T cell expansion and effector function, as well as equivalent generation of CD8 memory T cells responses. In addition, CD30 is dispensable for the generation of anti-influenza IgM and IgG responses. Upon rechallenge with influenza, CD30^{-/-} mice elicit antigenspecific CD8 T cell recall responses similar to those of WT mice. However, when infected with A/PR8, a strain of influenza that causes severe respiratory infection, CD30^{-/-} mice succumb to infection more readily than WT mice, suggesting that CD30 can confer protection from influenza-mediated death.

4.2 Introduction

CD30 is a member of the TNFR superfamily widely expressed in the immune system. Perhaps best studied for its overexpression in several lymphomas, CD30 is also expressed on B cells, NK cells, eosinophils, macrophages, and on activated T cells (5, 101). The ligand for CD30, CD30L or CD153, can be detected on dendritic cells, macrophages, resting B cells, neutrophils, eosinophils, activated T cells, as well as on a CD4⁺CD3⁻CD11c⁻ accessory cell implicated in the survival of CD4 memory Th2 cells (5, 101, 110). On T cells, CD30 is known to be a costimulatory molecule, and stimulation with agonistic anti-CD30 antibody or recombinant CD30L, in the presence of anti-CD3 or antigen primed dendritic cells, can enhance T cell activation, proliferation and cytokine production (111-114).

In vivo, much work has focused on the role of CD30 on CD4 T cells. It has been elegantly elucidated that CD30, in synergy with another costimulatory molecule OX40, is crucial for the survival of Th2 CD4 memory cells necessary to provide help to B cells for the generation of memory antibodies (110, 127, 128). Indeed, CD30^{-/-} mice were found to have defective memory antibody responses (128). In addition, CD30 has recently been implicated in the polarization of CD4 Th17 cells (117, 131), and has been found to play a role in several Th1 CD4 responses (115, 116, 130).

The role of CD30/CD30L signaling on CD8 T cells has been poorly characterized to date, although evidence is accumulating to suggest a role for CD30 in CD8 T cell activation and the maintenance of memory (100, 132, 133). Perhaps most convincing was one report demonstrating that CD30L^{-/-} mice have defective generation of long-term memory CD8 T cells following listeria infection, and further suggesting that CD30L is required for the formation of central

memory (132). In addition, like GITR, CD30 recruits the adaptor molecule TRAF1 upon T cell activation. Our lab has previously demonstrated that TRAF1 is required for both the survival of effector and memory CD8 T cells following influenza infection (34). While TRAF1 signaling downstream of 4-1BB has been implicated in having a role for CD8 T cell survival (302, 336), it remains to be elucidated whether other TRAF1 recruiting T cell costimulatory receptors are also important for this effect. Taken together, we postulate that CD30 is necessary for maximal TRAF1 dependent CD8 T cell survival.

As discussed in a recent review, the role of CD30 for anti-viral CD8 T cell responses remains poorly defined (89). While two preliminary studies using vesicular stomatitis virus (VSV) and murine cytomegalovirus (MCMV) as models failed to find a consistent role for CD30 on CD8 T cell and antibody responses (107, 133), an in depth analysis of CD30 in a viral response is lacking. Particularly necessary is the examination of longer time points to rule out a role for CD30 in CD8 T cell memory. Furthermore, it should be noted that both mousepox and cowpox viruses encode a soluble CD30 homologue which inhibits CD30L binding with its cellular receptor (134, 135). This suggests that CD30 signaling may play a role in subverting the immune response to these viruses. In this report, we study the role of CD30 in the immune response to influenza, with a particular focus on the antigen-specific CD8 T cell response. We find that CD30 is dispensable for maximal CD8 T cell numbers, CD8 T cell effector function, and the generation of antibodies in response to mild influenza infection. In addition, we show that CD30 does not impact on the generation of anti-influenza CD8 T cell memory, nor does it play a role in the recall response to influenza. However, when we infected WT and CD30^{-/-} mice with a severe and potentially lethal strain of influenza, the presence of CD30 in the mice elicited some protection from death. The cells responsible for this protection remain to be elucidated, and

this is currently under investigation in the lab.

4.3 Materials and Methods

Mice. C57BL/6 mice were obtained from Charles River Laboratories and used as WT mice. CD30^{-/-} mice were a generous gift from Dr. Tak Mak and have been previously described (107). CD30^{-/-} mice were backcrossed on to the C57BL/6 background for 10 generations. Mice were maintained in sterile microisolator cages under specific pathogen-free conditions. Animal studies were approved by the University of Toronto animal care committee in accordance with the regulations of the Canadian Council on Animal Care.

Influenza Virus Infection. Six to ten week old WT and CD30^{-/-} mice were infected intranasally (i.n.) with 5 hemagglutinin units (HAU) of influenza A/HKx31 (X31), a H3N2 virus. Thirty-five to ninety days after infection, some mice were challenged with 5 HAU of influenza A/PR8 (PR8), a H1N1 virus. These 2 viruses share the NP₃₆₆₋₇₄ conserved internal epitope, but differ in their hemagglutinin and neuraminidase envelope glycoproteins. For survival experiments using influenza A/PR8, mice were infected i.n. with either 0.5 or 2.5 HAU/mouse and were sacrificed when moribund. At the indicated times after infection, spleen, MLN, bone marrow and lungs were harvested. Lungs were perfused with PBS, and lymphocytes were enriched by isolation over an 80/40% Percoll gradient. Single-cell suspensions were prepared from all organs and were subjected to flow cytometry staining.

Flow Cytometry. MHC class I tetramers were obtained from the National Institute for Allergy and Infectious Diseases tetramer facility (Emory University, Atlanta, GA). Influenza NP₃₆₆₋₇₄specific CD8 T cells were surface stained with anti-CD8 α , anti-CD62L (eBioscience) and D^b/NP₃₆₆₋₇₄ tetramers. For intracellular IFN- γ staining, splenocytes were restimulated with 1 μ M of NP₃₆₆₋₇₄ for 6 hours with Golgi Stop (BD Biosciences) at 37^oC. Cells were surfaced stained as above, fixed and intracellularly stained for IFN-γ (BD Biosciences). Unstimulated samples (no peptide) were used as negative controls. Samples were analyzed using a FACScalibur (BD Biosciences) and FlowJo (TreeStar Inc.) software.

Cytotoxicity Assay. Splenocytes were harvested from WT and CD30^{-/-} mice at day 100 post-X31 influenza infection and restimulated in vitro with 100nm of D^b/NP₃₆₆₋₇₄ peptide for 5 days. On day 5 serial 3 fold dilutions of effector cells were performed and assayed for NP-specific killing against ⁵¹Cr-pulsed EL4 target cells pulsed with 50 μ M NP₃₆₆₋₇₄ peptide. Five hours later, the supernatant was harvested onto a 96 well plate and counted on a Topcount scintillation counter (Canberra Packard). The percentage of specific lysis was calculated from the equation: (experimental ⁵¹Cr release – spontaneous ⁵¹Cr release)/(maximum ⁵¹Cr release – spontaneous ⁵¹Cr release) X 100% = % specific lysis. Maximum and spontaneous specific lysis were calculated from control wells with 1% SDS or media alone.

Detection of influenza-specific Antibodies. Titers of IgM, IgG1, and IgG2a influenza-specific antibodies were determined from the sera of infected mice sacrificed either 10 or 100 days post-X31 infection. 96 well plates were coated with 500 HAU/ml of X31 in PBS for 1 hour at 37^{0} C, and then kept overnight at 4^{0} C. After blocking of plates for 2 hours with 5% skim milk in 0.1%PBS Tween, five fold serial dilutions of mouse serum were added to the plates and left for incubation overnight. After washing, HRP-conjugated anti-isotype antibodies (Caltag Laboratories, Burlingame, CA) were added to the wells and left for 2 hours before detection was mediated via $H_{2}O_{2}$ addition to the substrate ABTS (Sigma-Aldrish, St. Louis, MO) in a citrate phosphate buffer. After 20 minutes plates were read on an ELISA reader (Thermo Scientific, Waltham, MA) at OD₄₀₅.

Statistical Analysis. Where indicated, p values were obtained using the Student's t-test, unpaired, (two tailed, 95% confidence interval). The log rank test was used to determine significance for mouse survival experiments. Statistically significant differences are indicated as *, p < 0.05, **, p < 0.01 or ***, p < 0.001.

4.4 Results

CD30 is dispensable for primary influenza-specific CD8 T cell responses

In order to examine the role of CD30 in the immune response to influenza virus, WT and CD30^{-/-} mice were intranasally infected with Influenza A/HKx31 (X31). At day 10 postinfection, the peak of the primary response, the antigen-specific CD8 T cell response to the immunodominant epitope NP₃₆₆₋₇₄ was assessed using fluorescently labeled D^b/NP₃₆₆₋₇₄ MHC class I tetramers. Both the proportion and the number of NP-specific CD8 T cells in the spleens, mediastinal lymph nodes and lungs of CD30^{-/-} mice were equivalent to that observed in the WT controls (Figure 4.1A and 4.1B). The tetramer positive cells were CD62L^{lo}, indicating an effector phenotype (Figure 4.1A). Therefore, CD30 is dispensable for maximal primary CD8 T cell expansion to influenza virus.

CD30 is not required for the generation of CD8 memory T cells to influenza

A recent report demonstrated that following *L. monocytogenes* infection in CD30L^{-/-} mice, early antigen-specific CD8 T cell responses remained intact, but defects were present in all CD30L^{-/-} organs examined 84 days post-infection (132). This argued for an impairment in the generation or maintenance of CD8 T cell memory (132). We sought, therefore, to investigate whether CD30 might be necessary for maximal numbers of antigen-specific CD8 memory T cells at a much later time point following influenza infection. As the bone marrow has been shown to be a reservoir of memory T cells and the preferential organ for CD8 memory T cell



Figure 4.1: CD30 is dispensable for antigen-specific primary CD8 T cell responses to influenza virus and conversion to memory. WT and CD30^{-/-} mice were infected intranasally with 5 HAU of Influenza A/HK-X31 and mice were sacrificed at day 10 (B) and day 100 (C) post-infection. A. Representative gating strategy of the enumeration of D^b/NP₃₆₆₋₇₄ cells using fluorescently labeled tetramers from spleens at day 10 post-infection. **B** and C. The proportion and number of D^b/NP₃₆₆₋₇₄ antigen-specific CD8 T cells from various organs at day 10 (B) and day 100 (C) post-infection. Data in B are representative of 3 independent experiments with 4-5 mice each. Data in C are pooled from 2 independent experiments. 126
homeostasis (219, 236, 237), we analyzed this organ as well. However, when we infected mice and sacrificed them 100 days post-infection, there were still no significant differences observed between the number of NP-specific CD8 T cells in both the spleen and the bone marrow of WT and CD30^{-/-} mice (Figure 4.1C). CD30, therefore, is dispensable for the generation of CD8 memory T cells to influenza virus, as well as for their maintenance up to 100 days post-infection.

CD30 is not required for the generation of CD8 effector function

Although CD30 does not play a role in the generation of effector and memory antiinfluenza CD8 T cells, it was still possible that CD30 might regulate the effector function of these cells. Indeed, in vitro studies had shown that CD30 stimulation of T cells could enhance their production of IFN- γ (114), among other cytokines. As IFN- γ is abundantly produced in influenza infection, we used intracellular flow cytometry to analyze the proportion of CD8 T cells producing IFN- γ following a 6 hour restimulation with NP₃₆₆₋₇₄ peptide at both day 10 and day 100 post-infection. The proportion of CD30^{-/-} CD8 T cells producing IFN- γ was similar to that of WT CD8 T cells (Figure 4.2A and 4.2B). In addition, at day 100 post-infection we sought to examine the acquisition of CTL effector function. We restimulated splenocytes from WT or CD30^{-/-} mice for 5 days with NP₃₆₆₋₇₄ peptide and then performed an in vitro CTL assay. CD30 did not, however, affect the ability of the splenocytes to kill the target cells (Figure 4.2C). Therefore, not only does CD30 not play a role in the expansion of antigen-specific CD8 T cells, it is also dispensable for the formation of anti-influenza CD8 T cell effector function.



Figure 4.2: CD30 is dispensable for the generation of CD8 T cell effector function to influenza virus. WT and CD30^{-/-} mice were infected with Influenza X31 as described in Figure 4.1. Mice were sacrificed at either 10 or 100 days post-infection. A & B. Splenocytes were restimulated with NP₃₆₆₋₇₄ peptide for 6 hours and stained for IFN- γ expression. A. Representative FACS staining of CD8 producing IFN- γ cells at day 10 post-infection. Plots are gated on live CD8 T cells. B. % of splenic CD8 T cells producing IFN- γ at day 10 and day 100 post-infection. Data at day 10 are representative of 3 independent experiments, while data at day 100 are pooled from 2 independent experiments. C. Mice were sacrificed 100 days post-infection and splenocytes restimulated with NP₃₆₆₋₇₄ peptide for 5 days. A CTL assay was performed where cells were incubated with ⁵¹Cr-labeled EL4 lymphoma cells pulsed with NP₃₆₆₋₇₄ peptide and specific lysis was measured at four ratios of effector to target cells. Data are presented as mean ± SEM of 5 individual mice.

CD30 is dispensable for IgM and IgG antibody responses

To assess the role of CD30 on the humoral immune response to influenza virus, we quantified the anti-influenza antibodies in mouse sera by ELISA. At day 10 post-infection IgM levels were high compared to the naïve serum, and we observed that IgM responses were consistently slightly higher in serum from CD30^{-/-} mice at this time point (Figure 4.3A). In contrast, IgG1 and IgG2a serum antibodies were negligible at this time point in the sera of both WT and CD30^{-/-} mice (Figure 4.3A). Upon the examination of antibody levels at day 100 postinfection, not surprisingly, no IgM was present (Figure 4.3B), as these antibodies must have undergone isotype switching as the immune response progressed. Consistent with this, IgG1 responses were high at day 100 post-infection, although surprisingly, IgG2a responses were weak (Figure 4.3B). We had expected an IgG2a response to influenza at this time point and thus the minimal IgG2a signal is unexplained, and may be due to a technical issue, such as poor reagents. Nonetheless, the results show that CD30 does not affect the IgM or IgG1 or the weak IgG2a response. Therefore, not only is CD30 dispensable for antigen-specific CD8 T cell responses to influenza, it plays little, if any, role in the formation of anti-influenza IgM and IgG antibody responses, suggesting that B cell function is not impaired in CD30^{-/-} mice.

CD30 is dispensable for CD8 T cell recall responses to influenza virus

It has been demonstrated that initial infection with influenza X31 induces a mild immune response in mice, which elicits protection against challenge to a more lethal influenza virus A/PR8 (PR8). Influenza A/PR8 shares conserved internal epitopes, including NP₃₆₆₋₇₄, with X31, however it is a H1N1 virus, unlike X31 which is a H3N2 virus, and therefore infection with PR8 following X31 avoids neutralizing antibodies to these 2 envelope glycoproteins. Despite the lack



Figure 4.3: CD30 does not play a role in the generation of both primary and memory antiinfluenza antibody responses. Serum was taken from X31 infected WT and CD30^{-/-} mice at 10 days (A) or 100 days (B) post-infection. Influenza-specific IgM, IgG1 and IgG2a antibodies were measured in sera by ELISA. Sera from naïve mice was used as a negative control. Samples were assayed in the duplicate and the data presented are the average \pm SEM of 4-5 mice per group. Each graph is a representative of 2 independent experiments.

of a role for CD30 in the generation of antigen-specific CD8 T cell memory to influenza, it was possible that the costimulatory molecule might be needed to induce a maximal secondary response upon rechallenge with virus. To examine the role of CD30 in the recall response to influenza, we infected mice with X31 and allowed them to naturally clear the virus, which generally occurs at approximately 6-8 days post-infection (287). We then challenged the mice at either day 35 or day 90 post-infection with influenza PR8. The secondary CD8 T cell response has enhanced kinetics and therefore in both cases we enumerated the influenza-specific CD8 T cells at 8 days post-challenge. Again, no consistent differences were seen in the number or proportion of NP-specific CD8 T cells upon viral challenge (Figure 4.4A and 4.4B). Therefore, CD30 is not required for maximal CD8 T cell recall responses to influenza virus.

CD30 conveys protection against death following infection with a lethal influenza

We have previously seen that in primary infection of mice with PR8, a more pathogenic strain of influenza virus that results in severe respiratory infection, various TNFR costimulatory molecules play a role in disease outcome. Indeed, 4-1BBL was recently shown to be required for maximal CD8 T cell numbers in the lung in response to influenza PR8 and this correlated with enhanced lung function and viral clearance, as well as protection from death (287). Similarly, GITR on antigen-specific CD8 T cells protected mice from PR8 mediated death and augmented viral clearance upon infection with the virus (329). Therefore, despite CD30's lack of a role for CD8 T cell or antibody responses to X31, we sought to examine whether CD30 could influence influenza disease outcome upon PR8 infection. When we infected both WT and CD30^{-/-} mice with 0.5 HAU of PR8, both groups of mice had equivalent weight loss (Figure 4.5A). However, where all the WT mice survived, only 60% of the CD30^{-/-} mice were alive 16 days post-infection

A Day 35 + 8



Figure 4.4: CD30 is not needed for recall antigen specific CD8 T cell responses to influenza. WT and CD30^{-/-} mice were infected with Influenza X31 as described in Figure 4.1. Mice were challenged with 5 HAU of Influenza A/PR8 virus at 35 days post-infection (**A**) and 90 days post-infection (**B**) and sacrificed 8 days post-challenge. **A & B** show the proportion and number of D^b/NP₃₆₆₋₇₄ CD8 T cells in spleen, mediastinal lymph node (MLN) and lung 8 days post-challenge.

(Figure 4.5A). We further increased the dose of virus to 2.5 HAU of PR8 to examine whether this might give us even more striking results. At this viral dose, again weight loss was very similar between the two groups (Figure 4.5B), however the CD30^{-/-} mice were visibly sicker. Indeed, by day 8 post-infection all the WT mice were alive, but the majority of the CD30^{-/-} mice had succumbed to the virus, with only 25% surviving (Figure 4.5B). Therefore, the WT mice consistently had increased survival over the CD30^{-/-} mice, with more apparent differences occurring at higher doses of viral infection. Therefore, CD30 can protect mice from lethal influenza infection.



Α



Figure 4.5: CD30 is required for protection from death but not for weight loss following severe respiratory influenza infection. WT and CD30^{-/-} mice were intranasally infected with 0.5 HAU (**A**) or 2.5 HAU (**B**) of Influenza A/PR8. Mice were monitored post-infection and sacrificed when moribund. Body weights of mice were monitored daily, but this was ceased when several mice were moribund and had to be sacrificed. **A & B**. Percent of original body weight (left column) and percent of survival (right column) following PR8 infection.

4.5 Discussion

The role for CD30 in viral infections has previously been unclear. In this report, we examine the role of CD30 on the antigen-specific CD8 T cell response to influenza virus. We find that CD30 is dispensable for maximal CD8 T cell numbers and effector function in the primary response to mild influenza. CD30 is also not required for the generation of anti-influenza CD8 T cell memory or for the CD8 T cell recall response to influenza. Furthermore, CD30 did not play a significant role in the generation of ianti-influenza IgM and IgG antibody responses. However, upon infection with a more pathogenic strain of influenza, PR8, a greater proportion of CD30^{-/-} mice succumbed to virus, suggesting that CD30 can protect from lethal influenza infection.

As discussed above, infecting CD30^{-/-} mice with X31 influenza yielded no differences in antigen-specific CD8 T cell responses at every time point we observed. CD30 is found on multiple different types of immune cells, including but not limited to B cells, NK cells, eosinophils, macrophages and activated T cells (5, 101). Indeed, CD30 has even been shown to be expressed on regulatory T cells, and is important for regulatory T cell mediated suppression of allograft rejection and protection from graft versus host disease lethality in mice (337, 338). It is possible, therefore, that the role of CD30 on CD8 T cells may be masked by effects of the loss of CD30 on other cell types, such as on regulatory T cells. Interestingly, this was true when we examined GITR's role on CD8 T cells in an influenza model (329). While influenza-infected GITR^{-/-} mice had antigen-specific CD8 T cell defects only in certain organs at certain time points following infection, the use of a T cell transgenic adoptive transfer model where GITR was lacking only on CD8 T cells showed clear defects in every organ examined (329). This is not

always the case, though, as influenza-specific CD8 T cell defects observed in 4-1BBL^{-/-} mice nicely mirrored bone marrow chimera studies where 4-1BB was lacking only on α/β CD8 T cells (287, 300). In order to fully address whether CD30 has an intrinsic role on CD8 T cells specific for influenza, an adoptive transfer transgenic model will be employed. TCR transgenic OT-I mice have been crossed with CD30^{-/-} mice and a preliminary adoptive transfer study using X31-OVA suggests that CD30 does not seem to have an intrinsic role on CD8 T cells responding to influenza. These studies are being followed up further by another graduate student in the laboratory, Michael Wortzman.

An earlier report by Nishimura et al. suggested that CD30L was important for maximal numbers of long-lived memory CD8 T cells following *L. monocytogenes* infection (132). However, even at 100 days post influenza infection we did not see a defect in CD30^{-/-} antigen-specific CD8 memory T cell numbers. The discrepancy between our results and those seen by Nishimura et al. may, perhaps, be attributed to the differences in the models being used. Nishimura et al. show that CD30L was specifically required for the generation of central memory T cells in the bacterial model of *L. monocytogenes* (132). Even at 100 days post-influenza infection, however, a large proportion of antigen-specific CD8 T cells in WT mice were still CD62L¹⁰, and this was particularly evident in the bone marrow (data not shown). Therefore, at least at this time point, influenza elicits more effector than central memory. This leads us to speculate that CD30's role may be more or less prominent on CD8 T cell responses depending on the type of memory generated by different pathogens. It might be of interest to examine the role of CD30 on CD8 T cell responses to LCMV, which is known to elicit strong central memory.

Although not examined in detail, two other reports have discussed the role of CD30 in viral responses (107, 133). Our data showing that CD30 does not play a significant role in memory CD8 T cell responses to influenza is consistent with that seen for another virus, VSV. In that study, CD30^{-/-} memory CTL responses were unimpaired and similar protection from VSV challenge was seen in both CD30 sufficient and deficient mice (107). Furthermore, the study showed no defect in VSV-specific antibody production or class switching responses, suggesting normal T cell help for B cell responses (107), much like in our influenza model. In contrast to our data that CD30 is not required for the expansion of primary CD8 T cells, another report showed that OX40^{-/-}CD30^{-/-} mice had significant defects in antigen-specific CD8 T cell responses seven days following MCMV infection. Although many other models have shown OX40 and CD30 to be synergistic, this particular study did not examine the immune response to MCMV in single knockouts, so it is possible that OX40 may have played a larger role in this early CD8 T cell defect. Despite the early defect, however, both molecules proved dispensable for the generation and persistence of CD8 memory T cells (133). The authors point out that this may be epitope specific, as has been observed in studies of MCMV in OX40^{-/-} mice (339). Also consistent with our study, there was no defect in the proportion of antigen-specific CD8 T cells producing IFN- γ in the double knockout mice (133). Therefore, CD30 does not seem to play a large role in anti-viral responses to VSV, MCMV or from our data, a mild strain of influenza, influenza A/HK-X31. It might be of interest to examine the role of CD30 in the immune response to poxvirus, as both mouse and cowpox encode a soluble CD30 homologue, which when secreted binds CD30L and prevents binding and signaling of CD30 (134, 135). That the virus has evolved this immune evasion mechanism suggests that CD30 may be important for pox-mediated immunity.

We have observed that when mice were infected with a more severe strain of influenza, A/PR8, weight loss was similar but WT mice had a survival advantage over CD30^{-/-} mice. Influenza-mediated weight loss is induced by the early innate immune response to the virus (243), therefore implying that CD30 is not necessary for this portion of the response. Protection from influenza-mediated death, however, has been shown to be mediated by CD8 T cells, CD4 T cells or B cells (253, 264). Although our study did not examine CD4 T cell responses to influenza, it has been suggested that CD30 may play a larger role on CD4 T cells than on CD8 T cells. Indeed, CD30 works in synergy with OX40 to maintain the survival of CD4 memory Th2 cells, which are needed to help B cells generate memory antibody responses (128, 129). Recently, CD30 has also been implicated in having a role for maximal CD4 Th1 responses in several studies (115, 116, 130). Taken together that CD30 can enhance CD4 T cell responses, and that CD4 T cell responses can protect against lethal influenza (264), it is possible that the lack of CD30 on CD4 T cells may have negatively regulated the survival of PR8 infected CD30^{-/-} mice. Interestingly, primed CD4 T cells have been shown to protect against lethal influenza by 2 mechanisms: by enhancing anti-influenza antibody titers, and by the perforin-dependent killing of infected cells, independent of any CD4-mediated effects on B cells (264). We did not detect impaired antibody responses in influenza-infected CD30^{-/-} mice, suggesting that any survival defect in PR8 infected CD30^{-/-} mice attributed to CD4 T cells may be through direct CD4 mediated perforin-dependent killing. This will have to be investigated further. Currently, the laboratory is crossing transgenic OT-II mice with CD30^{-/-} mice to set up an adoptive transfer system to investigate whether CD30 on CD4 T cells can protect mice from lethal infection.

Alternatively, it is possible that CD30 on CD8 T cells may be required for protection from severe influenza, whereas it is dispensable for CD8 T cell responses to mild influenza infection. Indeed, 4-1BBL is only transiently induced to mild influenza and is dispensable for primary CD8 T cell responses to this virus, which is rapidly cleared from the host (287, 300). However, when the disease is more severe and potentially lethal, 4-1BB expression is sustained on CD8 T cells in the lung and 4-1BBL^{-/-} mice had defective accumulation of influenza-specific CD8 T cells to the lung (287). In addition, 4-1BBL was required for lowering PR8 viral titers in the lung and protecting mice from death (287). Similarly, as described in chapter 2 of this thesis, while GITR on CD8 T cells is necessary for optimal CD8 T cell responses to mild influenza infection, it is dispensable for disease outcome under these conditions. However, CD8 T cell intrinsic GITR lowers viral titers and protects mice from death following infection with lethal influenza. Therefore, the necessity of costimulatory molecules may have evolved to be dependent on the severity of disease (287, 329) and it is possible that CD30 may be necessary for CD8 T cell protection from death from severe influenza infection, but is dispensable for CD8 T cell responses to mild influenza infection.

Interestingly, CD30 has recently been implicated in the polarization of CD4 Th17 cells (117). Furthermore, it has been reported that both CD4 and CD8 T cells which produce IL-17 can be detected in the lung following influenza infection (340). The administration of an anti-IL-17 blocking antibody to virally infected mice resulted in decreased mouse survival, and the transfer of this novel subset of IL-17 producing CD8 T cells could protect from influenza-mediated death (340). Although the numbers of antigen-specific CD8 T cells were similar in CD30^{-/-} mice infected with influenza and we could detect no differences in the CD8 T cells. It is possible that the T cells in CD30^{-/-} mice could not generate adequate levels of IL-17, and this contributed to enhanced death of CD30^{-/-} mice.

We have shown in this report that CD30 is dispensable for CD8 T cell expansion and effector function to mild influenza virus. In addition, CD30 does not play a role in the generation or maintenance of CD8 memory T cells, nor in the recall CD8 T cell response to influenza. Therefore while TRAF1 is recruited to CD30 upon antigen activation, the presence of CD30 on the T cells does not seem to impact on the maintenance of CD8 memory T cells. Thus it is unclear whether CD30-mediated TRAF1 recruitment plays a redundant role in CD8 memory or no role at all. However, upon infection with a more pathogenic influenza virus, CD30 did offer some protection from death. The cells involved and the mechanism by which this occurs is currently under investigation in our laboratory.

Chapter 5

Summary and Future Directions

Several members of the TNFR superfamily are T cell costimulatory molecules and are known to enhance the magnitude and quality of T cell responses. Thus, the manipulation of these receptors and their signaling can be powerful tools for modulating immune responses. Currently, several TNFRs are promising candidates for immunotherapy and in certain cases clinical trials are underway, for example using agonistic antibodies targeting these receptors. However, in order to harness the full power of these costimulatory molecules for clinical manipulation, the cellular and biochemical mechanisms by which these receptors exert their effects on the immune response must be elucidated. To that effect, my thesis has focused specifically on 2 TNFR family costimulatory molecules, GITR and CD30, and their role on CD8 T cells. In chapter 2 and 4, I dissect the roles of these receptors on the CD8 T cell response to influenza, while in chapter 3, I discuss the expression and function of GITR for IL-15-mediated maintenance of CD8 memory T cells in the bone marrow.

Prior to the start of my thesis, little was known about the function of GITR and CD30 in anti-viral CD8 T cell responses. However, upon activation with their respective ligands, both these receptors recruit TRAF1, which our lab had previously shown to be crucial for the survival of effector and memory CD8 T cells specific for influenza (34). Therefore, I hypothesized that GITR and CD30 might mediate TRAF1 dependent CD8 T cell survival. Interestingly, while I showed that CD8 T cell intrinsic GITR was required for both maximal primary and secondary CD8 T cell responses to influenza, CD30 was dispensable for anti-influenza CD8 T cell responses at all time points examined. Further examination showed that GITR does not play a role on CD8 T cell proliferation, nor on homing, but instead mediates effects on the survival of CD8 T cells. Surprisingly, however, GITR signaling induces TRAF2/TRAF5 dependent, but TRAF1 independent, NF-κB activation, which causes the upregulation of the pro-survival



Figure 5.1. Model of the CD8 T cell intrinsic role of GITR during influenza infection. GITR on CD8 T cells has a profound effect on CD8 T cell expansion during influenza virus infection attributed to TRAF2/5-mediated NF- κ B survival signaling resulting in the upregulation of Bcl- x_L . In addition, CD8 T cell intrinsic GITR augments viral clearance and is essential for mouse survival during severe influenza infection.

molecule $Bcl-x_L$ (Figure 5.1). The role of TRAF1 is equally unclear downstream of CD30, as the receptor did not appear to affect the CD8 T cell response, at least in response to a mild influenza virus.

It is of interest to note that like GITR and CD30, another TNFR family member, 4-1BB, recruits TRAF1 as well. Our lab has demonstrated that downstream of 4-1BB signaling, TRAF1 deficient CD8 memory T cells fail to upregulate ERK, leading to an upregulation of the proapoptotic molecule Bim and defective upregulation of the pro-survival molecule $Bcl-x_L$ (34, 302). Therefore TRAF1 is crucial for 4-1BB mediated survival signaling in CD8 memory T cells. Although in this thesis I have shown that GITR, too, plays a role in the survival of CD8 T cells, TRAF1 is dispensable for DTA-1 mediated CD8 T cell expansion to influenza, and GITRmediated NF-κB survival signaling was not impaired in TRAF1^{-/-} T cells. Indeed, GITR induced NF- κ B signaling augments Bcl-x_L expression in as little as 3 hours post stimulation, but, unlike 4-1BB, I did not observe a role for Bim in the survival of CD8 T cells downstream of GITR (data not shown). While both receptors recruit TRAF1, TRAF2 and TRAF3, GITR also utilizes TRAF5 for its downstream signaling (55, 69, 72, 341-343). Upon T cell activation, TRAF1 and TRAF2 form heterotrimers downstream of 4-1BB while GITR may principally utilize TRAF2 and TRAF5 for survival signaling, rendering TRAF1 less important. In addition, there has been a report that TRAF4 can augment NF-kB signaling downstream of GITR in 293 T cell overexpression studies (35). This, however, should be confirmed in primary cells, and could be done using siRNA to knockdown TRAF4, as I have done for TRAF2 and TRAF5. It would be of interest to further elucidate the signaling downstream of 4-1BB and GITR, and to study the stoichiometry of the TRAFs at the cytoplasmic tails of these receptors to understand how similar recruitment of TRAFs to TNFRs can mediate such different signaling pathways.

Although I have demonstrated that CD8 T cell intrinsic GITR signaling is required for the survival of CD8 T cells specific to influenza virus, the origin of this signaling is unknown. Yet to be identified is the GITRL expressing cell which signals to GITR on CD8 T cells. While it is tempting to speculate that the GITRL signal is derived from an APC upon T cell priming, the expression of GITRL has also been detected on activated CD8 T cells (61, 62, 65, 66), and therefore a potential role for T-T interactions can not be ruled out. These studies will be greatly facilitated by the generation and characterization of a GITRL^{-/-} mouse, which to our knowledge does not exist at the present moment.

In this thesis, I demonstrate that GITR on CD8 T cells can lower viral titers in the lungs and protect mice from mortality upon infection with a pathogenic, and potentially lethal strain of influenza. Similarly, 4-1BBL has also been shown to be required for maximal CD8 T cell numbers in the lungs following severe influenza infection, and this correlates with increased mouse survival (287). Indeed, 4-1BBL^{-/-} mice succumb to lethal influenza infection in a similar fashion to WT mice injected with CD8 T cells lacking GITR. Interestingly, the lack of GITR on CD8 T cells and the lack of 4-1BBL in the entire mouse gave a similar fold enhancement in the viral titers of influenza in the lungs. (287, 329) Whereas GITR is an early T cell costimulatory receptor present on naïve cells and is very quickly upregulated upon T cell activation, 4-1BB is an inducible molecule, with peak expression reported between 48 and 72 hours (60, 336). Because of the difference in kinetics of these 2 receptors, it is tempting to speculate that they protect mice temporally, with GITR playing a role early and 4-1BB being more important late in the immune response. In addition, our lab has recently shown that GITR is required for maximal 4-1BB expression on CD8 memory T cells in the bone marrow, where these cells obtain signals for homeostatic proliferation, as well as for survival (Lin, unpublished). Though the dependence on GITR for 4-1BB expression has not been tested in the influenza model, this may also be the

case, implying that there may be some overlap in the survival signaling by these 2 receptors. It would be informative to conduct studies where both molecules are lacking, for instance the transfer of GITR^{-/-}OT-I cells into 4-1BBL^{-/-} mice to observe if the mice had even higher mortality or higher viral titers than when only one receptor was lacking.

As GITR is a fairly recently identified member of the TNFR family and has not yet been extensively characterized, it will be informative to elucidate its role on T cells in other infectious contexts including bacterial responses, as well as acute and chronic viral responses. In addition, little is known as to GITR's role on human T cells, and this too is paramount to investigate. While agonistic antibody studies have identified that GITR augments T cell responses, to our knowledge my study was the first to identify a CD8 intrinsic role for GITR. Having crossed GITR^{-/-} mice with OT-I mice, we now have a valuable tool for the study of the role of GITR specifically on CD8 T cells, and these mice could easily be used in a multitude of different adoptive transfer models. Similarly, it will be of interest to examine the CD4 intrinsic role of GITR. This, too, could be achieved by crossing the GITR^{-/-} mice to OT-II mice and setting up similar adoptive transfer models.

As discussed above, while studies using GITR agonistic antibodies have shown enhancement of T cell responses, the cellular targets for the antibody have been unclear in vivo. As humanized monoclonal GITR antibodies are entering clinical trials, determining the mechanism of action of the antibody is of considerable importance. In this thesis I have examined the cellular targets for the agonistic GITR antibody, DTA-1. In our model of influenza infection, I have shown that the antibody seems to work primarily on CD8 T cells, having little effect on the proliferation of regulatory T cells. Similar results were obtained shortly after in a report demonstrating that GITR on CD8 T cells is required for DTA-1 induced concomitant immunity in a melanoma model (92). However, other tumor models have shown a role for the costimulation of Tregs by DTA-1 (91, 94). This may be dependent on the model and the ratio of local effector to regulatory T cells. More investigation is warranted to more clearly comprehend this issue. To date, treatment with agonistic GITR antibody has not shown considerable pathology in mouse models, as has been seen for agonistic antibodies to other costimulatory TNFR family members, such as 4-1BB (344). This makes it a promising candidate for immunotherapy and currently a phase I trial using agonistic anti-GITR antibody for malignant melanoma is recruiting patients. Additionally, another trial for melanoma is using GITRL to modify dendritic cells (http://clinicaltrials.gov/; search for key word: GITR). However, it remains to be seen whether these therapies will be successful in humans with limited pathology. Regardless, the clearer our understanding of the mechanism of these antibodies to induce potent immune responses, the more we can modulate the formulation or delivery of these TNFR family signals to better target the cells of interest, with minimum pathology.

In chapter 3 of this thesis, I show that a proportion of memory phenotype CD8 T cells in the bone marrow express higher than basal levels of GITR, not seen in the spleen and peripheral lymph nodes. I further determine that this is not a unique subset of T cells, but rather IL-15 in the local bone marrow environment can upregulate GITR expression on CD8 memory T cells upon their trafficking into the organ. Therefore, GITR expression serves as a marker of CD8 memory T cells that have recently received an IL-15 signal. In addition, we further show that aged unimmunized GITR^{-/-} mice had a slight, but statistically significant defect in CD8 memory phenotype cells in the bone marrow. This could not be attributed to a defect in homeostatic proliferation, but rather seemed likely to be a defect in CD8 memory T cell survival.

Although it is well understood that homeostatic cytokines, such as IL-7 and IL-15, drive the maintenance of T cell memory, recently a role for various TNFR family members has also come to the forefront. Peter Lane's group demonstrated that the expression of OX40 and CD30 on CD4 Th2 memory cells is necessary for the survival of these cells (128). Survival signaling was further shown to be derived through interaction of CD4 Th2 memory cells with a CD4⁺CD3⁻ CD11c⁻ accessory cell that expresses CD30L and OX40L (110, 128) (Figure 5.2). Of interest, OX40 upregulation on CD4 memory T cells was dependent on IL-7, (128) and CD4 memory T cells have been shown to interact with IL-7 producing stromal cells in the bone marrow, where it is inferred they receive maintenance signals (225).

Our lab has previously shown that 4-1BBL is necessary for the maintenance of CD8 memory T cells in vivo (308) and CD27 has also been implicated for the survival of memory T cells (77). This thesis now adds GITR to the list of TNFR family members that are required for the persistence of CD8 memory T cells (Figure 5.2). Interestingly, however, while GITR expression was upregulated by IL-15 in the bone marrow, we have recently discovered that 4-1BB expression on CD8 memory T cells in vivo is independent of IL-15, but rather is dependent on GITR expression (Lin, unpublished). In addition, the defect in memory phenotype CD8 T cells in 4-1BBL^{-/-} mice is similar in magnitude to the defect seen in GITR^{-/-} mice (308). Therefore, it is possible that GITR's role for the maintenance of CD8 memory T cells in the bone marrow may be at least in part through the augmentation of 4-1BB on CD8 memory T cells. Experiments with a lack of both receptors, as described above for antigen-dependent studies, may shed light on this possibility.

While I have demonstrated that IL-15 upregulates GITR to higher than basal levels on CD8 memory T cells in the bone marrow, and that GITR is necessary for the persistence of

CD4 memory T cells



Figure 5.2 The Role of TNFRs in CD8 and CD4 memory T cell maintenance. It is thought that when antigen is absent or limiting, CD4 memory T cells migrate to the bone marrow, where IL-7, which is produced by VCAM1⁺ stromal cells, can induce OX40 and perhaps CD30 expression on CD4 memory T cells. These cells can then receive survival signals from OX40L and CD30L expressing CD4⁺CD3⁻ accessory cells. Similarly, we have shown that GITR can be induced by IL-15 on CD8 memory phenotype T cells upon entry into the bone marrow. We have also shown that GITR is required for the persistence of these cells in the bone marrow. In addition, our lab has demonstrated that 4-1BB: 4-1BBL signaling is necessary for the survival of CD8 memory T cells. Interestingly, 4-1BB expression on CD8 memory T cells has been shown to be dependent on GITR, and therefore the effects of GITR may be partially through regulation of 4-1BB expression. As discussed in the text, however, 4-1BB levels are independent of IL-15, suggesting that basal GITR expression may suffice for 4-1BB upregulation.

memory phenotype CD8 T cells in the bone marrow, we have not conclusively proven that it is this IL-15 induced upregulation of GITR which is functionally important for the maintenance of these cells. Indeed, IL-15^{-/-} mice have basal expression of GITR but normal levels of 4-1BB (Lin, unpublished). Therefore, basal expression of GITR can induce 4-1BB, and may be adequate enough for survival signaling. Indeed, it is possible that high levels of GITR in the bone marrow may simply mark a population of CD8 memory T cells which have recently received IL-15 signaling. Whether or not the upregulation of GITR in the bone marrow is functionally relevant for persistence of CD8 memory T cells is very difficult to prove experimentally, as GITR^{-/-} mice have all GITR ablated, not just this hi population. Although technically difficult because the populations are so small, it is theoretically possible to sort GITR^{hi} versus GITR^{basal} cells and upon adoptive transfer into mice determine whether the GITR^{hi} cells have enhanced survival. However, the IL-15 signal is most likely transient, and any length of time needed to determine survival would give the GITR^{basal} cells opportunity to home to the bone marrow and receive IL-15 signals themselves. Although one could use IL-15^{-/-} mice as recipients, given the small GITR^{hi} population it is likely that the effects detected would be too marginal to give definitive conclusions. Indeed one of the difficulties of studying effects of the TNFR family on memory cell maintenance is that as one takes away one of several molecules contributing to memory T cell survival, homeostatic mechanisms may compensate, rendering effects rather small.

Although in this thesis I have not shown that the effect of GITR on the maintenance of CD8 memory T cells is CD8 T cell intrinsic, our lab has demonstrated that this is the case, using an adoptive transfer model with GITR sufficient or deficient memory CD8 T cells (Lin, unpublished). The cell that expresses GITRL and interacts with these CD8 memory T cells to induce survival signaling in the bone marrow remains to be characterized. It is possible that like

the CD4⁺CD3⁻CD11c⁻ accessory cell that expresses both OX40L and CD30L, the same cell may express both GITRL and 4-1BBL. Our lab has determined that a radioresistant stromal cell in the bone marrow provides 4-1BBL dependent signals to CD8 memory T cells (Lin, unpublished). Once this cell type is characterized further it will be of interest to see whether it expresses GITRL as well. Alternatively, because GITR expression seems to induce 4-1BB, the ligand expressing cells could be different and could interact with CD8 memory T cells at different time points.

In chapter 4, I examined the role of CD30 in the CD8 T cell response to influenza. Although, two previous reports demonstrated that CD30 was important for the survival of CD8 memory T cells (100, 132), I did not find evidence for this in the influenza model. I showed that CD30 did not play a role for either primary or recall CD8 T cell responses to mild influenza virus, nor for anti-viral CD8 T cell effector function. I also examined primary influenza-specific antibody responses, although I did not find a significant role for CD30 in this regard, either. Upon infection with a more pathogenic strain of influenza, however, CD30^{-/-} mice exhibited a defect in survival. This is most likely due to a defective arm of the adaptive immune response, as it has been reported that CD8 T cells, CD4 T cells, or B cells can protect against lethal influenza (240). In addition, the weight loss between the two groups of mice was equivalent, and this is typically due to innate responses (243), thereby suggesting that the early innate responses of CD30^{-/-} mice are intact. Although I did not examine viral titers in these mice, it would be of interest to see if the decrease in survival is associated with defective control of the virus, particularly in the lungs.

As mentioned earlier, a role for both 4-1BBL and GITR was elucidated for disease protection upon infection of mice with influenza A/PR8. While I demonstrate in this thesis that

GITR on CD8 T cells is protective from lethal flu, it was inferred but not directly shown that it was intrinsic 4-1BB signaling on the CD8 T cells that protected against mortality. Interestingly, however, GITR and 4-1BBL were not required for the clearance of virus upon infection with mild influenza, and 4-1BBL was found to be important for maximal primary CD8 T cell responses in the lungs only when mice were infected with the pathogenic strain of influenza, and not the mild strain. Therefore, while we saw no impairment in anti-influenza CD8 T cell responses to mild influenza in CD30^{-/-} mice, we did not examine CD8 T cell responses to pathogenic influenza in these mice. Thus, we cannot formally rule out that CD30 on CD8 T cells is dispensable for the protection seen in this model of lethal influenza. To address this, the influenza-specific CD8 T cell response should be quantitated, particularly in the lung, following severe influenza infection of WT and CD30^{-/-} mice. Additionally, an adoptive transfer model could be set up where OT-I or CD30^{-/-} OT-I cells are transferred into host mice that are then infected with pathogenic influenza. The ability of CD30^{-/-} OT-I cells to confer protection against death could then be determined.

It is possible that CD30 on B cells may be protective in our model of lethal influenza. However, because of the near normal anti-viral antibody responses in CD30^{-/-} mice, we feel that this is unlikely. Because of the known role of CD30 on CD4 T cells, it is perhaps more likely that CD4 T cells play a role in protection. CD4 T cells have been shown to promote survival to influenza virus by enhancing antibody responses, as well as by directly lysing infected cells via a perforin dependent mechanism (264). In addition, recently a role for CD8 and CD4 Th17 cells has been revealed as protective in influenza infection (340), and as CD30 has been implicated in the polarization of CD4 Th17 cells (117), it is possible that these cells may play an important role for protection. Our studies were initially contrived to focus on CD8 T cells and we neglected to examine the CD4 T cell response to influenza, even in mild infection. This would be of interest to examine, as defects may very well be evident. Additionally, the lab is currently crossing CD30^{-/-} mice on to the OT-II TCR transgenic background in order to test the intrinsic role of CD30 on CD4 T cells. Peter Doherty and Paul Thomas have generously provided strains of recombinant influenza virus which have incorporated into their neuraminidase stalks the class II OVA epitope (X31-OVA2 and PR8-OVA2), and adoptive transfer studies could be carried out using OT-II and CD30^{-/-}OT-II cells in conjunction with infection of these viruses. In addition, such an adoptive transfer model could be set up to directly test whether CD30^{-/-}OT-II cells can protect mice from lethal influenza. These studies are being carried out to completion by another graduate student in the lab, Michael Wortzman.

In conclusion, GITR appears to be a good candidate for immunotherapy and a humanized monoclonal agonistic antibody to GITR is currently in phase I clinical trials. My work has elucidated that intrinsic GITR signaling on CD8 T cells is crucial for the survival of CD8 T cells following influenza infection. Similarly, in unimmunized mice GITR is required for the persistence of CD8 memory phenotype T cells in the bone marrow, where GITR expression is upregulated on these cells by local sources of IL-15. Furthermore, CD8 T cells and not regulatory T cells appeared to be the primary targets of agonistic anti-GITR antibody in the immune response to influenza. Although, CD30 did not play a role in the CD8 T cell response to influenza to be important for the generation of long-term CD8 T cell memory. This may be model dependent, however, and warrants more investigation into the conditions whereby CD30 may exert an effect on the T cell response.

Chapter 6

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