

**REGULATION OF ALLOREACTIVE CD8 T CELL RESPONSES BY  
COSTIMULATION AND INFLAMMATION**

A Dissertation Presented

By

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## **Abstract**

CD8 T lymphocytes are a crucial component of the adaptive immune system and mediate control of infections and malignancy, but also autoimmunity and allograft rejection. Given their central role in the immune system, CD8 T cell responses are tightly regulated by costimulatory signals and cytokines. Strategies targeting signals that are critical for T cell activation have been employed in a transplantation setting to impede alloreactive T cell responses and prevent graft rejection. The goal of my thesis is to understand how costimulatory signals and inflammation regulate alloreactive CD8 T cell responses and how to target these pathways to develop more effective tools to prevent graft rejection.

Costimulation blockade is an effective approach to prolong allograft survival in murine and non-human primate models of transplantation and is an attractive alternative to immunosuppressants. I describe a novel murine anti-CD40 monoclonal antibody that prolongs skin allograft survival across major histocompatibility barriers and attenuates alloreactive CD8 T cell responses. I find that the pro-apoptotic proteins Fas and Bim function concurrently to regulate peripheral tolerance induction to allografts. Activation of the innate immune system by endogenous molecules released during surgery or infections in transplant recipients can modulate T cell responses. However, the direct impact of inflammation on alloreactive CD8 T cell responses is not clear. Using a T cell receptor (TCR) transgenic mouse model, I demonstrate that inflammatory stimuli bacterial lipopolysaccharide (LPS) and the viral dsRNA mimetic poly(I:C)

differentially regulate donor-reactive CD8 T cell responses by generating distinct cytokine milieus. Finally I demonstrate the role of pro-inflammatory cytokines stem cell factor (SCF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) in improving human B cell development in humanized NOD-*scid*  $IL2R\gamma^{null}$  (NSG) mice.

## Table of Contents

<b>Acknowledgements .....</b>	<b>iii</b>
<b>Abstract .....</b>	<b>vi</b>
<b>Table of Contents .....</b>	<b>viii</b>
<b>List of Tables.....</b>	<b>xiii</b>
<b>List of Figures .....</b>	<b>xiv</b>
<b>List of copyrighted material.....</b>	<b>xvi</b>
<b>List of Abbreviations .....</b>	<b>xvii</b>
<b>Preface.....</b>	<b>xxiv</b>
 <b>CHAPTER I .....</b>	 <b>1</b>
<b>Introduction.....</b>	<b>1</b>
<b>Rationale and thesis outline .....</b>	<b>2</b>
<b>CD8 T cell activation: signal 1 (antigen recognition) .....</b>	<b>3</b>
<b>CD8 T cell activation: signal 2 (costimulation) .....</b>	<b>6</b>
CD28/B7/CTLA4 .....	6
CD40/CD154.....	7
OX40/OX40L.....	8
LFA-1/ICAM-1 and VLA-4/VCAM-1 .....	9
ICOS/ICOS-L .....	9
4-1BB/4-1BBL .....	10
CD27/CD70.....	10
TIM family members .....	10
PD-1/PD-L1.....	11
<b>CD8 T cell activation: signal 3 (cytokines).....</b>	<b>12</b>
<b>Innate immunity: cross-talk with adaptive immunity .....</b>	<b>13</b>
Pattern recognition receptors .....	14
Toll-like receptors (TLRs).....	14
C type lectin receptors (CLRs).....	15
RIG-I-like receptors (RLRs) .....	15
NOD-like receptors (NLRs) .....	15
AIM2-like receptors (ALRs).....	16
LPS and poly(I:C).....	16
Regulation of T cell responses by PRRs.....	17
<b>T cell apoptosis.....</b>	<b>21</b>
<b>Transplant rejection .....</b>	<b>24</b>
Hyperacute rejection .....	25
Acute rejection .....	26

Chronic rejection .....	28
Mechanisms of T cell allorecognition .....	29
Direct allorecognition .....	29
Indirect allorecognition .....	30
Semi-direct allorecognition.....	31
<b>Transplantation tolerance: why and how? .....</b>	<b>33</b>
Immunosuppressants: old and new .....	33
Central and peripheral tolerance .....	35
Deletion .....	37
Anergy.....	38
Immunoregulation .....	38
Costimulation blockade .....	40
CD28/B7 .....	40
CD40/CD154.....	41
OX40/OX40L.....	42
LFA-1/ICAM-1 and VLA-4/VCAM-1 .....	43
Alternative approaches to tolerance induction .....	44
Blocking co-inhibitory pathways.....	44
Establishment of mixed chimerism .....	45
Regulatory T cell infusion.....	46
Myeloid suppressor cell infusion .....	46
Mesenchymal stem cell infusion .....	47
Apoptotic cell infusion .....	48
Infusion of exosomes .....	48
<b>Barriers to transplantation tolerance.....</b>	<b>49</b>
Sterile inflammation.....	49
Infections .....	51
Memory alloreactive T cells.....	52
Memory alloreactive B cells and alloantibodies .....	53
Homeostatic proliferation .....	53
Pre-existing autoimmunity .....	54
<b>Humanized mice .....</b>	<b>54</b>
 <b>CHAPTER II .....</b>	 <b>60</b>
<b>A novel murine anti-CD40 monoclonal antibody prolongs skin allograft survival .....</b>	<b>60</b>
<b>Abstract .....</b>	<b>61</b>
<b>Introduction.....</b>	<b>62</b>
<b>Materials and Methods .....</b>	<b>64</b>
Generation and characterization of the anti-CD40 mAb .....	64
Animals .....	65
Generation of CD8 <sup>+</sup> KB5 TCR-transgenic synchimeric mice.....	65
Flow cytometry, antibodies and intracellular cytokine staining.....	66
VPD450 labeling and adoptive transfer.....	66



In vivo cytotoxicity assay .....	67
Skin transplantation and tolerance induction .....	67
Measurement of alloantibodies .....	68
Statistics .....	68
<b>Results.....</b>	<b>69</b>
Characterization of the anti-CD40 monoclonal antibody.....	69
Anti-CD40 mAb combined with DST prolongs skin allograft survival.....	71
Anti-CD40 mAb combined with DST inhibits alloreactive CD8 T cell expansion and function.....	75
Anti-CD40 antibody does not deplete CD40 expressing antigen presenting cells ..	82
Foxp3 <sup>+</sup> Tregs are necessary for the tolerogenic effects of DST/anti-CD40 costimulation blockade.....	82
Administration of LPS at the time of DST and anti-CD40 mAb shortens skin allograft survival .....	86
<b>Discussion.....</b>	<b>91</b>
 <b>CHAPTER III .....</b>	 <b>99</b>
<b>Proapoptotic proteins Fas and Bim concurrently regulate costimulation blockade induced tolerance to allografts.....</b>	<b>99</b>
<b>Abstract .....</b>	<b>100</b>
<b>Introduction.....</b>	<b>100</b>
<b>Materials and Methods .....</b>	<b>102</b>
Mice.....	102
Transplantation .....	102
Antibodies, flow cytometry and intracellular cytokine staining .....	103
Statistics .....	103
<b>Results.....</b>	<b>103</b>
Peripheral tolerance induction by costimulation blockade is dependent on Fas and Bim .....	104
<b>Discussion.....</b>	<b>106</b>
 <b>CHAPTER IV .....</b>	 <b>109</b>
<b>Differential regulation of alloreactive CD8 T cell responses by inflammation .....</b>	<b>109</b>
<b>Abstract .....</b>	<b>110</b>
<b>Introduction.....</b>	<b>111</b>
<b>Materials and Methods .....</b>	<b>113</b>
Mice.....	113
Processing of tissues and leukocyte isolation.....	114

Reagents.....	115
Apoptosis assay .....	115
Proliferation assay.....	116
Intracellular cytokine staining .....	116
In vivo cytotoxicity assay .....	116
Plasma cytokine analysis .....	116
Phospho-STAT3 (Tyr705) staining.....	117
Statistical analyses.....	118
<b>Results.....</b>	<b>118</b>
LPS and poly(I:C) generate distinct cytokine milieus .....	118
LPS promotes survival of activated alloreactive CD8 T cells .....	121
LPS and poly(I:C) alter the phenotype of alloreactive CD8 T cells in response to the alloantigen .....	130
LPS suppresses alloreactive CD8 T cell IFN- $\gamma$ production and cytotoxicity .....	133
LPS signaling through recipient and not donor cells is critical for suppression of alloreactive CD8 T cell function.....	140
IL-6, and not IL-10, is necessary for LPS mediated suppression of alloreactive CD8 T cell function .....	143
<b>Discussion.....</b>	<b>150</b>
 <b>CHAPTER V .....</b>	<b>160</b>
<b>Improved B cell development in humanized NOD-<i>scid</i> <i>IL2R<math>\gamma</math></i><sup>null</sup> mice transgenically expressing human stem cell factor, granulocyte-macrophage colony-stimulating factor and interleukin-3.....</b>	<b>160</b>
<b>Abstract .....</b>	<b>161</b>
<b>Introduction.....</b>	<b>161</b>
<b>Materials and Methods .....</b>	<b>164</b>
Mice.....	164
Generation of BLT mice .....	165
Antibodies and flow cytometry .....	165
Infections and ELISAs.....	166
Statistical analyses.....	167
<b>Results.....</b>	<b>167</b>
NSG-SGM3 BLT mice show accelerated human cell chimerism as compared to NSG BLT mice .....	167
NSG-SGM3 BLT mice support human T cell development.....	170
NSG-SGM3 BLT mice support human B cell development .....	170
NSG-SGM3 BLT mice support enhanced myeloid cell development compared to NSG BLT mice .....	172
NSG-SGM3 BLT mice show improved engraftment of CD4 <sup>+</sup> regulatory T cells as compared to NSG BLT mice .....	175
NSG-SGM3 BLT mice develop higher levels of mature naïve B cells compared to NSG BLT mice .....	177

NSG-SGM3 BLT mice show an improved ability to generate IgG antibodies .....	180
<b>Discussion.....</b>	<b>182</b>
 <b>CHAPTER VI .....</b>	 <b>189</b>
<b>Discussion.....</b>	<b>189</b>
 <b>CHAPTER VII .....</b>	 <b>202</b>
<b>References .....</b>	<b>202</b>

## **List of Tables**

Table 2.1. DST/anti-CD40 treatment inhibits expansion of alloreactive CD8 T cells in blood.....	78
Table 4.1. LPS and poly(I:C) differentially regulate kinetics of CD8 T cells in blood upon alloantigen challenge.....	122

## List of Figures

Fig. 1.1. LPS and poly(I:C) are recognized by distinct receptors. ....	19
Fig. 1.2. Mechanisms of allorecognition .....	32
Fig. 2.1. Anti-CD40 mAb is a fully antagonistic antibody. ....	69
Fig. 2.2. Anti-CD40 mAb is equivalent to anti-CD154 mAb at prolonging allograft survival. ....	72
Fig. 2.3 Anti-CD40 mAb combined with DST reduces the generation of graft-reactive antibodies and IFN- $\gamma$ producing effector/memory CD8 T cells. ....	74
Fig. 2.4. DST/anti-CD40 mAb treatment inhibits cytolysis of allogeneic target cells. ....	76
Fig. 2.5. DST/anti-CD40 mAb treatment inhibits alloreactive CD8 <sup>+</sup> T cell expansion and function. ....	79
Fig. 2.6. Anti-CD40 mAb does not deplete CD40 expressing cells. ....	83
Fig. 2.7. CD4 <sup>+</sup> Foxp3 <sup>+</sup> Tregs are necessary for tolerance induction by DST/anti-CD40 mAb treatment. ....	85
Fig. 2.8. LPS prevents DST/anti-CD40 mediated tolerance induction. ....	87
Fig. 2.9. LPS challenge increases levels of donor-reactive IFN- $\gamma$ producing CD8 T cells, but not alloantibodies in response to DST/anti-CD40. ....	89
Fig. 3.1. Costimulation blockade induced tolerance to allografts is dependent on Bim and Fas. ....	105
Fig. 4.1. LPS and poly(I:C) generate distinct cytokine milieus. ....	120
Fig. 4.2. LPS enhances survival of alloreactive CD8 T cells in response to antigen challenge. ....	123
Fig. 4.3. Trafficking to other compartments does not account for the early loss of alloreactive CD8 T cells from the spleen in response to alloantigen. ....	125
Fig. 4.4. LPS challenge does not induce division of alloreactive CD8 T cells within 24 hours of alloantigen challenge. ....	127
Fig. 4.5. LPS challenge generates higher levels of memory alloreactive CD8 T cells. ....	129
Fig. 4.6. LPS and poly(I:C) alter the phenotype of alloreactive CD8 T cells in response to antigen challenge. ....	131
Fig. 4.7. LPS suppresses alloreactive CD8 T cell function. ....	134
Fig. 4.8. The endogenous alloreactive CD8 T cell kinetics are similar to those of the KB5 transgenic system. ....	137
Fig. 4.9. Poly(I:C) challenge enhances the levels of granzyme B producing effector alloreactive CD8 T cells. ....	139
Fig. 4.10. TLR4 expression on recipient cells is necessary for LPS mediated suppression of CD8 T cell function. ....	141
Fig. 4.11. IL-10 signaling is not necessary for LPS to suppress IFN- $\gamma$ production by effector alloreactive CD8 T cells. ....	144
Fig. 4.12. IL-6 is necessary but not sufficient for LPS mediated suppression of CD8 T cell function. ....	146

Fig. 4.13. Summary: LPS and poly(I:C) differentially regulate alloreactive CD8 T cell kinetics and function in response to donor alloantigen (DST).....	159
Fig. 5.1. Human CD45 <sup>+</sup> cell engraftment kinetics in the peripheral blood, spleen and bone marrow of NSG BLT mice and NSG-SGM3 BLT mice. ....	169
Fig. 5.2. Human CD3 <sup>+</sup> T cell engraftment kinetics in the peripheral blood, spleen and bone marrow of NSG BLT mice and NSG-SGM3 BLT mice. ....	171
Fig. 5.3. Human CD20 <sup>+</sup> B cell engraftment kinetics in the peripheral blood, spleen and bone marrow of NSG BLT mice and NSG-SGM3 BLT mice. ....	173
Fig. 5.4. Human CD33 <sup>+</sup> myeloid cell engraftment kinetics in the peripheral blood, spleen and bone marrow of NSG BLT mice and NSG-SGM3 BLT mice ...	174
Fig. 5.5. Characterization of human CD3 <sup>+</sup> T cells in the peripheral blood of NSG BLT and NSG-SGM3 BLT mice at 12 week-post transplantation .....	176
Fig. 5.6. Characterization of human CD20 <sup>+</sup> B cells in the peripheral blood, spleen and bone marrow of NSG BLT and NSG-SGM3 BLT mice at 12 weeks post-transplantation .....	178
Fig. 5.7. Evaluation of total antibody titers and dengue virus specific antibody responses in NSG BLT and NSG-SGM3 BLT mice .....	181
Fig. 6.1. Model .....	195

### List of copyrighted material

Some sections in this thesis appear/will appear in the following publications:

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### List of Abbreviations

ADCC	antibody-dependent cell-mediated cytotoxicity
AICD	activation-induced cell death
AIM2	absent in melanoma 2
ALR	AIM2-like receptor
Apaf-1	apoptotic protease activating factor 1
AMR	antibody-mediated rejection
APC	antigen presenting cell
AP-1	activator protein-1
ATG	anti-thymocyte globulin
Bad	Bcl-2 associated death protein
BAFF	B cell activating factor
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2 associated X protein
Bcl-xL	B-cell lymphoma extra-large
Bcl-2	B-cell lymphoma 2
BCR	B cell receptor
Bfl-1	Bcl2 related gene expressed in fetal liver 1
BH3	Bcl-2 homology domain3
Bik	Bcl-2 interacting killer
Bim	Bcl-2 interacting molecule
BLT	bone marrow/liver/thymus



BTLA	B and T lymphocyte attenuator
CAR	chimeric antigen receptor
CD	cluster of differentiation
CDC	complement-dependent cytotoxicity
CFSE	Carboxyfluorescein succinimidyl ester
CLR	C type lectin receptor
CNS	central nervous system
CTL	cytotoxic T lymphocyte
CTLA4	cytotoxic T lymphocyte-associated antigen 4
cGy	centiGray
DAMP	damage associated molecular pattern
DC	dendritic cell
DISC	death-inducing signaling complex
ds	double-stranded
DSA	donor-specific antibodies
DST	donor specific transfusion
DT	diphtheria toxin
EC	endothelial cell
ECDI	ethylenecarbodiimide
EGFP	enhanced green fluorescence protein
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum

FACS	Fluorescence-activated cell sorting
FBS	fetal bovine serum
Foxp3	forkhead box P3
GM-CSF	granulocyte macrophage colony stimulating factor
GvHD	graft versus host disease
HIV-1	human immunodeficiency virus 1
HLA	human leukocyte antigen
HMGB1	high mobility group box 1
HMW	high molecular weight
HRP	horseradish peroxidase
HSC	hematopoietic stem cell
HSP	heat shock protein
HSV	Herpes simplex virus
ICAM-1	intercellular adhesion molecule
ICOS	inducible costimulatory molecule
IELs	intraepithelial lymphocytes
IFI16	interferon gamma inducible protein 16
IFN	interferon
Ig	immunoglobulin
IL	interleukin
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal

IRF	interferon regulatory factor
IRI	ischemia reperfusion injury
ITAM	immunoreceptor tyrosine-based activation motif
iTreg	induced Treg
i.v.	intravenous
JAK	Janus kinase
LBP	LPS binding protein
LCMV	lymphocytic choriomeningitis virus
LFA-1	lymphocyte function-associated antigen 1
LGP2	laboratory of genetics and physiology 2
LPS	lipopolysaccharide
mAb	monoclonal antibody
MAVS	mitochondrial antiviral-signaling protein
MFI	mean fluorescence intensity
Mcl-1	myeloid leukemia cell differentiation protein 1
MDA5	melanoma differentiation-associated protein 5
MDSC	myeloid derived suppressor cells
MHC	major histocompatibility complex
6-MP	6-mercaptopurine
MPECs	memory precursor effector cells
Mreg	regulatory macrophage
MSC	mesenchymal stem cell

MST	median survival time
mTOR	mammalian target of rapamycin
MyD88	myeloid differentiation primary response gene 88
NFAT	nuclear factor of activated T cells
NF- $\kappa$ B	nuclear factor kappa B
NHP	non-human primate
NK	natural killer
NKG2D	natural-killer group 2, member D
NLR	NOD-like receptor
NOD	nucleotide-binding oligomerization domain
NSG	NOD-scid IL2R $\gamma$ null
OVA	ovalbumin
PAMP	pattern associated molecular pattern
PBL	peripheral blood leukocyte
PBS	phosphate buffer saline
poly(I:C)	polyinosinic:polycytidylic acid
PD-1	programmed death-1
PKR	protein kinase R
PML	progressive multifocal leucoencephalopathy
PRR	pattern recognition receptor
Puma	p53 upregulated modulator of apoptosis
Rag	recombination activating gene

RBC	red blood cell
RIG-I	retinoic acid-inducible gene I
RLR	RIG-I-like receptor
SCF	stem cell factor
scid	severe combined immunodeficiency
SIRP $\alpha$	signal regulatory protein alpha
SLECs	short-lived effector cells
SRC	SCID repopulating cell
STAT	signal transducer and activator of transcription
TBI	total body irradiation
TCR	T cell receptor
TGF	transforming growth factor
TIM	T cell immunoglobulin and mucin domain
TIR	Toll/interleukin-1 receptor
TIRAP	TIR-containing adaptor protein
TLR	Toll like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	tumor necrosis factor
TRAM	TRIF-related adaptor molecule
Treg	regulatory T cell
TRIF	TIR domain-containing adaptor inducing IFN $\beta$
UV	ultraviolet

VCAM-1	vascular cell adhesion molecule 1
VLA-4	very late antigen 4
VPD 450	Violet Proliferation Dye 450
WT	wild type

## **Preface**

Experiments in Fig. 2.1 have been performed by Kerry L.M. Ralph.

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Experiments in Fig. 5.7C and 5.7D have been performed by Dr. Anuja Mathew.

# **CHAPTER I**

## **Introduction**



## **Rationale and thesis outline**

T lymphocytes of the adaptive immune system protect the host from infections with pathogens and malignancies. An important feature of the immune system is the ability to differentiate 'self' from 'non-self' or 'altered self'. Mechanisms such as negative selection of self-reactive thymocytes are in place to ensure that no immune response is mounted to the host's own tissues. Dysregulation of these mechanisms can result in autoimmunity and hence, aberrant activation of T cells needs to be prevented. In the context of infections, an uncontrolled T cell response can result in immunopathology and tissue damage. T cell responses are therefore carefully regulated.

In the context of organ transplantation graft-reactive T cell responses need to be suppressed to facilitate transplant survival. Organ transplantation is the only treatment option available for end stage organ failure, but successful organ survival requires chronic immune suppression in order to prevent allograft rejection. CD8 T cells are crucial mediators of acute rejection and kill target cells directly by secreting cytotoxic proteins perforins and granzymes. Understanding how CD8 T cells are activated in the context of transplantation will aid in the development of therapies to dampen their responses.

The goal of my thesis research is to examine how costimulation and inflammation impact alloreactive CD8 T cell responses. Costimulation blockade therapies have shown success in prolonging allograft survival in rodent and non-human primate models of transplantation (Ford, 2016). Using a well-established

skin graft model of transplant rejection, I investigated the role of CD40-CD154 costimulation in generating alloreactive CD8 T cell responses across major histocompatibility barriers. Inflammation resulting from tissue damage during organ procurement and surgery as well as infections in the peri-transplant period can impact graft survival. Using a TCR transgenic model to study alloreactive CD8 T cells in vivo, I examined the impact of two inflammatory agents LPS and poly(I:C) on alloreactive CD8 T cell responses. Finally, I studied how cytokines influence B cell development and antibody responses using a humanized mouse model.

The following introduction will provide a background on the topics that are relevant for this thesis. Although the focus of the thesis is transplantation immunology, I have referenced some studies in the fields of viral infection, cancer immunity and vaccines since knowledge obtained from these studies has directly helped transplantation research. I have provided a brief overview of signals required for CD8 T cell activation to set the stage for subsequent topics. I have then described the mechanisms of graft rejection, the strategies employed by researchers and clinicians to improve graft survival, and the hurdles in achieving transplantation tolerance. I have ended with an overview of humanized mice and their advantages and limitations in studying human immunity.

### **CD8 T cell activation: signal 1 (antigen recognition)**

CD8 T cell activation requires antigen processing and presentation by antigen presenting cells (APCs). Dendritic cells (DCs), macrophages and B cells are the professional APCs of the immune system. DCs sample antigen in the local environment and migrate to draining lymph nodes where they present the processed antigen to naïve CD8 T cells to stimulate their activation. Upon activation, naïve CD8 T cells undergo proliferation and differentiate into effector cells or cytotoxic T cells (CTLs) that kill infected cells through secretion of perforins and granzymes. Effector CD8 T cells also produce cytokines such as IFN- $\gamma$  and TNF- $\alpha$ . Following elimination of the invading pathogen, effector CD8 T cells undergo contraction where 90-95% of the antigen-specific cells die and the remaining cells survive to become long-lived memory CD8 T cells (Kaech and Cui, 2012). Memory CD8 T cells are highly sensitive to restimulation and the memory or secondary T cell response is faster than the primary T cell response (Russ et al., 2012).

Optimal CD8 T cell activation requires 3 signals. The first signal is the interaction between the T cell receptor (TCR) and cognate MHC-peptide complex on the antigen-presenting cell. The second signal is delivered by costimulatory and coinhibitory molecules on the APC and the balance between these costimulatory and coinhibitory signals determines the outcome of the CD8 T cell-APC interaction. The third signal is a cytokine signal. CD8 T cell activation by the integration of these three signals drives their differentiation to fully functional effector T cells and eventually memory T cells.

CD8 T cells recognize foreign peptides derived from pathogens or tumors in the context of self-MHC class I. In the context of tissue transplantation, CD8 T cells can recognize foreign peptides in the context of foreign MHC, a process termed allorecognition (Afzali et al., 2008), which I have described in a subsequent section. Classical MHC class I antigen presentation involves peptides that are generated in the cytosol upon degradation of proteins by the proteasome. These peptides are translocated into the endoplasmic reticulum (ER) by transporter associated with antigen processing (TAP) where they may undergo further trimming by aminopeptidases to generate 8-10 amino acid long peptides that are then loaded onto MHC class I molecules. The peptide-MHC complex is transported to the cell surface through the Golgi complex and exocytic vesicles. This pathway thus allows CD8 T cells to respond to infected or neoplastic cells displaying foreign or modified self-peptides respectively (Blum et al., 2013). Alternatively, APCs may capture exogenous antigen by endocytosis and present it on MHC class I to CD8 T cells, a process known as cross-presentation. This mechanism is important for pathogens that do not directly infect the APC. DCs are the main cross-presenting APCs in vivo. There are two pathways for cross-presentation of exogenous antigens: cytosolic and vacuolar (Joffre et al., 2012). In the cytosolic pathway, antigen is transferred to the cytosol where it is processed by the classical MHC I antigen presentation machinery. The vacuolar pathway involves antigen processing in endocytic compartments

where the antigen is cleaved by endosomal proteases and loaded onto MHC-I directly within the endosome.

The  $\alpha:\beta$  heterodimeric TCR associates with the CD3 complex consisting of CD3 $\gamma$ , CD3 $\delta$  and two CD3 $\epsilon$  chains along with a homodimer of  $\zeta$  chains to form the T cell receptor complex. The T cell receptor complex has ITAMs (immunoreceptor tyrosine-based activation motifs) that are phosphorylated by Src kinases Lck and Fyn. The co-receptor CD8 binds MHC-I and clusters with the TCR complex to facilitate phosphorylation of the ITAMs and subsequent signaling into the cell. The signal transduction cascade results in the activation of transcription factors NF- $\kappa$ B, NFAT and AP-1 that stimulate transcription of genes involved in T cell proliferation and differentiation (Smith-Garvin et al., 2009).

### **CD8 T cell activation: signal 2 (costimulation)**

The recognition of cognate MHC-peptide complexes by the T cell receptor triggers TCR signaling, but the cumulative effect of costimulatory and coinhibitory signals regulates T cell function and fate. Most costimulatory and coinhibitory molecules belong to the CD28 family or the tumor necrosis factor receptor (TNFR) family (Ford, 2016). Since the focus of my thesis is alloreactive T cells, I have described in this section the costimulatory and coinhibitory molecules that are most relevant in the context of transplantation.

#### CD28/B7/CTLA4

CD28 was the first costimulatory molecule identified and was shown to augment proliferation and IL-2 production by T cells after TCR stimulation thus amplifying the response (June et al., 1987). CD28 is constitutively expressed on T cells and binds B7 molecules B7-1 or CD80 (Linsley et al., 1990) and B7-2 or CD86 (Azuma et al., 1993; Hathcock et al., 1993) expressed on the surface of APCs. The expression of B7 molecules is upregulated when the APC senses pathogen associated molecular patterns (PAMPs) through innate immune receptors. Ligation of CD28 with B7 molecules activates several signaling pathways and delivers a positive signal for T cell survival and growth. The coinhibitory receptor CTLA4 (cytotoxic T lymphocyte-associated antigen 4) was subsequently identified (Linsley et al., 1991). CTLA4 is induced on T cells upon activation, binds CD80 and CD86 with higher affinity than CD28 and negatively regulates T cell responses. After the CD28-B7 interaction was characterized, the 'two signal' model of T cell activation was accepted, which entailed that both signal 1 (the specific signal between TCR and peptide-MHC complex) and signal 2 (the non-specific signal between CD28 and B7) are required for T cell proliferation and function (June et al., 1994).

#### CD40/CD154

CD40, a TNFR family member, was first identified on bladder carcinoma cells and found to be expressed on B cells (Paulie et al., 1985). The CD40 ligand (CD154) was identified on activated CD4 helper T cells and its interaction with CD40 on B cells was shown to promote T-dependent humoral immunity (Noelle

et al., 1992). Subsequent studies defined roles for the CD40-CD154 signal in promoting T cell immunity (Caux et al., 1994; Cella et al., 1996). CD40 is constitutively expressed on B cells, macrophages and DCs and expression is inducible on parenchymal cells such as epithelia, endothelia, fibroblasts and keratinocytes upon inflammation (Larsen and Pearson, 1997). CD154, the ligand for CD40, is expressed predominantly on activated CD4 T cells, although expression has also been demonstrated on activated B cells, smooth muscle cells, DCs and platelets (Ma and Clark, 2009). Ligation of CD154 on activated CD4 T cells with CD40 on APCs promotes APC maturation by increasing expression of CD80, CD86, MHC class I and II and production of cytokines. Moreover, CD40 signaling into DCs increases DC life span by promoting upregulation of the anti-apoptotic factor Bcl-xL in an NF- $\kappa$ B dependent manner (Wong et al., 1997). Activated DCs can then effectively prime naïve CD8 T cells. Some studies have reported CD154 expression on activated CD8 T cells (Han et al., 2007; Hernandez et al., 2007; Wong et al., 2008). In the absence of CD4 T cells, interaction between CD8 T cell expressed CD154 and DC expressed CD40 contributes to the generation of a maximal primary CD8 T cell response (Hernandez et al., 2007).

#### OX40/OX40L

Originally identified as a T cell activation marker, OX40 was discovered to have costimulatory function (Calderhead et al., 1993). OX40 is a member of the TNFR superfamily and inducible expressed on both activated CD4 and CD8 T cells.

Although TCR signaling is sufficient to induce OX40 expression, CD28-B7 interactions enhance and sustain its expression (Walker et al., 1999). OX40L is expressed on APCs upon CD40 stimulation or activation of Toll-like receptors (TLRs) (Godfrey et al., 1994). OX40 signaling directly promotes proliferation and survival of CD8 T cells after antigen encounter and generation and maintenance of memory CD8 T cells (Croft et al., 2009).

#### LFA-1/ICAM-1 and VLA-4/VCAM-1

The expression of the integrin  $\alpha_L\beta_2$  or LFA-1 (lymphocyte function-associated antigen 1) on T cells is crucial for cell arrest and migration on surfaces expressing its ligand ICAM-1 (intercellular adhesion molecule 1) and for forming immunological synapses with APCs reducing the amount of antigen necessary for T cell activation (Hogg et al., 2011). TCR stimulation activates LFA-1, which in turn enhances CD8 T cell responses by triggering Erk1/2 signaling (Li et al., 2009). LFA-1 signaling is critical for effective memory CD8 T cell responses (Cox et al., 2013; Scholer et al., 2008).

VCAM-1 (vascular cell adhesion molecule 1) is expressed on endothelial cells at low levels and upregulated upon inflammation. T cell activation increases expression of  $\alpha_4\beta_1$  integrin or VLA-4 (very late antigen 4) that promotes T cell migration to sites of inflammation as well as interactions with proteins of the extracellular matrix that can enhance TCR signaling. VLA-4 also contributes to the immunological synapse (Denucci et al., 2009).

#### ICOS/ICOS-L



ICOS (inducible costimulatory molecule), a CD28 family member, is inducibly expressed on T cells upon activation and has overlapping functions with CD28 in regulating CD8 T cell proliferation and function (Nurieva et al., 2009).

#### 4-1BB/4-1BBL

4-1BB, a TNFR superfamily member, is inducibly expressed upon T cell activation and CD28 signaling and its interaction with 4-1BBL promotes CD8 T cell survival by upregulating anti-apoptotic proteins Bcl-xL and Bfl-1 (Lee et al., 2002) and downregulating pro-apoptotic protein Bim (Sabbagh et al., 2008). This interaction also promotes CD8 T cell proliferation and amplifies CTL responses in vivo (Shuford et al., 1997).

#### CD27/CD70

CD27, a TNFR superfamily member, is constitutively expressed on naïve CD4 and CD8 T cells (Nolte et al., 2009). Although TCR stimulation elevates CD27 expression, CD27 is downregulated after T cells undergo multiple rounds of division and differentiate to effector cells. CD70 is transiently expressed on activated T cells and DCs following TCR stimulation and CD40/TLR signaling respectively. CD27/CD70 interactions promote effector and memory T cell generation by inducing proliferation and survival of T cells and are critical for the quality of memory CD8 T cell responses (Nolte et al., 2009)

#### TIM family members

The TIM (T cell immunoglobulin mucin) family member TIM-1 is expressed on activated CD4 T cells and promotes T cell proliferation and cytokine production

upon interaction with its ligand TIM-4 on APCs (de Souza et al., 2005; Meyers et al., 2005). TIM-3 is a coinhibitory molecule expressed on activated CD4 T cells and interacts with its ligand galectin-9 to inhibit T cell proliferation and cytokine production (Sabatos et al., 2003).

#### PD-1/PD-L1

PD-1 (programmed death 1), a member of the CD28 family, is expressed on activated T cells and functions in the effector phase of an immune response to negatively regulate the response. The ligand PD-L1 is widely expressed in peripheral tissues whereas expression of the ligand PD-L2 is restricted to DCs and monocytes and can be induced on other cell types depending on the local microenvironment. PD-1 has a higher binding affinity for PD-L2. Ligation of PD-1 with its ligands reduces the magnitude of an ongoing T cell response (Buchbinder and Desai, 2016). Elevated and sustained expression of PD-1 is a hallmark of T cell 'exhaustion', a state characterized by progressive loss of T cell effector function and proliferative potential, resulting from persistent exposure to antigen and inflammation observed in chronic infections and cancer (Wherry and Kurachi, 2015). PD-1 blockers nivolumab and pembrolizumab have shown therapeutic benefits in many cancer patients by reinvigorating the anti-tumor T cell response (Buchbinder and Desai, 2016).

Costimulatory and coinhibitory molecules regulate specific aspects of T cell activation such as survival, proliferation, differentiation to effector or memory

cells and primary or recall responses and contribute to the quality of the response.

### **CD8 T cell activation: signal 3 (cytokines)**

Although signal 1 (TCR stimulation) and signal 2 (costimulation) can initiate proliferation of naïve CD8 T cells, they are not sufficient to develop optimal effector function and formation of an effective memory population. In vitro experiments demonstrated that IL-12 (Curtsinger et al., 1999) and type I interferons (Curtsinger et al., 2005) provide a signal directly to CD8 T cells to stimulate clonal expansion and differentiation to effector CD8 T cells. Using the adoptive transfer of antigen-specific T cells deficient in receptors for IL-12 and IFN $\alpha/\beta$  into wild type mice, several investigators reported the requirement for these two cytokines in generating optimal CD8 T cell responses to viral, bacterial and parasitic pathogens as well as tumors and transplants (Curtsinger and Mescher, 2010). These studies also illustrated that these cytokines are important for generating optimal memory CD8 T cells. The 'three signal' model of T cell activation is widely accepted and includes signal 3 as a cytokine signal with IL-12 and type I IFN being signal 3 for CD8 T cells. IL-1 is thought to be the signal 3 cytokine for the activation of naïve CD4 T cells (Curtsinger and Mescher, 2010).

Conceptually, signal 3 cytokines could promote accumulation of effector CD8 T cells by increasing survival or proliferation during the expansion phase of the T cell response. Recently Harty and colleagues demonstrated that IL-12 and

type I IFN extend the division of CD8 T cells in response to IL-2 by sustaining the expression of the high affinity IL-2 receptor CD25 (Starbeck-Miller et al., 2014). Gene expression analysis of CD8 T cells stimulated with signal 1+2 or signal 1+2+3 revealed significant changes between the two groups that included genes involved in CD8 T cell proliferation, survival, function, migration as well as fate determining transcription factors T-bet and Eomes (Starbeck-Miller et al., 2014). Histone deacetylase inhibitors mimicked the effects of IL-12 and type I IFN signaling indicating that signal 3 cytokines sustain gene expression by promoting histone acetylation (Agarwal et al., 2009). CD4 T cells provide help to CD8 T cells by stimulating DCs to produce signal 3 cytokines.

### **Innate immunity: cross-talk with adaptive immunity**

The innate immune system forms the first line of defense against infections and responds rapidly to invading pathogens. Cells are well-equipped with molecules that serve as sensors of conserved structures of pathogens termed 'pathogen associated molecular patterns' or PAMPs. These sensors, known as 'pattern recognition receptors' or PRRs are germline-encoded and upon sensing PAMPs, transduce signals that communicate to the cells the presence of a pathogen and trigger the production of interferons and inflammatory cytokines and alert the adaptive immune system to eliminate the pathogen.

### *Pattern recognition receptors*

Based on protein domain homology, PRRs are grouped into 5 families (Brubaker et al., 2015).

#### Toll-like receptors (TLRs)

TLRs are the best-characterized family of pattern recognition receptors and contain the TIR (Toll/interleukin-1 receptor) domain (Brubaker et al., 2015). They are transmembrane proteins located either on the cell surface where they sense extracellular pathogens or on endosomal membranes where they sense pathogens within the endosome. There are 10 known TLRs in humans (TLR1-TLR10) and 12 known TLRs in mice (TLR1-TLR9 and TLR11-TLR13). Cell surface TLRs include TLR1, TLR2, TLR4, TLR5, TLR6 and human TLR10 and endosomal TLRs include TLR3, TLR7, TLR8, TLR9 and mouse TLR11, TLR12 and TLR13 (Kawasaki and Kawai, 2014). TLR2 heterodimerizes with TLR1 or TLR6 to recognize distinct lipopeptides, peptidoglycan, lipoteichoic acids, zymosan and mannan. TLR4 recognizes bacterial lipopolysaccharide as well as several endogenous molecules released by stressed cells known as DAMPs (damage-associated molecular patterns) (Politorak et al., 1998). TLR5 binds flagellin from flagellated bacteria (Hayashi et al., 2001). TLR3 and TLR7 recognize viral nucleic acids double-stranded RNA (dsRNA) and single-stranded RNA (ssRNA) respectively (Alexopoulou et al., 2001; Hemmi et al., 2002). TLR9 predominantly recognizes unmethylated CpG DNA motifs that are not common in mammalian genomes (Hemmi et al., 2000). Human TLR8 and TLR13 respond to

viral and bacterial RNA (Heil et al., 2004; Oldenburg et al., 2012). TLR11 and TLR12 recognize profilin from *Toxoplasma gondii* (Koblansky et al., 2013; Yarovinsky et al., 2005).

#### C type lectin receptors (CLRs)

CLRs contain the C-type lectin-like domain and are soluble or transmembrane proteins. They recognize fucose, mannose and glucan carbohydrate structures of diverse pathogens (Geijtenbeek and Gringhuis, 2009) CLR signaling activates NF- $\kappa$ B and regulates TLR induced gene transcription.

#### RIG-I-like receptors (RLRs)

RLRs are cytosolic RNA sensors containing a DExD/H-box helicase core (Wu and Chen, 2014). These receptors differentiate 'non-self' from 'self' RNA by detecting 5' triphosphate RNA, long double stranded RNA and viral genome specific sequences such as the poly-uridine region of hepatitis C virus. RIG-I (retinoic acid-inducible gene I), MDA5 (melanoma differentiation-associated protein 5) and LGP2 (laboratory of genetics and physiology 2) are the three known members of this family. dsRNA sensing by RIG-I and MDA-5 is responsible for IFN production in response to viral infection (Yoneyama et al., 2004).

#### NOD-like receptors (NLRs)

NLRs are intracellular sensors characterized by a nucleotide-binding oligomerization domain (NOD). NLR signaling activates NF- $\kappa$ B, MAPKs and

caspase-1 to stimulate production of inflammatory mediators such as IL-1 $\beta$  (Chen et al., 2009a).

#### AIM2-like receptors (ALRs)

ALRs are intracellular DNA sensors containing a pyrin domain for protein-protein interactions and a HIN-200 DNA-binding domain. AIM2 (absent in melanoma 2) and IFI16 (interferon gamma inducible protein 16) are the known ALRs. These sensors are a component of inflammasomes, cytosolic multiprotein complexes that trigger production of IL-1 $\beta$  and IL-18 and a form of inflammatory cell death known as pyroptosis (Vanaja et al., 2015).

#### *LPS and poly(I:C)*

LPS and poly(I:C) are recognized by distinct cellular receptors (Fig. 1.1). TLR4 signaling upon LPS binding involves a multireceptor complex including the proteins LBP (LPS binding protein) (Tobias et al., 1986), CD14 (Wright et al., 1990) and MD2 (O'Neill et al., 2013). Upon LPS transfer to TLR4-bound MD2, TLR4 dimerizes and initiates a signal transduction cascade that requires adaptor proteins. The sorting adaptor TIRAP (TIR-containing adaptor protein) recruits the signaling adaptor MyD88 (myeloid differentiation primary response gene 88) to trigger signaling culminating in the activation of transcription factors NF- $\kappa$ B and AP-1 and the resultant transcription of proinflammatory cytokine genes. Once MyD88 signaling is initiated, TLR4 is endocytosed with the help of CD14. The sorting adaptor TRAM (TRIF-related adaptor molecule) recruits the signaling

adaptor TRIF (TIR domain-containing adaptor inducing IFN $\beta$ ) to trigger signaling culminating in the activation of transcription factors NF- $\kappa$ B and IRF3/IRF7 and transcription of type I interferon genes. Recently it was found that the murine non-canonical inflammasome caspase-11 directly binds intracellular LPS (Shi et al., 2014). When LPS reaches high concentrations in endotoxemia, its aberrant localization to cytosol activates caspase-11 that mediates pyroptotic cell death (Hagar et al., 2013).

Poly(I:C) is a synthetic dsRNA analog that binds TLR3 within endosomes and signals through the adaptor TRIF to activate NF- $\kappa$ B and IRF3/IRF7. Poly(I:C) is also recognized by RIG-I or MDA-5 depending on its length. MDA5 prefers long fragments (>4 kb) whereas RIG-I prefers enzyme digested shorter fragments (~300bp) (Kato et al., 2008). Both RNA sensors use the adaptor protein MAVS (mitochondrial antiviral-signaling protein) to initiate a signaling cascade that results in NF- $\kappa$ B and IRF3 activation and subsequent type I IFN production. Poly(I:C) is also recognized by the serine/threonine kinase PKR (protein kinase R) that suppresses the initiation of translation by phosphorylating eukaryotic initiation factor 2 and inhibits viral replication (Balachandran et al., 2000). Moreover, a triad of helicases DDX1, DDX21 and DHX36 was shown to bind poly(I:C) in the cytosol and use the TRIF pathway to stimulate type I IFN production (Zhang et al., 2011b).

*Regulation of T cell responses by PRRs*



The innate and adaptive immune systems work in a co-ordinated fashion to protect the host from pathogens. PRR signaling regulates the adaptive immune response at multiple stages of the response.

PRR signaling on DCs initiates DC maturation and migration to lymph nodes. TLR stimulation of immature DCs reduces the expression of the inflammatory chemokine receptor CCR6 and increases expression of lymphoid chemokine receptor CCR7 that enables DC migration from tissues to draining lymph nodes. (Dieu et al., 1998; Sallusto et al., 1998). TLR signaling through MyD88 increases expression of costimulatory molecules CD80 and CD86 and MHC-II on DCs and production of cytokines thereby facilitating efficient priming of naïve T cells (Pasare and Medzhitov, 2004; Schnare et al., 2001). As DCs mature, they lose their phagocytic potential. TLR4 stimulation with LPS was reported to transiently enhance antigen capture and subsequent cross-presentation if antigen was delivered at the same time as LPS (West et al., 2004). However, pre-treatment with LPS or poly(I:C) downregulated antigen uptake and cross-presentation (Weck et al., 2007).

Fig. 1.1. LPS and poly(I:C) are recognized by distinct receptors.

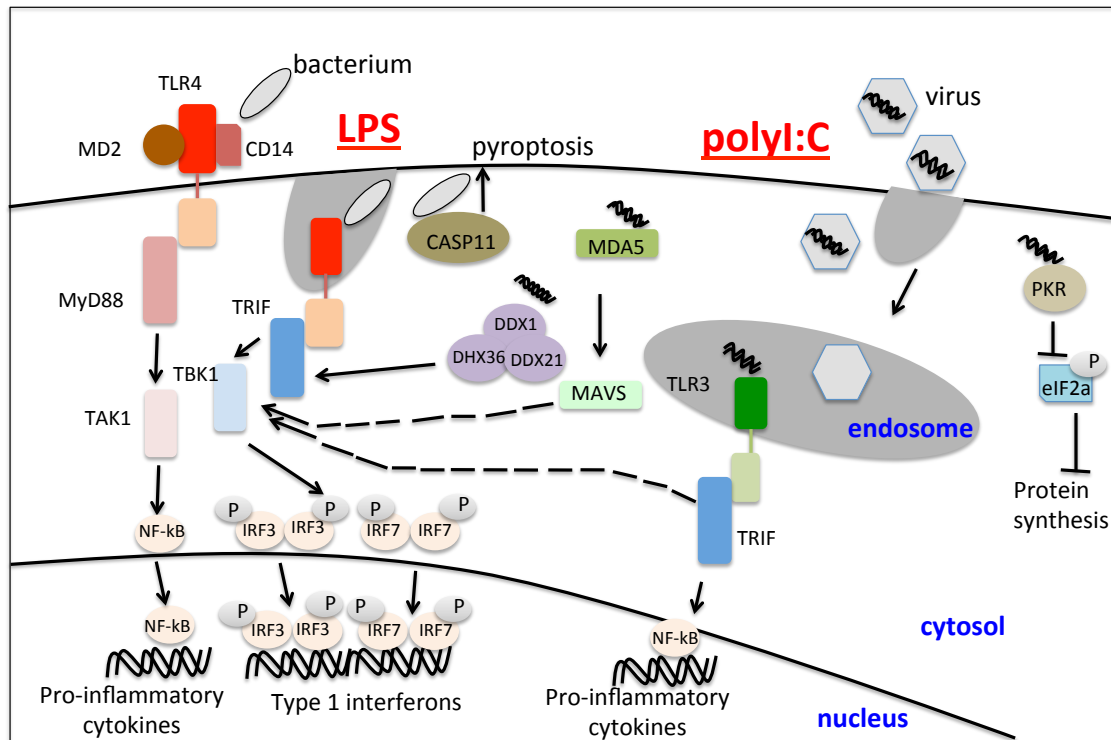


Fig. 1.1. LPS and poly(I:C) are recognized by distinct receptors. LPS from Gram negative bacterial membrane binds to CD14/TLR4/MD2 complex and signals through the adaptor MyD88 to activate NF-κB and transcription of inflammatory cytokine genes. Endocytosed LPS signals through the adaptor TRIF to phosphorylate and activate IRF3/IRF7 and transcription of type I interferon genes. Poly(I:C), a viral dsRNA mimetic, binds the endosomal receptor TLR3 and signals through TRIF. The triad comprising DDX1, DDX21 and DHX36 helicases sense poly(I:C) in the cytoplasm and use the TRIF pathway. Cytosolic MDA5 uses the adaptor MAVS and signals through TRIF to stimulate transcription of type I interferon genes. The cytoplasmic sensor PKR upon recognizing dsRNA, inhibits translation by phosphorylating eIF2α.

CD4 T cells may differentiate into Th1, Th2, Th9, Th17, Th22 or Tfh cells depending on the cytokines they encounter which in turn depends on the kind of microbial infection that is sensed by PRRs (DuPage and Bluestone, 2016). Th1 cells are induced upon intracellular bacterial and viral infection; Th2 and Th9 cells are induced upon extracellular parasite infections and Th17 cells are induced upon fungal and extracellular bacterial infections. A seminal study showed that TLR4 stimulation of DCs relieves conventional CD4 T cells from Treg-mediated suppression in an IL-6 dependent manner (Pasare and Medzhitov, 2003). Besides regulating CD4 T cell differentiation and function, TLR ligands regulate CTL function as well as antibody responses. TLR3 and TLR9 ligands are potent stimulators of cytotoxic CD8 T cell responses (Jelinek et al., 2011). Because of the ability of TLR ligands to costimulate T as well as B cell responses, they are desirable adjuvants for peptide or soluble protein based vaccines (Dowling and Mansell, 2016).

Although PRR signaling promotes the generation of T cell responses, emerging evidence indicates that it can also inhibit responses. TLR2 and TLR4 signaling has been associated with suppression of CD8 T cell responses. TLR4 engagement abrogated TLR3 induced priming (Mandraju et al., 2014) and function (Bogunovic et al., 2011) of CD8 T cells by altering the recruitment of DC subsets to draining lymph nodes and promoting secretion of the suppressive cytokine IL-10 respectively. Moreover, LPS has been shown to induce IL-10-producing Tregs (Tr1 cells) (den Haan et al., 2007) and immature myeloid

derived suppressor cells (MDSCs) (De Wilde et al., 2009) that suppress CD8 T cell immunity. TLR2-TLR6 binding lipopeptides were recently shown to suppress T cell immunity by inducing MDSCs in an IL-6 dependent manner (Skabytska et al., 2014). IL-6 can also act directly on CD8 T cells to inhibit their function (Wu et al., 2015). Clinical and experimental sepsis inhibits T cell immunity by inducing apoptosis of naïve T cells and lymphopenia (Danahy et al., 2016).

### **T cell apoptosis**

Apoptotic cell death is characterized by cell shrinkage, plasma membrane blebbing and formation of apoptotic bodies, chromatin condensation and DNA fragmentation (Elmore, 2007). Apoptosis of T and B lymphocytes is a tightly regulated process. Lymphocytes are deleted during development in the thymus and bone marrow due to unproductive rearrangements of genes that form the T and B cell receptors, inefficient recognition of self-ligands by the TCRs/BCRs (positive selection) and high affinity binding of developing lymphocytes to self-ligands (negative selection). Deletion of lymphocytes in the primary lymphoid organs serves to eliminate non-functional and potentially autoreactive cells and prevent autoimmunity. Clonal deletion of lymphocytes occurs in the periphery after an invading pathogen has been cleared from the system as the immune response wanes. Deletion at this stage is important to prevent immunopathology and establish homeostasis. Just as suboptimal death of lymphocytes is undesirable since it can result in autoimmunity, excessive pathology or

lymphoma depending on the context, uncontrolled death of lymphocytes can manifest as immunodeficiency that can be detrimental to the host. Hence, checkpoints exist in the life of lymphocytes to ensure that apoptosis occurs in a controlled manner. Two distinct pathways mediate apoptosis of T cells: extrinsic (death-receptor mediated) and intrinsic (mitochondrial) (Krammer et al., 2007). These pathways are not mutually exclusive and members of one pathway can regulate the other.

The extrinsic pathway is activated upon binding of death ligands to their cognate death receptors. The best-characterized death receptor/ligand pairs include Fas/FasL, TNFR1/TNF, DR3/Apo3L, DR4/Apo2L and DR5/Apo2L (Pobezinskaya and Liu, 2012). The death receptors belong to the TNF receptor superfamily and have an intracellular 'death domain' that transduces signals into the cell. Activation of the death receptor is followed by the formation of the death-inducing signaling complex (DISC) that includes an adaptor protein and procaspase-8. Formation of the DISC triggers autoproteolytic activation of procaspase-8. The resulting caspase-8 is an initiator caspase that activates executioner caspases caspase-3 and caspase-7 resulting in apoptosis.

The intrinsic pathway is activated by a variety of stimuli such as growth factor deprivation, cytotoxic drugs, irradiation and extracellular stresses and is mediated by Bcl-2 family members (Youle and Strasser, 2008). Bcl-2 family proteins are grouped into three classes: the pro-survival proteins Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and A1, the pro-apoptotic proteins Bax, Bak and Bok, and the BH3

domain-only proteins such as Bim, Bik, Bad, Noxa and Puma that are regulators of anti-apoptotic proteins. Activation of Bim by death stimuli activates pro-apoptotic proteins Bax and Bak that disrupt the outer mitochondrial membrane resulting in a loss of mitochondrial transmembrane potential and the release of cytochrome *c*. Cytochrome *c* binds Apaf-1 and procaspase-9 to form an apoptosome. Upon activation, the initiator caspase-9 activates executioner caspase-3, caspase-6 and caspase-7. Thus both extrinsic and intrinsic apoptotic pathways converge at the executioner caspases that cleave cellular substrates resulting in cell death. Anti-apoptotic proteins Bcl-2 and Bcl-xL repress Bax and Bak. BH3-only proteins relieve this repression by direct binding and inhibition of Bcl-2 and Bcl-xL.

The death receptor Fas is involved in activation induced cell death (AICD), a form of death induced upon restimulation of the TCR (Green et al., 2003). Mice deficient in functional Fas (*Fas<sup>lpr</sup>* mice) (Watanabe-Fukunaga et al., 1992) and functional FasL (*Fas<sup>gld</sup>* mice) (Takahashi et al., 1994) develop a lymphoproliferative disorder characteristic of humans with autoimmune lymphoproliferative syndrome caused by mutations in the *Fas* gene (Rieux-Laucat et al., 1995). Likewise, deficiency of the mitochondrial apoptosis inducer Bim results in autoimmunity in mice (Bouillet et al., 1999). These results suggest that Fas and Bim play a role in negative selection and/or peripheral deletion of autoreactive T cells that escape negative selection. Bim is indispensable for T cell contraction following acute HSV (Herpes simplex virus) or LCMV infection

because of a dearth of cytokines occurring after the antigen is cleared (Pellegrini et al., 2003; Weant et al., 2008). On the other hand, Fas is necessary for contraction following LCMV but not HSV infection (Pellegrini et al., 2003; Weant et al., 2008). It has been proposed that the strength of the signal received through the TCR in the periphery determines the ensuing response and its contraction (Bouillet and O'Reilly, 2009). Mice with combined deficiency in Fas and Bim exhibit autoimmunity and blocks in contraction of antiviral immune responses larger than those of mice with single Fas or Bim deficiency suggesting the cooperation of these proteins in regulating death of autoreactive and viral antigen-specific T cells (Hughes et al., 2008; Hutcheson et al., 2008; Weant et al., 2008).

### **Transplant rejection**

When cells or tissues are transplanted between genetically different individuals of the same species, the immune system of the recipient recognizes the donor graft as 'foreign', a process termed as allorecognition. Alloantigens comprise both major and minor histocompatibility antigens. The major histocompatibility complex (MHC) of humans, located on chromosome 6, is known as the HLA complex. In mice, the MHC is located on chromosome 17 and known as the H-2 complex. Minor histocompatibility antigens are proteins that are polymorphic and hence, may vary between the donor and recipient even if they are matched at the MHC. In recipients that are otherwise untreated, the immune system rejects the

donor graft. Rejection is classified as hyperacute, acute and chronic based on the time it takes to reject the graft.

### *Hyperacute rejection*

Hyperacute rejection develops within minutes to hours of organ transplantation (Moreau et al., 2013). This form of rejection is mediated by pre-existing antibodies that originate in the graft recipient from previous exposure to donor antigen through blood transfusions, previous transplantation or pregnancy. Pre-formed antibodies bind alloantigen expressed on the endothelium of the donor graft vasculature triggering the complement cascade and stimulating endothelial cells to secrete von Willebrand procoagulant factor resulting in thrombosis, recruitment of neutrophils and macrophages, parenchymal injury and graft necrosis (Moreau et al., 2013). This form of rejection is now an uncommon event because of routine pretransplant checking for ABO compatibility and cross-matching between donor cells and recipient sera for pre-formed donor-specific antibodies (Mulley and Kanellis, 2011). Hyperacute rejection can be modeled in rodents using passive transfer of high-titer antibodies to *Rag*<sup>-/-</sup> xenograft recipients (Ding et al., 2008) or high-titer antibodies with xenograft complement to *Rag*<sup>-/-</sup> allograft recipients (Koene et al., 1973).



### *Acute rejection*

Acute rejection in graft recipients occurs between 1 week and several months after transplantation and results in rapid graft loss (Moreau et al., 2013). This form of rejection is mediated by donor-reactive T cells (cellular rejection) and/or B cells (humoral rejection).

Upon activation by alloantigen presenting DCs, naïve CD4 T cells differentiate into Th1 and Th17 cells in the proinflammatory environment (Moreau et al., 2013). Similarly, naïve CD8 T cells are activated by DCs and differentiate into cytolytic effectors with help from CD4 T cells. The CTLs migrate to the graft where they recognize their allogeneic targets and induce apoptosis thereby mediating graft rejection. Besides aiding CTL differentiation, CD4 helper T cells secrete cytokines that stimulate B cells to trigger humoral rejection and recruitment and activation of other cell types such as macrophages and NK cells.

B cells, upon terminal differentiation to plasma cells, secrete alloantibodies that play an important role in acute rejection. Acute antibody-mediated rejection (AMR) is dependent on complement activation and diagnostic features of AMR include donor-specific antibodies (DSA) in the serum and C4d deposition (Stegall et al., 2012). Immunoglobulin (Ig) knockout mice show delayed rejection of cardiac allografts and passive transfer of complement activating antibodies promotes acute rejection mediated by T cells (Wasowska et al., 2001). Acute rejection can be achieved in the absence of T cells and B cells

in *Rag*<sup>-/-</sup> allograft recipients by the passive transfer of sera containing extremely high-titer alloantibodies (Nozaki et al., 2007).

Other immune cell types contribute to acute graft rejection but are neither necessary nor sufficient to mediate rejection (LaRosa et al., 2007). NK cells may promote graft rejection by directly inducing apoptosis in the graft epithelial cells via the NKG2D receptor (Zhang et al., 2008), amplify intragraft inflammation by producing chemokines (Kondo et al., 2000), sensitize endothelial cells (ECs) to CTL attack by inducing upregulation of MHC-I and MHC-II on the ECs in an IFN- $\gamma$  dependent manner (Ayalon et al., 1998; McDouall et al., 1997) or directly mediate graft rejection upon maximal stimulation by IL-15 (Kroemer et al., 2008). Macrophages comprise a significant proportion of the graft infiltrate in rejecting grafts and promote tissue damage by producing inflammatory cytokines as well as effector molecules such as reactive oxygen and nitrogen species and may act as APCs to primed CD4 T cells (Wyburn et al., 2005). Neutrophils migrate into the grafts early after transplantation (Celli et al., 2011) and produce chemokines that recruit T cells to the graft (Morita et al., 2001). Eosinophils infiltrate into the graft and mediate tissue damage by releasing inflammatory effectors and cationic granule proteins when the Th2 alloresponse predominates (Goldman et al., 2001).

### *Chronic rejection*

Chronic rejection is a gradual process occurring over months or years after transplantation (Moreau et al., 2013). With improvements in immunosuppressive medications lowering the risk of acute rejection, chronic rejection is now the leading cause of graft failure. The main feature of chronic rejection is progressive occlusion or intimal hyperplasia of blood vessels along with fibrosis of the graft tissue, processes that culminate in ischemia, cell death and graft failure (Chong et al., 2013). Several immunological and non-immunological factors such as multiple acute rejection episodes, immunosuppressant side effects, viral infection, hypertension and hyperlipidemia contribute to the pathogenesis of chronic rejection (Li and Yang, 2009). T cells and donor specific antibodies are the main immunological mediators of chronic rejection, although innate immune cells also play a role in the process. Alloantibodies activate endothelial cells to produce growth factors that promote the accumulation of extracellular matrix, a lesion of fibrosis. Endothelial cell activation also stimulates proliferation of smooth muscle cells and recruitment of inflammatory cells through release of chemokines that further contribute to graft rejection (Libby and Pober, 2001).

### *Mechanisms of T cell allorecognition*

T cells, predominant mediators of acute graft rejection, can recognize alloantigens by 3 pathways: direct, indirect and semi-direct (Fig. 1.2).

#### Direct allorecognition

APCs from the donor are co-transferred with the graft during organ transplantation. These APCs, termed as 'passenger leukocytes' migrate out of the graft to the secondary lymphoid tissues where they activate recipient T cells specific for the alloantigen, a process termed 'direct recognition' (Talmage et al., 1976). The principal passenger leukocytes capable of activating recipient T cells are DCs (Talmage et al., 1976). Approximately 1-10% of T cells in any individual are specific for foreign MHC-peptide complexes (Suchin et al., 2001; Veerapathran et al., 2011) whereas approximately 0.01% of the T cells recognize a foreign peptide bound to a self-MHC. Two models have been proposed to explain this unusually high precursor frequency of T cells with direct allospecificity. The peptide-centric model or the 'binary complexes hypothesis' postulates that foreign MHC structurally similar to self-MHC will bind an entire pool of foreign peptides that will activate recipient T cells with a wide range of specificities (Matzinger and Bevan, 1977). The MHC-centric model or the 'high determinant density' hypothesis postulates that TCRs will recognize foreign MHC with polymorphic amino acid residues in the TCR docking sites regardless of the bound peptide and the high density of these cognate MHC molecules on donor

APCs will activate high numbers of recipient T cells (Bevan, 1984). Direct recognition by CD8 T cells triggers a potent cytolytic response that destroys the graft. Studies have also showed that CD4 cells with direct allospecificity are sufficient to mediate acute rejection (Pietra et al., 2000). Since donor-derived APCs die over time, the direct pathway is believed to predominate during the early phase of acute rejection.

#### Indirect allorecognition

When allopeptides are presented to recipient T cells by recipient APCs in a self-MHC restricted manner, the process is termed 'indirect allorecognition'. In this scenario, the allopeptide may be derived from the foreign MHC molecule or a minor alloantigen. There are three possible mechanisms by which recipient APCs can acquire donor antigens. First, alloantigens from the graft may be shed into the circulation and engulfed by recipient APCs in secondary lymphoid tissue. Second, donor cells may be engulfed by recipient APCs in secondary lymphoid tissue. Third, recipient APCs may migrate to the graft, capture alloantigen and then migrate out to secondary lymphoid tissue. Although CD4 T cells are predominantly activated by allopeptides presented in the context of self-MHC II, CD8 T cells activated by cross-presentation can also contribute to graft rejection (Valujskikh et al., 2002). The requirement for antigen processing and presentation in the indirect pathway correlates with slower graft-reactive responses compared to those generated by the direct pathway. Since the

precursor frequency of T cells with indirect specificity is orders of magnitude less than T cells with direct specificity, the indirect pathway dominates in the later part of acute rejection after the passenger leukocytes have been eliminated and in chronic rejection.

#### Semi-direct allorecognition

Recipient APCs can acquire intact donor MHC-peptide complexes by direct cell-to-cell contact (trogocytosis) (Joly and Hudrisier, 2003) or endocytosis of donor derived vesicles or exosomes (Thery et al., 2002), a process termed 'cross-dressing' (Herrera et al., 2004). The process whereby recipient APCs acquire donor MHC-peptide complexes and activate CD8 T cells directly is referred to as semi-direct recognition. This mechanism allows the same recipient APC to activate CD8 T cells by semi-direct pathway and CD4 T cells by indirect pathway and thus, linked help to occur. The semi-direct pathway is now known to activate direct specificity CD8 T cells long after the passenger leukocytes have been eliminated (Smyth et al., 2017).

Fig. 1.2. Mechanisms of allorecognition

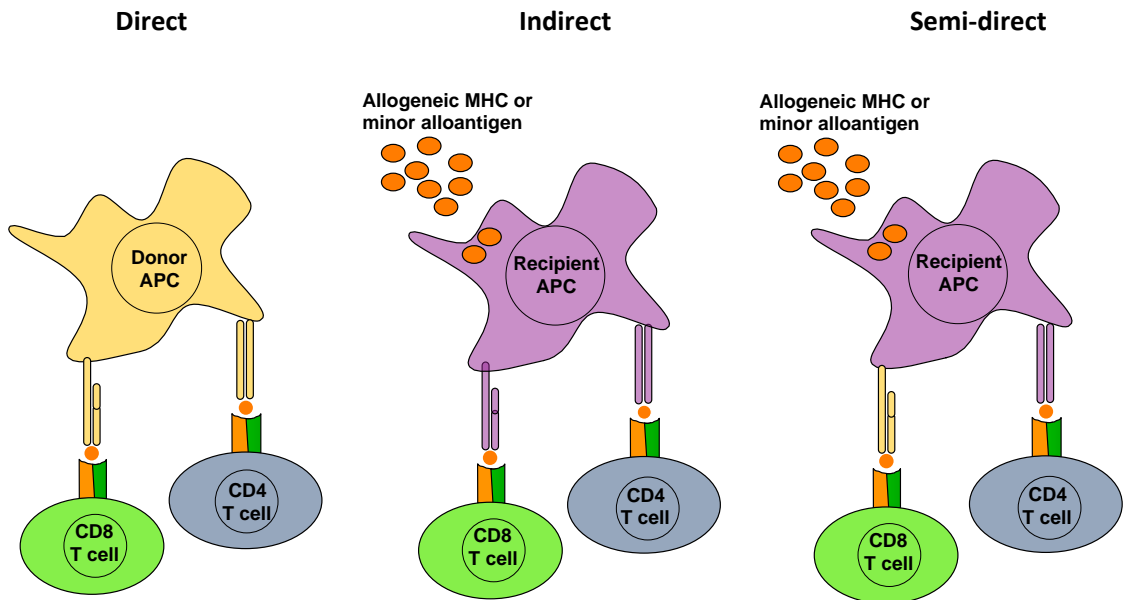


Fig. 1.2. Mechanisms of allorecognition. The direct pathway involves recognition of donor MHC-peptide complexes on donor APCs by recipient T cells. The indirect pathway involves uptake, processing and presentation of donor-derived alloantigen by recipient APCs to recipient T cells. The semi-direct pathway involves acquisition of donor MHC-peptide complexes by recipient APCs and direct presentation to CD8 T cells. In this pathway, CD4 T cells recognize donor derived alloantigen presented on recipient MHC by the indirect pathway.

### **Transplantation tolerance: why and how?**

#### *Immunosuppressants: old and new*

Transplant recipients receive immunosuppressants in order to prevent graft rejection by the immune system. The first successful renal transplantation was performed in 1954 at the Peter Bent Brigham Hospital in Boston under the direction of Joseph Murray between identical twins in the absence of immunosuppression (Merrill et al., 1956). This event opened up the possibility of extending transplantation to non-identical twins and stimulated research into immunosuppressants. The first immunosuppressant identified was cortisone, a glucocorticosteroid, which was used in combination with sub-lethal doses of total body irradiation (TBI) in the 1950s (Sayegh and Carpenter, 2004). Bone marrow aplasia developing as a result of TBI made the patients extremely susceptible to infections and death. After Robert Schwartz and William Dameshek reported the efficacy of the antimetabolite 6-mercaptopurine (6-MP) in rabbits in 1959 (Schwartz et al., 1959), variants of 6-MP were synthesized and screened and the candidate drug azathioprine was tested in clinical trials along with corticosteroids in 1962. This combination improved one-year allograft survival rate to 40-50%. The introduction of the depleting reagent anti-thymocyte globulin (ATG) in late 1960s further increased the one-year allograft survival rate to 70% (van Sandwijk et al., 2013). The advent of the calcineurin inhibitor cyclosporine in early 1980s marked the beginning of an era in clinical transplantation increasing one-year survival rates to over 80% (Kahan, 1989).



Based on the mechanism of action, immunosuppressants are classified as glucocorticosteroids, antimetabolites, calcineurin inhibitors, mTOR inhibitors, depleting and non-depleting antibodies (van Sandwijk et al., 2013). Glucocorticosteroids cause complex changes in immune cell populations and inhibit synthesis of inflammatory mediators. Antimetabolites such as azathioprine and mycophenolate mofetil inhibit DNA synthesis. Mycophenolate mofetil, first used in early 1990s, inhibits lymphocyte proliferation by blocking purine synthesis via inhibition of inosine monophosphate dehydrogenase and is more selective than azathioprine (Allison and Eugui, 2005). Calcineurin inhibitors such as cyclosporine and tacrolimus inhibit calcineurin and thereby block translocation of the transcription factor NFAT into the nucleus and expression of cytokine genes. mTOR inhibitor rapamycin (Sirolimus) blocks signal transduction by IL-2 and other cytokines by inhibiting the mTOR complex and has anti-proliferative effects on T and B cells. ATG and OKT3 are T cell depleting immunoglobulins. Alemtuzumab (Campath) is another depleting antibody that targets CD52 expressed on T, B and NK cells. Non-depleting antibodies in clinical use in transplantation include basiliximab that targets the IL-2 receptor alpha chain CD25.

Induction regimens for transplant recipients involve drugs with the highest intensity of immunosuppression since the risk for graft rejection is the highest in the early post-operative period (van Sandwijk et al., 2013). Lymphocyte depleting drugs and corticosteroids are usually the preferred drugs for induction therapy.

Maintenance regimens involve drugs with lower intensity of immunosuppression and lower toxicity and are given throughout the patient's life to prevent acute and chronic rejection (van Sandwijk et al., 2013). Calcineurin and mTOR inhibitors, antimetabolites along with corticosteroids are the preferred maintenance immunosuppressants. Despite strict adherence to immunosuppressive regimens, patients are at a risk of acute or chronic allograft rejection (Heemann and Lutz, 2013). The complications associated with the use of immunosuppressants such as toxicity and increased risk of infections and malignancy necessitate the development of safe and efficacious therapies (Christians et al., 2011; Gallagher et al., 2010; Jha, 2010). Achieving transplantation tolerance in the clinic is indeed the 'Holy Grail' for transplantation immunologists.

#### *Central and peripheral tolerance*

'Immunologic tolerance' is a state in which the immune system does not mount a response to a particular antigen in the absence of exogenous immunosuppression (Li et al., 2001). Self-tolerance is of utmost importance in order to prevent development of autoimmunity. There are two mechanisms of self-tolerance: central and peripheral. Central T cell tolerance is established in the thymus when developing T cells with high affinity for self-antigen/MHC complexes are eliminated (negative selection) (Hogquist et al., 2005). Since negative selection does not eliminate all autoreactive T cells, mechanisms must exist in the periphery to control the cells that escaped negative selection. These

mechanisms include deletion, anergy and immunoregulation and constitute peripheral tolerance (Mueller, 2010).

Spontaneous operational tolerance has been observed in liver and renal transplant patients where they maintain normal graft function after being weaned off immunosuppression, although this is a rare event (Benitez et al., 2013; Sagoo et al., 2010). The concept of 'actively acquired tolerance' was first introduced by Peter Medawar's group in early 1950s when they observed that injecting allogeneic tissue into mouse fetuses made the adult mice tolerant to skin grafts from the original donor strain (Billingham et al., 1953). This observation opened up the possibility of inducing tolerance to a foreign antigen by exposing the immature or developing immune system to the antigen. Transplantation immunologists have tried to induce tolerance to allografts based on the principle that strategies/therapies to induce and maintain transplantation tolerance will exploit the natural mechanisms of self-tolerance. Central tolerance induction approaches in animal models involve direct introduction of allogeneic cells into the thymus accompanied by anti-lymphocyte depleting serum (Odorico et al., 1993) or creation of mixed hematopoietic chimerism by injecting T cell depleted syngeneic and allogeneic bone marrow into irradiated hosts (Ildstad and Sachs, 1984). Deletion of the donor antigen specific T cells in the thymus by apoptosis is the major mechanism operating in central tolerance. Peripheral tolerance induction is more complex with multiple mechanisms operating simultaneously. Blocking of costimulatory molecules that are required for productive T cell

activation, termed as 'costimulation blockade', is an effective means of inducing peripheral tolerance to allografts. Deletion, anergy and immunoregulation are the major mechanisms that constitute peripheral tolerance.

### Deletion

Several studies in MHC-mismatched transplantation have indicated a requirement for T cell apoptosis by costimulation blockade therapies for successful peripheral tolerance induction. Both extrinsic and intrinsic apoptotic pathways play a role in peripheral tolerance induction. The resistance of IL-2 deficient mice to tolerance induction by costimulation blockade was ascribed to the failure of T cells to undergo Fas-mediated AICD (Dai et al., 1998; Wells et al., 1999). Moreover, costimulation blockade-induced deletion of alloreactive CD4 T cells was significantly impaired in *lpr* mice that lack functional Fas (Wekerle et al., 2001). Bcl-xL transgenic mice were also resistant to tolerance induction (Wells et al., 1999) and impaired costimulation blockade induced alloreactive CD4 T cell deletion (Wekerle et al., 2001). However, other studies revealed that tolerance induction can occur when either apoptotic pathway is dysfunctional suggesting that there may be a redundancy in the execution of T cell apoptosis upon costimulation blockade treatment (Li et al., 1999; Wagener et al., 2000). Since the frequency of alloreactive T cells in the periphery may be as high as 1 in 20 T cells (Suchin et al., 2001), apoptotic deletion may aid in controlling this extremely large pool of cells capable of mediating rapid graft destruction. Li et al. proposed a model in which apoptosis is required to reduce the unusually large size of the

alloreactive T cell pool to a level at which other mechanisms of tolerance, such as anergy and immunoregulation can operate to control the otherwise potent alloresponse (Li et al., 2001).

### Anergy

Gustav Nossal and Beverley Pike first described 'clonal anergy' as functional inactivation of B cells after injecting mice with tolerogenic doses of antigen (Nossal and Pike, 1980). Ronald Schwartz has defined anergy as a state in which 'a lymphocyte is intrinsically functionally inactivated following antigen encounter, but remains alive for an extended period of time in the hyporesponsive state' (Schwartz, 2003). In the context of transplantation tolerance, TCR activation in the absence of costimulation results in hyporesponsiveness of T cells to proliferate and produce cytokines such as IL-2 upon in vitro restimulation. Costimulation blockade induced CD4 T cell anergy characterized by reduced ability to proliferate and produce cytokines upon in vitro restimulation and inability to reject a graft when transferred into another recipient (Phillips et al., 2006; Quezada et al., 2005; Quezada et al., 2003). Recently, CD4 and CD8 T cell anergy has been demonstrated in mice treated with a tolerogenic anti-CD3 antibody (Baas et al., 2016; Besancon et al., 2017). CD8 anergy was characterized by reduced proliferation, IFN- $\gamma$  production and donor-responsiveness and was dependent on PD-1/PD-L1 and TGF $\beta$ /TGF $\beta$ RII pathways.

### Immunoregulation

Regulatory T cells are indispensable for the maintenance of self-tolerance and have an important role in peripheral tolerance induction. These cells suppress self-reactive and foreign antigen specific effector T cell responses. Richard Gershon first described a T cell subset capable of suppressing immune responses and mediating tolerance induction (Gershon and Kondo, 1970, 1971). However it was Shimon Sakaguchi and his group who established the identity of suppressor cells as regulatory T cells (Tregs), a distinct T cell lineage characterized as CD4<sup>+</sup> CD25<sup>+</sup> T cells in mice (Sakaguchi et al., 1995) and later by the transcription factor Foxp3 (Hori et al., 2003). Tregs are known to mediate immunosuppression by secreting anti-inflammatory cytokines IL-10, IL-35, TGF $\beta$ , releasing exosomes carrying miRNAs that silence T cell response genes, modulating APC maturation and function through CTLA4, directly inducing T cell apoptosis, suppressing IL-2 consumption by effector T cells or suppressing metabolism (Schmidt et al., 2012; Vaikunthanathan et al., 2017). Operationally tolerant liver recipients have an increased frequency of Tregs in peripheral blood compared to patients on immunosuppression (Li et al., 2004). In animal models of transplantation, Tregs play a vital role in the induction and maintenance of tolerance to allografts (Ferrer et al., 2014; Quezada et al., 2005; Walsh et al., 2004). Scientists are now exploiting the tolerogenic potential of Tregs to develop Treg cell-based therapies for tolerance induction in the clinic. I have discussed the therapeutic potential of Tregs in a subsequent section of the thesis.

### *Costimulation blockade*

Given the central role of costimulation in the optimal generation of T cell responses, blocking costimulatory signals therapeutically is a logical approach to prevent graft rejection mediated by T cells. I have described in brief some of the costimulatory pathways that have been targeted and shown success in animal models of transplant rejection.

#### CD28/B7

The first successful costimulation blockade reagent developed was CTLA4-Ig, a fusion protein consisting of the extracellular domain of CTLA4 and IgG (Linsley et al., 1991). CTLA4-Ig blocks B7 signaling and inhibits alloreactive T cell responses as well as T-dependent B cell antibody production. CTLA4-Ig (abatacept) showed significant activity in rheumatoid arthritis patients (Kremer et al., 2005) and was the first costimulation blockade agent to be approved by the FDA in 2005. Since abatacept did not show efficacy in non-human primate (NHP) transplant models (Levisetti et al., 1997), a high variant with increased affinity and avidity towards both B7 molecules CD80 and CD86 called belatacept was developed that showed better outcomes than abatacept (Larsen et al., 2005). Belatacept was approved by the FDA in 2011 and has shown improved renal function in kidney recipients compared to the current standard of care calcineurin inhibitor cyclosporine, although belatacept was associated with higher incidence and severity of acute rejection (Durrbach et al., 2016; Vincenti et al., 2010; Vincenti et al., 2016). It is speculated that belatacept may negatively impact

Tregs by inhibiting CD28 (Golovina et al., 2008) and CTLA4 signaling (Sojka et al., 2009), which has been shown to be important for Treg homeostasis and function. It was recently reported that long-term belatacept-treated kidney transplant patients exhibit reduced Treg suppressor function compared to healthy individuals and it may be a result of epigenetic regulation of *Foxp3* locus (Alvarez Salazar et al., 2017). Antibodies selectively targeting CD28 have been developed in order to maintain CTLA4 signaling and have shown superior outcomes in NHP transplant models relative to belatacept (Liu et al., 2014; Ville et al., 2016).

#### CD40/CD154

CD40/CD154 pathway has a tremendous potential for tolerance induction due to its critical impact on both T cell activation and generation of antibody responses. Antibodies targeting CD154 showed great outcomes in murine and NHP transplant models (Pinelli and Ford, 2015); however their clinical translation was precluded by the unanticipated development of thromboembolic complications in early clinical trials (Kawai et al., 2000). The prothrombotic effect of the antibody was thought to be associated with the expression of CD154 on platelets and destabilization of platelet plugs by anti-CD154 and/or the crosslinking of the Fc receptor FcγRIIa on platelets by anti-CD154-soluble CD154 immune complexes (André et al., 2002; Robles-Carrillo et al., 2010). After the clinical trials with the anti-CD154 antibodies were halted, researchers directed their efforts towards developing Fc-silent anti-CD154 antibodies and reagents targeting the receptor CD40. Fc-silent anti-CD154 domain antibodies (Kim et al., 2017; Pinelli et al.,



2013) and CD40 targeting antibodies (Adams et al., 2005; Aoyagi et al., 2009; Badell et al., 2012; Cordoba et al., 2015; Gilson et al., 2009; Haanstra et al., 2003; Lowe et al., 2012; Oura et al., 2012; Page et al., 2012; Pearson et al., 2002) have shown efficacy in murine and NHP transplant models with currently the CD40 antagonists ASKP1240 in phase 2a (NCT01780844) and CFZ533 in phase1/phase 2 (NCT02217410) clinical trials for use in kidney transplant recipients.

Our lab employs a two-component costimulation blockade protocol consisting of donor specific transfusion (DST) and anti-CD154 mAb (Clone MR1). Historically, living donor kidney recipients have been treated with whole blood transfusions from the donor (DST) in addition to immunosuppressants and patients receiving DST have shown better outcomes than patients receiving immunosuppressants alone (Marti et al., 2006). Tolerance induction by DST/anti-CD154 costimulation blockade involves all three mechanisms of peripheral tolerance, namely deletion, anergy and immunoregulation.

#### OX40/OX40L

The finding that OX40 stimulation abrogates the suppressor function of Tregs and prevents induction of Tregs from conventional CD4 T cells is of immense importance for tolerance induction (Vu et al., 2007). Although OX40 blockade as a monotherapy did not induce tolerance to murine allografts, it prolonged allograft survival by inhibiting accumulation of CD4 and CD8 T cells in the draining lymph nodes and graft infiltration and enhancing potency of Tregs (Kinnear et al.,

2013b; Kinnear et al., 2010). Moreover, OX40 blockade inhibited CD28/CD154 independent rejection and in wild type mice, synergized with CD28/CD154 blockade to induce long-term skin allograft acceptance (Demirci et al., 2004). Studies in NHP models will reveal the robustness of OX40 blockade and determine if these agents go to clinical trials for transplantation.

#### LFA-1/ICAM-1 and VLA-4/VCAM-1

Antibodies targeting LFA-1 and VLA-4 have shown efficacy in both murine and NHP models of transplantation (Kean et al., 2017). These reagents are of particular interest because they prevent CD28/CD154 blockade resistant rejection by memory alloreactive T cells by impairing T cell trafficking and graft infiltration and/or memory recall effector function (Badell et al., 2010; Kitchens et al., 2012; Setoguchi et al., 2011). FDA approved anti-LFA-1 antibody efalizumab for psoriasis was tested in clinical trials for islet and renal transplantation (Posselt et al., 2010; Turgeon et al., 2010; Vincenti et al., 2007). However, it was taken off the market because of the development of progressive multifocal leucoencephalopathy (PML), a central nervous system (CNS) infection caused by JC polyoma virus, in some patients (Carson et al., 2009). Anti-VLA-4 antibody natalizumab, which was FDA approved for multiple sclerosis and Crohn's disease, is also associated with PML development. The incidence of PML development, owing to these antibodies causing CD4 T lymphopenia and altered trafficking of T cells into the CNS, has dampened interest in targeting these adhesion molecules for transplantation.

Other costimulatory pathways targeted include ICOS/ICOS-L (Harada et al., 2003; Kosuge et al., 2003; Lo et al., 2015; Ozkaynak et al., 2001), 4-1BB/4-1BBL (Cho et al., 2004; Wang et al., 2003) and CD27/CD70 (Demirci et al., 2004), LFA-3/CD2 (Lo et al., 2013; Weaver et al., 2009a), TIM-1 (Ueno et al., 2008) and TIM-4 (Yeung et al., 2013). Targeting these pathways has shown variable success in different transplant models. Reagents targeting these costimulatory molecules may work better as combination therapies and need to be tested with CD28 and CD154 pathway blockers.

#### *Alternative approaches to tolerance induction*

##### Blocking co-inhibitory pathways

Blocking the co-inhibitory receptor PD-1 or its ligand PD-L1 accelerated acute graft rejection (Ito et al., 2005) and prevented induction (Wang et al., 2007) and maintenance (Koehn et al., 2008) of tolerance by CD28/CD154 blockade by increasing the accumulation of IFN- $\gamma$  secreting effector CD8 T cells and decreasing graft infiltration of Tregs. Stimulating PD-1 might aid in inducing tolerance along with blocking costimulation; however no PD-1 agonists are available. Similarly, activating CTLA4 is desirable in the context of transplantation but is precluded by the lack of availability of CTLA4 agonists. Agonistic antibody against the newly identified co-inhibitory molecule PD-1H (VISTA) protected mice from graft versus host disease (GvHD) by arresting donor alloreactive T cell activation and expansion and promoting donor Treg expansion (Flies et al.,

2015). Targeting the coinhibitory molecule BTLA prolonged murine allograft survival when combined with CTLA4-Ig, but the mechanism of the anti-BTLA antibody was not clearly defined in these studies (Truong et al., 2007a; Truong et al., 2007b). While blocking the coinhibitory receptor TIM-3 accelerated graft rejection by increasing frequencies of alloreactive effector T cells and attenuating iTreg generation (Boenisch et al., 2010), activating TIM-3 by treatment with the ligand galectin-9 combined with rapamycin induced tolerance to allografts (Cai et al., 2013).

#### Establishment of mixed chimerism

Mixed chimerism is a state in which donor and recipient cells co-exist after allogeneic bone marrow transplantation. More than 1% donor cells of total cells should be detectable in the recipient's blood to be considered 'mixed chimerism'. Donor-specific T cells get eliminated in the thymus by clonal deletion (negative selection) because the donor antigens originating from the donor bone marrow are now 'self' for the recipient. Conditioning treatments are necessary for donor bone marrow acceptance. Non-myeloablative conditioning regimens include immunosuppressants (depleting antibodies, costimulation blockade) and thymic or total body irradiation at low doses to prevent eradication of all recipient bone marrow (Ruiz et al., 2013). Three groups have reported induction of mixed chimerism and operational tolerance in renal transplant patients using distinct conditioning regimens (Kawai et al., 2008; Kawai et al., 2013; Leventhal et al., 2012; Leventhal et al., 2016; Scandling et al., 2008; Scandling et al., 2012).

### Regulatory T cell infusion

Cell-based therapies have become popular over the past decade because they permit tolerance induction without the adverse effects of pharmacological immunosuppression. Considering the prominent role of Tregs in the induction and maintenance of tolerance, several laboratories have explored ways to utilize these cells to prolong graft survival in the clinic. Tregs may be obtained directly from the recipient peripheral blood and expanded ex vivo followed by autologous transfer; they may be obtained from umbilical cord blood followed by ex vivo expansion; they may be induced in vitro from naïve T cells (iTregs) under specific tissue culture conditions or they may be induced in vitro by allogeneic stimulation or polyclonal activation (Tang and Bluestone, 2013). Several clinical trials are ongoing with Tregs in solid organ transplantation and treatment of GvHD following hematopoietic stem cell transplantation (Romano et al., 2016). Overall, Tregs seem to be well tolerated even at high doses; their efficacy will become clear when results from these trials are published. A recent study employed a new approach to generate antigen-specific Tregs. Using the synthetic fusion protein chimeric antigen receptor (CAR) technology, the authors generated HLA-A2 specific CAR Tregs and showed their potency in a human skin xenograft transfer model (Boardman et al., 2017).

### Myeloid suppressor cell infusion

Several kinds of suppressor cells of the myeloid lineage have been identified and developed for cell-based therapies for tolerance induction. Tolerogenic DCs (tol-

DCs), originally described as regulatory DCs (DCregs), are immature DCs that express low levels of MHC-II and costimulatory molecules. Tol-DCs promote tolerance induction by various mechanisms, including T cell deletion and anergy, induction of Tregs and production of immunosuppressive factors (Li and Shi, 2015; Riquelme et al., 2012). Regulatory macrophages (Mregs) suppress proliferation and induce apoptosis of activated T cells (Riquelme et al., 2012). Myeloid derived suppressor cells (MDSCs) are immature cells of the monocytic (mMDSCs) and granulocytic (gMDSCs) origin. They express arginase-1 and iNOS (inducible nitric oxide synthase), produce reactive oxygen species and peroxynitrite and mediate immunosuppression by inhibiting T cell proliferation and function, inducing T cell apoptosis and supporting Treg induction and proliferation (Gabrilovich and Nagaraj, 2009). The ONE Study, a collaborative effort to test and compare these myeloid derived cell-based therapies in renal transplantation in patients, should reveal which of these populations is effective at promoting tolerance induction (Geissler, 2012).

#### Mesenchymal stem cell infusion

Mesenchymal stem cells are a heterogeneous population found in the bone marrow and connective tissue. These cells are known to mediate immunosuppression by increasing the numbers of Tregs and tol-DCs and impairing alloreactive T cell homing (Scalea et al., 2016). Clinical trials using MSCs in renal transplantation have shown their feasibility and safety and suggest

that infusing MSCs may facilitate reduction of pharmacological immunosuppression (Mudrabettu et al., 2015; Peng et al., 2013).

#### Apoptotic cell infusion

Phagocytosis of apoptotic cells by APCs may reduce APC maturation and stimulate secretion of immunosuppressive factors thereby inducing Tregs and inhibiting T cell function (Griffith and Ferguson, 2011). Intravenous transfusion of UV-B- or  $\gamma$ -irradiation treated early apoptotic cells before transplantation prolongs allograft survival in mouse transplant models (Morelli and Larregina, 2016). Although apoptotic cell therapy has not been clinically tested for solid organ transplantation, infusing allogeneic early apoptotic cells prior to HLA-matched hematopoietic stem cell transplantation reduced the severity of GvHD in a clinical trial (Mevorach et al., 2014).

#### Infusion of exosomes

Exosomes are extracellular vesicles formed by the inward budding of endosomal membranes and are secreted by many cell types. Intravenous delivery of donor-derived exosomes purified from peripheral blood (Song et al., 2016) or bone marrow derived DC culture supernatants (Peché et al., 2003) prolonged allograft survival and induced tolerance when combined with short-term immunosuppression (Peché et al., 2006) in rodent models of transplantation by inhibiting alloreactive T cell responses. Exosomes purified from syngeneic Tregs were also able to modestly prolong allograft survival (Yu et al., 2013). Clinical

trials in cancer have shown safety of exosomes (Viaud et al., 2010), testing in organ transplantation may not be far off.

Another approach that employs strategically exposing the recipient to donor antigens before transplantation is the delivery of donor splenocytes cross-linked with ethylenecarbodiimide (ECDI) (Luo et al., 2008). This approach induced donor-specific tolerance to murine allografts via multiple mechanisms, including clonal deletion, anergy and immunoregulation (Kheradmand et al., 2012).

### **Barriers to transplantation tolerance**

Although tolerance induction is possible in rodents using the strategies I described in the previous section, several barriers have been recognized that may prevent tolerance induction and abrogate tolerance once it has been established. Since rodents are housed in specific pathogen-free environments in laboratory settings, they are not exposed to the environmental antigens that humans are exposed to on a daily basis, making it difficult to identify precisely the factors that prevent tolerance in clinical settings. However, knowledge gained from patient studies and the creation of elegant mouse models has made it feasible to conduct mechanistic studies in the laboratory. I have described some of the hurdles in transplantation tolerance reported in the literature.

#### *Sterile inflammation*



In the context of solid organ transplantation, antigen-independent injury occurs to the graft before and during transplantation (Mori et al., 2014). Organ damage occurs during brain death of the donor as well as during cold storage of the organ prior to transplantation. After the organ is transplanted, subsequent damage occurs due to restoration of blood flow, known as ischemia reperfusion injury (IRI). These processes cause localized activation of the innate immune system and inflammation in the graft known as 'sterile inflammation'. Intracellular and extracellular molecules that are normally sequestered become exposed to the immune system upon necrotic cell death and trigger sterile inflammation. These endogenous triggers of inflammation are called damage associated molecular patterns (DAMPs). Intracellular DAMPs include nuclear proteins such as HMGB1 and cellular chaperones such as heat shock proteins; extracellular DAMPs include low molecular weight form of the extracellular matrix component hyaluronan and acute phase protein haptoglobin. Many of these DAMPs are recognized by TLR4 and TLR2 and are associated with acute and chronic rejection in clinical transplantation as well as mouse transplant models (Mori et al., 2014). Functional polymorphisms in TLR4 that reduce TLR4 signaling are associated with improved graft outcomes in patients undergoing lung and kidney transplantation (Ducloux et al., 2005; Fekete et al., 2006; Palmer et al., 2003). Moreover, genetic deficiency or knockdown of TLR4 or the adaptors MyD88 and TRIF facilitates peripheral tolerance induction to murine allografts (Walker et al.,

2006; Zhang et al., 2010; Zhang et al., 2012) illustrating the significant barrier that innate immune signaling represents to tolerance induction.

### *Infections*

Certain infections in the peri-transplant period are known to interfere with tolerance induction in mouse models. PAMPs from the infecting microbes bind their PRRs on the recipient's cells and stimulate production of inflammatory cytokines that induce APC maturation and trigger alloimmunity overriding the effects of tolerance induction therapies. Bacterial infections such as *Listeria monocytogenes* (Wang et al., 2008), and *Staphylococcus aureus* (Ahmed et al., 2011) and viral infections such as LCMV and Pichinde (Turgeon et al., 2000) in the peri-transplant period prevent tolerance induction by costimulation blockade. Our lab and others have demonstrated that injecting mice with PAMPs such as LPS, Pam<sub>3</sub>CysK<sub>4</sub>, poly(I:C) and CpG on the day of transplantation prevents long-term acceptance of allografts (Chen et al., 2006; Thornley et al., 2006b). Once tolerance is induced, it is difficult to breach in mouse models of transplantation (Alegre et al., 2014). *Listeria* infection can abrogate established tolerance in some graft recipients although it requires 15-fold higher dose of *Listeria* to reverse tolerance compared to the dose required to prevent induction of tolerance (Wang et al., 2010). In a patient study of kidney transplant recipients who were operationally tolerant, 30% of the patients rejected their grafts after a mean duration of 10±5 years and these patients displayed a higher incidence of

bacterial and viral infections relative to the ones who maintained their tolerance (Brouard et al., 2012).

### *Memory alloreactive T cells*

Memory alloreactive T cells may develop in an individual as a result of prior exposure to alloantigens or through microbial infections (heterologous immunity). Memory T cells have a low activation threshold and hence can be stimulated by low doses of antigen and limited costimulation and hence are resistant to costimulation blockade protocols of tolerance induction. Both CD4 and CD8 memory T cells contribute to allograft rejection. Memory alloreactive CD4 T cells provide help to alloreactive B cells to generate alloantibodies and to alloreactive effector CD8 T cells that then drive allograft rejection (Chen et al., 2004). Memory alloreactive CD8 T cells directly mediate costimulation blockade-resistant graft rejection (Su et al., 2014). Studies investigating the impact of alloreactive memory T cells on transplant rejection are based on generating cross-reactive memory T cells by infection of the recipient, priming the recipient to donor antigen or adoptive transfer of donor-antigen primed memory T cells (Benichou et al., 2017). Using these strategies, studies have demonstrated that memory alloreactive T cells present a formidable barrier to tolerance induction by costimulation blockade and hematopoietic chimerism (Adams et al., 2003; Brehm et al., 2003; Pantenburg et al., 2002). NHPs with higher frequencies of endogenous alloreactive memory T cells exhibit shorter allograft survival

compared to those with lower frequencies of memory T cells (Nadazdin et al., 2011). As a result of exposure to environmental antigens, humans have cross-reactive memory T cells despite no previous exposure to alloantigens. The frequency of donor-reactive memory T cells was shown to correlate with the risk of developing acute rejection episodes post kidney transplantation (Heeger et al., 1999). Recently, an 'incognito' form of immune memory was described where memory T cells specific for a non-MHC antigen prevent tolerance induction to an allograft provided the presentation of the non-MHC antigen is linked to the alloantigen (Nelsen et al., 2017).

#### *Memory alloreactive B cells and alloantibodies*

Similar to memory alloreactive T cells, memory alloreactive B cells and donor-specific antibodies may arise in the recipient as a result of previous exposure to alloantigens or through infections. Memory alloreactive B cells and alloantibodies prevent costimulation blockade-induced tolerance by promoting T cell alloimmunity (Burns and Chong, 2011; Burns et al., 2009).

#### *Homeostatic proliferation*

Homeostatic proliferation refers to the proliferation of residual non-depleted lymphocytes following lymphodepletion. T cell depleting agents ATG and alemtuzumab skew the repopulating T cells to allo-specific effector memory phenotype in kidney transplant recipients (Zwang and Turka, 2014). Moreover,

studies in mouse models have revealed that T cells undergoing homeostatic proliferation developed memory phenotype and function and rendered the mice resistant to costimulation blockade induced tolerance to allografts (Wu et al., 2003). Transient lymphopenia was also sufficient to abrogate established tolerance (Iida et al., 2013).

#### *Pre-existing autoimmunity*

Patients with T cell autoreactivity may have a higher risk of post-transplant graft injury (Burlingham et al., 2007; Jurcevic et al., 2001; Kalache et al., 2011). It has been demonstrated in mouse transplant models that pre-sensitization to autoantigen prevents costimulation blockade mediated tolerance induction to allografts expressing the autoantigen (Kalache et al., 2014). In this scenario, memory autoreactive T cells provided help to activate and expand alloreactive effector T cells that rejected the graft. The authors did not find cross-reactivity between autoreactive and alloreactive T cells indicating that heterologous immunity was not the mechanism. Treating recurrent autoimmunity by depleting the primed autoreactive T cell pool allowed long-term survival of grafts (Shi et al., 2004).

### **Humanized mice**

Animal models are used as surrogates of human biology due to the logistical and ethical restrictions of working with cell and tissue samples from human donors and the biological limitations of culture systems. Small animals

such as mice and rats are widely used mammalian model systems due to their small size, ease of maintenance and handling, short reproductive cycle, sharing of genomic and physiological properties with humans, and ability to be readily manipulated genetically. Despite the vast amount of basic biology obtained from mouse studies, there are limitations to mouse models when investigating human biology. Several components of mouse biological systems are incongruent with those of humans, particularly their immune system. For example, there are many differences in innate immune molecules, including the lack of a functional TLR10 in mice and the expression of TLR11, TLR12, and TLR13 in mice that are not present in the human genome (Zschaler et al., 2014). Furthermore, many drugs and pathogens are species specific. The nature and pathogenesis of immune responses mounted against pathogens that can infect only human cells may markedly differ from that of murine infectious agents or human infectious agents that have been murine-adapted (Shultz et al., 2007). These issues underscore the need for better small animal models that can more faithfully recapitulate human biological systems.

Humanized mice have begun to fill this gap and have become important preclinical tools for biomedical research. Humanized mice, also known as human immune system mice, are immunodeficient mice that have been engrafted with hematopoietic cells and tissues of human origin to produce a functional human immune system. There has been a continuous improvement of immunodeficient recipients used to generate humanized mice over the last 25 years (Shultz et al.,

2012). The key breakthrough was the development of immunodeficient mice bearing mutations in the IL-2 receptor common gamma chain (*IL2rg<sup>null</sup>*) in the early 2000s. The common gamma chain constitutes an important component of receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 and is indispensable for high affinity binding and signaling of these cytokines. When combined with either the protein kinase DNA-activated catalytic polypeptide mutation (*Prkdc<sup>scid</sup>* or *scid*) or with recombination activating gene (*Rag*) 1 or 2 (*Rag1<sup>null</sup>* or *Rag2<sup>null</sup>*) mutations, these mice lack adaptive immunity and exhibit severe deficiencies of innate immunity, including absence of murine natural killer (NK) cells.

Three strains of immunodeficient *IL2rg<sup>null</sup>* mice are widely used today: NSG (NOD.Cg-*Prkdc<sup>scid</sup>IL2rg<sup>tm1Wjl</sup>*) (Ito et al., 2002), NOG (NOD.Cg-*Prkdc<sup>scid</sup>IL2rg<sup>tm1Sug</sup>*) (Shultz et al., 2005) and BRG (C;129S4-*Rag2<sup>tm1Flv</sup>IL2rg<sup>tm1Flv</sup>*) (Traggiai et al., 2004). NOG mice have a truncated cytoplasmic domain of the gamma chain that binds cytokines but lacks the signaling domain, whereas NSG and BRG mice completely lack the gamma chain. When engrafted with human cells, tissues, and immune systems, the biological responses in humanized *IL2rg<sup>null</sup>* mice more faithfully recapitulate those seen in humans than in any previous models of humanized mice.

There are three general approaches to engraft human immune systems into immunodeficient *IL2rg<sup>null</sup>* mice. The first model, known as Hu-PBL-SCID, is created by injection of human peripheral blood leukocytes (PBLs) (Mosier et al., 1988). This model results in rapid engraftment of human CD3<sup>+</sup> T cells by the end

of the first week. This model is excellent for studying human T cell function *in vivo* but has only a short experimental window due to the development of lethal xenogeneic graft-versus-host disease (GVHD), usually within 4–8 weeks. However, this experimental window can be extended using NSG recipients lacking murine MHC class I or II (King et al., 2009). The second model, known as Hu-SRC-SCID, encompasses the intravenous or intrafemoral injection of human severe combined immunodeficiency (SCID) repopulating cells, that is, human CD34<sup>+</sup> hematopoietic stem cells (HSCs) derived from bone marrow, umbilical cord blood, fetal liver, or G-CSF–mobilized peripheral blood. This model (also referred to as HSC-engrafted) supports engraftment of a complete human immune system. Although B, T, myeloid, and antigen-presenting cells are present in the peripheral hematopoietic tissues, granulocytes, platelets, and red blood cells (RBCs) that are generated in the bone marrow are observed at only very low levels in the blood. In addition, the human T cells are educated in the mouse thymus and are thus H2-, not HLA-restricted (Watanabe et al., 2009). The third model is the bone marrow/liver/thymus (BLT) model that is established by transplantation of human fetal liver and thymus under the kidney capsule and *i.v.* injection of autologous fetal liver HSCs (Lan et al., 2006; Melkus et al., 2006). As in the Hu-SRC-SCID model, all lineages of human hematopoietic cells develop. However, BLT mice also develop a robust mucosal human immune system, and the human T cells are educated on an autologous human thymus and are HLA-restricted. One of the main caveats of the BLT model is that in most laboratories,



the mice develop a wasting GVHD-like syndrome that limits the time window for experimentation. Each model has its advantages and limitations, and therefore it is important for researchers to select a model appropriate for their specific biological questions.

There are several limitations associated with these humanized mouse models (Shultz et al., 2012). Residual mouse immune system precludes complete human cell engraftment. Functional responses of human immune cells are impaired due to the differences between human and mouse cytokines. Humoral responses are limited because of impaired class switching and affinity maturation and poorly organized secondary lymphoid structures. Several modifications have been made to the existing strains by knocking out specific mouse genes or knocking in or transgenically expressing specific human genes to overcome these limitations. The result is an improved array of humanized mice with higher human immune cell engraftment levels and enhanced functionality (Shultz et al., 2012). These improved humanized mouse models are now being used to study many human biological responses and diseases and are increasingly employed as preclinical tools to evaluate drugs and identify underlying mechanisms in a broad array of diseases. In particular, humanized mice are playing an increasing role in the study of human-specific infectious agents such as HIV and are widely used as preclinical models in cancer biology. Moreover, humanized mice are being increasingly utilized as translational models in many additional areas of biomedical research, including regenerative

medicine, transplantation, and immunity.

## **CHAPTER II**

**A novel murine anti-CD40 monoclonal antibody prolongs skin allograft survival**

### **Abstract**

The CD40-CD154 pathway regulates key aspects of immune system activation, and blockade of this pathway with antibodies targeting CD154 has been used to induce transplantation tolerance and suppress autoimmune disease. The efficacy of anti-CD154 antibody in prevention of allograft rejection and suppression of autoimmunity in murine and non-human primate models accelerated efforts to develop this strategy for clinical use. However, clinical trials with anti-CD154 antibody resulted in thromboembolic complications in patients and limited clinical application. To develop an alternative approach to anti-CD154 antibody, recent efforts have focused on targeting its receptor, CD40. In this study I evaluated the effects of a novel murine antagonistic, non-depleting antibody targeting CD40 on alloreactive immune responses. Costimulation blockade consisting of donor splenocytes (DST) and anti-CD40 mAb significantly prolonged skin graft survival in the stringent BALB/c to B6 MHC mismatch model with an efficacy similar to anti-CD154 mAb. Moreover DST/anti-CD40 treatment attenuated donor-specific antibody production and alloreactive CD8 T cell responses as shown by inhibition of expansion, decreased cytokine production and decreased killing of allogeneic targets. However tolerance induced by anti-CD40 antibody was abrogated by induction of systemic inflammation with bacterial lipopolysaccharide, as previously described with strategies targeting CD154. These results demonstrate that targeting CD40 has similar efficacy to anti-CD154 antibody for the induction

of transplantation tolerance and support the application of anti-CD40 antibodies in clinical settings.

## **Introduction**

Contemporary immunosuppressive medications, particularly those that rely on calcineurin inhibitors, are highly effective at reducing acute rejection rates, but improvement of long term allograft survival has been modest (Webber and Vincenti, 2016). Costimulation blockade is a potential calcineurin-inhibitor free approach that has emerged as one of the most promising methods for induction of transplantation tolerance in the absence of chronic immunosuppression (Kinnear et al., 2013a). Studies in mouse and non-human primates (NHP) have demonstrated the effectiveness of costimulation blockade therapies to diminish alloreactive T cell responses and improve graft survival (Ford, 2016; Shiao et al., 2005). Costimulation blockade strategies targeting human-specific molecules have been successfully modeled in humanized mice, preventing the rejection of human stem cell derived pancreatic endoderm (Szot et al., 2015). Several costimulatory molecules including CD80, CD86, CD28, CD154, ICOS, OX-40, 4-1BB, LFA-3, LFA-1 and TIM-1 have been targeted as monotherapies or combination therapies and have shown some degree of success in transplant models (Demirci et al., 2004; Kitchens et al., 2012; Larsen et al., 1996; Ozkaynak et al., 2001; Ueno et al., 2008; Wang et al., 2003; Weaver et al., 2009b; Zhang et al., 2011a).

CD40-CD154 interactions have been extensively studied in the context of alloimmunity and transplantation. After the clinical trials with the anti-CD154 antibodies were halted, researchers directed their efforts towards developing Fc-silent anti-CD154 antibodies and reagents targeting the receptor CD40. As an alternative to targeting CD154, recent efforts have focused on CD40 for the induction of transplantation tolerance (Pinelli and Ford, 2015). Early studies showed that mice genetically deficient in CD40 were amenable to tolerance induction to islet and cardiac, but not skin allografts, from CD40 deficient donors (Nathan et al., 2004; Phillips et al., 2003). A murine anti-CD40 antibody with intact Fc binding activity (7E1-G2b) had comparable efficacy to anti-CD154 in promoting allogeneic bone marrow chimerism and skin graft survival (Gilson et al., 2009). Several investigators have developed and tested anti-CD40 antibodies in NHP models and shown significant efficacy in kidney, liver, bone marrow and islet transplantation either alone or in combination with CD28 pathway blockers (Adams et al., 2005; Aoyagi et al., 2009; Badell et al., 2012; Cordoba et al., 2015; Haanstra et al., 2003; Lowe et al., 2012; Oura et al., 2012; Page et al., 2012; Pearson et al., 2002). The promising results achieved in these murine and NHP models paved the way for anti-CD40 reagents to be tested in human subjects (Goldwater et al., 2013).

Understanding the mechanism of action for anti-CD40 antibodies and how they compare to anti-CD154 antibodies is critical. In this chapter, I describe costimulation blockade and transplantation tolerance with a novel, Fc-mutated,

non-depleting, fully antagonistic anti-CD40 monoclonal antibody. Anti-CD40 mAb in combination with DST was as effective in prolonging survival of fully mismatched skin allografts as anti-CD154 mAb. DST/anti-CD40 treatment attenuated the generation of alloantibodies and IFN- $\gamma$  producing effector/memory CD8 T cells, and impaired the expansion of alloreactive CD8 T cells. Inflammation induced by bacterial lipopolysaccharide (LPS) precipitated allograft rejection despite DST/anti-CD40 costimulation blockade. My findings suggest that reagents targeted towards CD40 are attractive candidates for translation in clinical transplantation.

## **Materials and Methods**

### *Generation and characterization of the anti-CD40 mAb*

Wistar rats were immunized with recombinant mouse CD40/hFc (R&D Systems) in alum adjuvant in the footpad and boosted i.p and/or i.v. Sera positive animals were sacrificed and hybridomas were created by fusion with the mouse myeloma cell line Sp2/0 (ATCC). Hybridomas that exhibited selective molecular and cellular CD40 binding and capable of blocking molecular CD40-CD40L interaction were subcloned, grown in serum-free medium and purified. The antagonistic and agonistic activity of purified mAbs was assessed using proliferation assays. Briefly, mouse splenocytes ( $4 \times 10^4$  cells) cultured with recombinant mouse IL-4 were treated with varying concentrations of mAb alone (agonism) or for 1 hour prior to stimulation with 100 ng/ml of MegaMouse CD40L (Enzo Life Sciences) (antagonism). Proliferation was measured after 72 hrs via

<sup>3</sup>[H]-thymidine incorporation. Anti-mouse CD40 clones FGK4.5 and 3/23 were used as full agonist and partial/weak agonist controls, respectively. The Vgene sequence of the sole clone exhibiting pure antagonistic activity was recovered and engineered onto a mouse IgG2a Fc containing mutations to abrogate Fcγ receptor binding.

### *Animals*

C57BL/6J ( $H2^b$ ), BALB/cJ ( $H2^d$ ), CBA/J ( $H2^k$ ) and B6.129(Cg)-*Foxp3*<sup>tm3(DTR/GFP)*Ayr*/J</sup> mice were obtained from The Jackson Laboratory. (CBA/J X KB5.CBA) F1 CD8<sup>+</sup> TCR-transgenic mice were bred at the animal facility of University of Massachusetts Medical School. The TCR transgene is expressed in CBA ( $H2^k$ ) mice by CD8 T cells and specifically recognizes native H-2K<sup>b</sup> (Iwakoshi et al., 2001). All animals were housed in a specific pathogen free facility in microisolator cages and given autoclaved food. All experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

### *Generation of CD8<sup>+</sup> KB5 TCR-transgenic synchimeric mice*

To generate KB5 TCR transgenic hematopoietic chimeras, male CBA/J non-transgenic mice at 4-5 weeks of age were treated with 200cGy whole body gamma irradiation and injected intravenously (i.v.) with  $0.5 \times 10^6$  bone marrow cells from KB5 TCR-transgenic mice (Iwakoshi et al., 2001). Hematopoietic



chimerism was allowed to develop for 8 weeks post bone marrow transfer. The resulting 'synchimeras' were used in experiments between 12-16 weeks of age.

*Flow cytometry, antibodies and intracellular cytokine staining*

At the specified times, mice were sacrificed by cervical dislocation. Single cell suspensions of splenocytes were prepared by grinding spleens between glass microscope slides. Erythrocytes were lysed with 0.84% ammonium chloride. Single cell suspensions were stained with the antibodies: CD16/CD32 (2.4G2), CD3 (145-2C11), CD4 (RM4-5), CD8 $\alpha$  (53-6.7), CD44 (IM7), B220 (RA3-6B2), CD19 (1D3), CD11c (HL3), CD11b (M1/70), F4/80 (BM8), Ig (multiple adsorption), IFN- $\gamma$  (XMG1.2) or isotype controls purchased from BD Biosciences, Ebioscience or BioLegend. For intracellular cytokine staining,  $2 \times 10^6$  RBC lysed splenocytes from recipient mice were incubated with  $1 \times 10^6$  LPS matured, irradiated syngeneic or allogeneic splenocytes in complete RPMI and monensin (eBioscience) for 4 hrs at 37°C and assessed for intracellular IFN- $\gamma$  production as described previously (Brehm et al., 2007). Samples were run on LSR II flow cytometer (BD Biosciences). At least 300000 events were collected. Data were analyzed using FlowJo software (Tree Star).

*VPD450 labeling and adoptive transfer*

Splenocytes were harvested from KB5 transgenic mice, RBC lysed and resuspended in PBS and labeled with 1  $\mu$ M Violet Proliferation Dye (VPD450)

(BD Biosciences) for 12 mins at 37°C. Cells were washed twice in PBS + 10% FBS, and splenocytes comprising  $2 \times 10^6$  labeled DES<sup>+</sup> CD8<sup>+</sup> T cells resuspended in PBS were injected i.v. into non-transgenic CBA mice.

#### *In vivo cytotoxicity assay*

Splenocytes were harvested from B6 and CBA mice, RBC lysed, resuspended in PBS and labeled with 2.6  $\mu$ M and 0.2  $\mu$ M CFSE (Sigma) respectively for 4 mins at 37°C. Cells were washed twice in PBS + 10% FBS and combined in equal proportions.  $20 \times 10^6$  cells were adoptively transferred intravenously into the indicated recipient B6 mice. Spleens from recipient mice were harvested 3 hours later and the survival of each CFSE labeled population was assessed by flow cytometry. Specific lysis was calculated as:  $[1 - (\text{ratio immune} / \text{ratio naive})] \times 100$ , where the ratio = % allogeneic population / % syngeneic population.

#### *Skin transplantation and tolerance induction*

Skin grafts were obtained from the flanks of BALB/c mice and transplanted onto the dorsal flanks of B6 mice on day 0. The recipient B6 mice were given a DST of  $10 \times 10^6$  BALB/c splenocytes i.v. on day -7 and 0.5 mg of anti-CD154 mAb, clone MR1 (BioXCell) or 1 mg of the anti-CD40 mAb or its isotype control i.p. on days -7, -4, 0 and +4 relative to transplantation. 0.5 mg of CLTA4-Ig (BioXCell) was injected i.p. on days 0, 2, 4 and 6 where specified. Recipient mice were injected with 50 or 100  $\mu$ g of Ultrapure LPS from *E.coli* 0111:B4 (InvivoGen) i.p. on day -7

where indicated. Diphtheria toxin (DT) obtained from List Biological Laboratories, Inc. was given i.p. daily from day -8 to day -2 at a dose of 100 ng per mouse.

#### *Measurement of alloantibodies*

Alloantibody levels were measured by flow cytometry as described previously (Leenaerts et al., 1990). Briefly, B6 mice were bled two weeks after skin transplantation and plasma was isolated by centrifugation. BALB/c splenocytes were used as allogeneic target cells.  $1 \times 10^6$  BALB/c splenocytes were resuspended in 100ul PBS, incubated with Fc block™ and then incubated with 100 ul of B6 plasma diluted 1:10, 1:100 and 1:1000 for 30 mins at 4°C. Cells were washed twice to remove excess plasma and incubated with PE-conjugated polyclonal anti-mouse Ig for 20 mins at 4°C. B cells stained with anti-CD19 and anti-B220 were excluded by gating and mean fluorescence intensity (MFI) of the remainder cells represented the relative concentration of alloantibodies in the plasma.

#### *Statistics*

Statistical analyses were performed using GraphPad PRISM software. Allograft survival curves were generated by the Kaplan-Meier method and compared by the log-rank test. Three or more means were compared using one-way ANOVA and Tukey's multiple comparisons test. p values < 0.05 were considered statistically significant.

## Results

### Characterization of the anti-CD40 monoclonal antibody

The chimeric anti-CD40 monoclonal antibody with mutated mouse Fc regions was tested for specificity of binding to mouse CD40, inhibition of CD40L binding to CD40 and antagonistic activity. The anti-CD40 mAb bound to plate-bound recombinant murine CD40-hFc fusion protein with an  $EC_{50}$  of 0.07 nM whereas binding to the control RANK-hFc fusion protein was negative (Fig. 2.1A). In addition anti-CD40 mAb bound to B6 splenocytes with an  $EC_{50}$  of  $0.42 \pm 0.08$  nM with no binding to *Cd40*<sup>-/-</sup> splenocytes observed (Fig. 2.1B). Next we tested the ability of the anti-CD40 mAb to block binding of soluble CD154 to plate bound CD40. The anti-CD40 mAb blocked MegaMouseCD40L-FLAG from binding to plate bound recombinant mouse CD40/hFc (10 ng/well) by ELISA ( $IC_{50}$  of 0.25nM). Anti-mouse CD40 clone 3/23 was used as a positive control (Fig. 2.1C). Finally, we tested the anti-CD40 mAb for the ability to antagonize proliferation of mouse B cells induced by MegaCD40L, a soluble construct of two artificially linked CD40L molecules. FGK4.5, a complete CD40 agonist, induced maximal proliferation of B cells (Fig. 2.1D). The anti-CD40 mAb completely inhibited cell proliferation, whereas 3/23 showed partial agonistic effects (Fig. 2.1D). These data demonstrate that the anti-CD40 mAb is a fully antagonistic antibody.

Fig. 2.1. Anti-CD40 mAb is a fully antagonistic antibody.

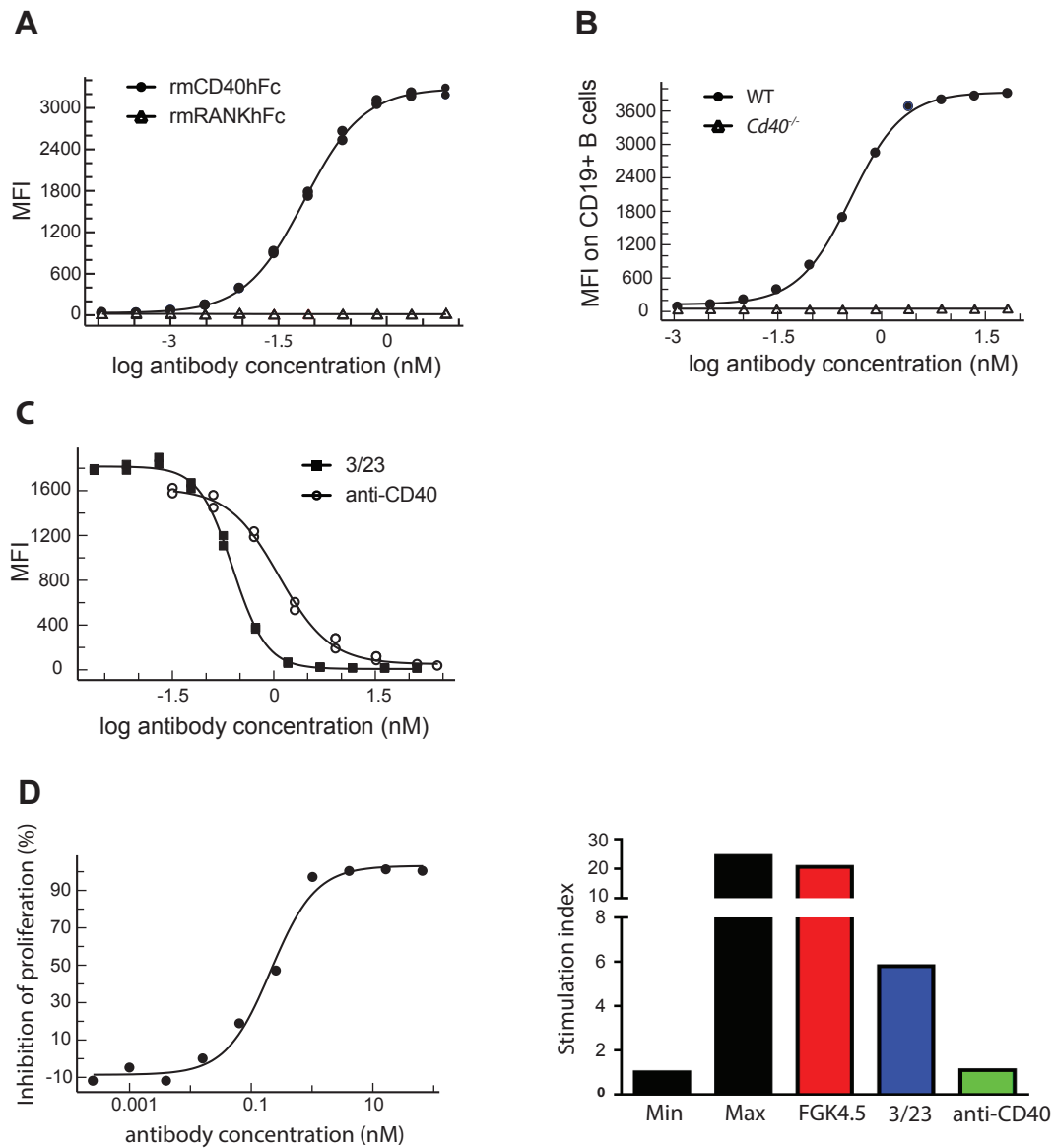
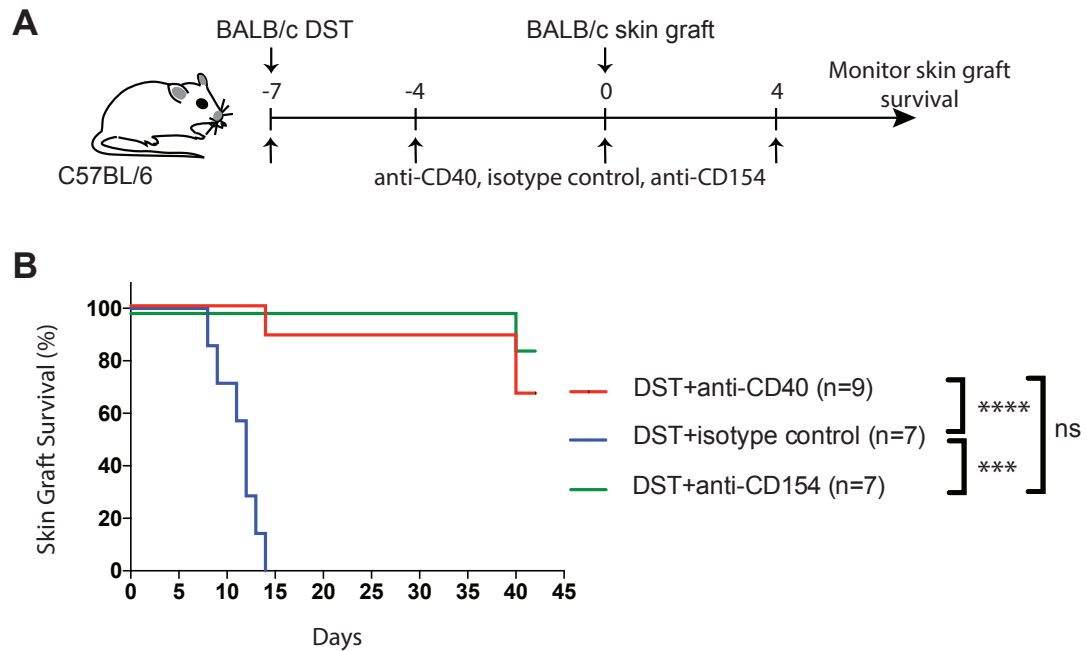


Fig. 2.1. Anti-CD40 mAb is a fully antagonistic antibody. (A) The anti-CD40 mAb was tested for binding to plate-bound recombinant CD40-hFc and RANK-hFc fusion proteins. (B) The anti-CD40 mAb was tested for binding to WT B6 and *Cd40*<sup>-/-</sup> splenocytes. (C) Anti-CD40 mAb and control 3/23 antibody were tested for blocking soluble CD154 binding to plate-bound CD40. (D) The anti-CD40 mAb and control antibodies FGK4.5 and 3/23 were tested for inhibition of proliferation of B cells induced by MegaCD40L.

### **Anti-CD40 mAb combined with DST prolongs skin allograft survival**

A two-element protocol comprising DST and anti-CD154 mAb has been shown to prolong the survival of murine skin, islet and cardiac allografts (Kishimoto et al., 2004; Markees et al., 1997; Parker et al., 1995). Considering the complete antagonistic nature of the anti-CD40 mAb (Fig. 2.1), I hypothesized that the anti-CD40 mAb will work efficiently in costimulation blockade therapies aimed at prolonging survival of allogeneic tissues in mice. I therefore tested DST/anti-CD40 combination therapy in the stringent BALB/c to B6 mouse model of skin transplantation (Fig. 2.2A). Untreated mice reject skin grafts between 10-15 days in this model (Iwakoshi et al., 2000). Mice that received DST/isotype control treatment rapidly rejected skin grafts with a median survival time (MST) of 12 days (Fig. 2.2B). DST/anti-CD40 treatment, on the other hand, significantly prolonged allograft survival over the duration of the experiment similar to DST/anti-CD154 (Fig. 2.2B).

**Fig. 2.2. Anti-CD40 mAb is equivalent to anti-CD154 mAb at prolonging allograft survival.**



**Fig. 2.2. Anti-CD40 mAb is equivalent to anti-CD154 mAb at prolonging allograft survival.** (A) C57BL/6 mice were grafted with BALB/c skin on day 0 and treated with a DST of  $10 \times 10^6$  BALB/c splenocytes i.v. on day -7 and 0.5 mg of anti-CD154 mAb or 1 mg of anti-CD40 mAb or its isotype control i.p. on days -7, -4, 0 and +4 where indicated. Graft survival curves are shown in (B). Data are representative of three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$ .

Next I tested the impact of these treatments on alloreactive B cell and T cell function. Mice were bled two weeks post skin transplantation and the plasma levels of alloantibodies were determined. Untreated/naive mice were used as negative controls. Mice that received DST/isotype control treatment had high levels of circulating alloantibodies (Fig. 2.3A). Anti-CD40 mAb, similar to anti-CD154 mAb, blunted the alloantibody levels to the extent of untreated mice (Fig. 2.3A). Next I investigated the ability of alloreactive CD8 T cells to produce IFN- $\gamma$  upon ex vivo allogeneic (BALB/c) stimulation 6 weeks post skin transplantation. In agreement with our previous studies, DST/anti-CD154 treatment resulted in a low frequency of IFN- $\gamma$  producing alloreactive effector/memory CD8 T cells (Fig. 2.3B) (Brehm et al., 2007; Thornley et al., 2006a). Mice that received DST/isotype control had significantly higher levels of IFN- $\gamma^+$  CD8 T cells, whereas DST/anti-CD40 treatment reduced the frequency of IFN- $\gamma$  producers (Fig. 2.3B). These results indicate that anti-CD40 mAb in combination with DST inhibits alloantibody production by B cells and generates low levels of IFN- $\gamma$  producing alloreactive CD8 T cells and is thus, functionally equivalent to the anti-CD154 mAb.



Fig. 2.3 Anti-CD40 mAb combined with DST reduces the generation of graft-reactive antibodies and IFN- $\gamma$  producing effector/memory CD8 T cells.

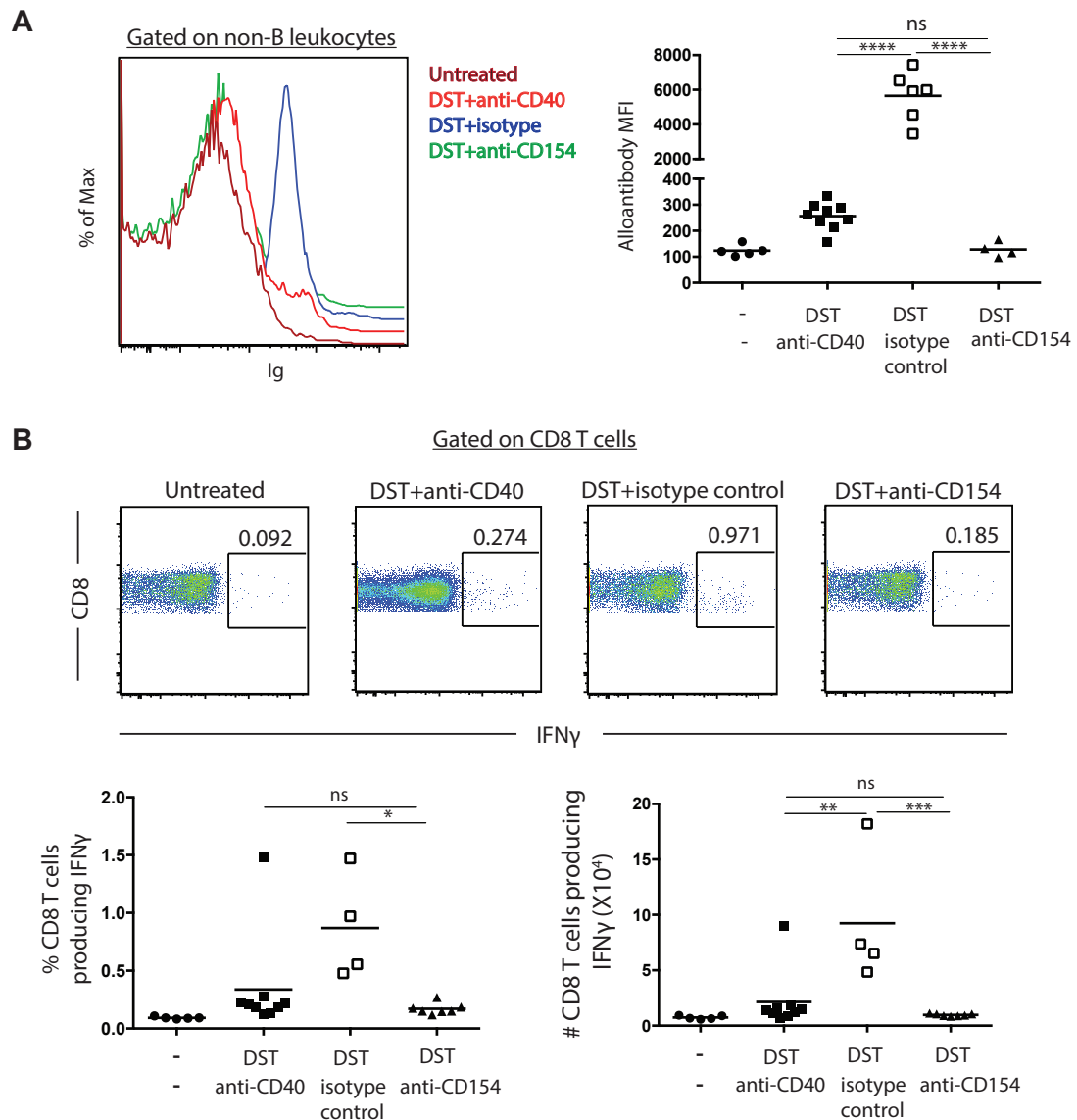


Fig. 2.3. Anti-CD40 mAb combined with DST reduces the generation of graft-reactive antibodies and IFN- $\gamma$  producing effector/memory CD8 T cells. C57BL/6 mice were treated as in Fig. 2.2A. (A) Alloantibodies in the plasma of the mice were measured by flow cytometry 2 weeks post-transplantation. Plasma from untreated mice served as negative control for the alloantibody assay. (B) Splenocytes were harvested from mice 6 weeks post-transplantation and stained for intracellular IFN- $\gamma$  upon allogeneic BALB/c stimulation. The percentages and numbers of IFN- $\gamma$  producing CD8<sup>+</sup> T cells are shown. Data are cumulative of two independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$ .

### **Anti-CD40 mAb combined with DST inhibits alloreactive CD8 T cell expansion and function**

I next evaluated the effects of anti-CD40 mAb treatment on alloreactive CD8 T cell function, levels, and phenotype. I assessed the cytotoxic potential of alloreactive CD8 T cells in C57BL/6 mice using an in vivo cytotoxicity assay. Recipient mice were administered allogeneic (H2<sup>k</sup>) CBA DST along with a single dose of anti-CD154, anti-CD40 or the isotype control Ab and tested for their ability to reject allogeneic CBA splenocytes 3 days later. NK cells from B6 mice do not reject CBA splenocytes in vivo and the rejection observed is entirely a result of CD8 T cell cytotoxicity (Brehm et al., 2005). Untreated mice did not reject allogeneic cells since they have not acquired cytolytic capability in the 3 hour time frame of this assay (Fig. 2.4). Mice in the DST/isotype control group demonstrated 81% killing of allogeneic CBA targets confirming alloreactive CD8 T cell cytotoxic functionality. Anti-CD40 mAb, similar to anti-CD154 mAb, reduced the cytolysis to background levels. The results of the in vivo cytotoxicity assay suggested two possibilities: blocking CD40-CD154 interactions 1) inhibits cytotoxicity of CD8 T cells on a per cell basis and/or 2) depletes alloreactive CD8 T cells effectively reducing the net cytotoxicity of the remainder cells.

To determine if DST/anti-CD40 treatment results in deletion of alloreactive CD8 T cells, I utilized a TCR transgenic mouse model on the H-2<sup>k</sup> background where CD8 T cells have a TCR specific for the alloantigen H-2K<sup>b</sup>.

Fig. 2.4. DST/anti-CD40 mAb treatment inhibits cytolysis of allogeneic target cells.

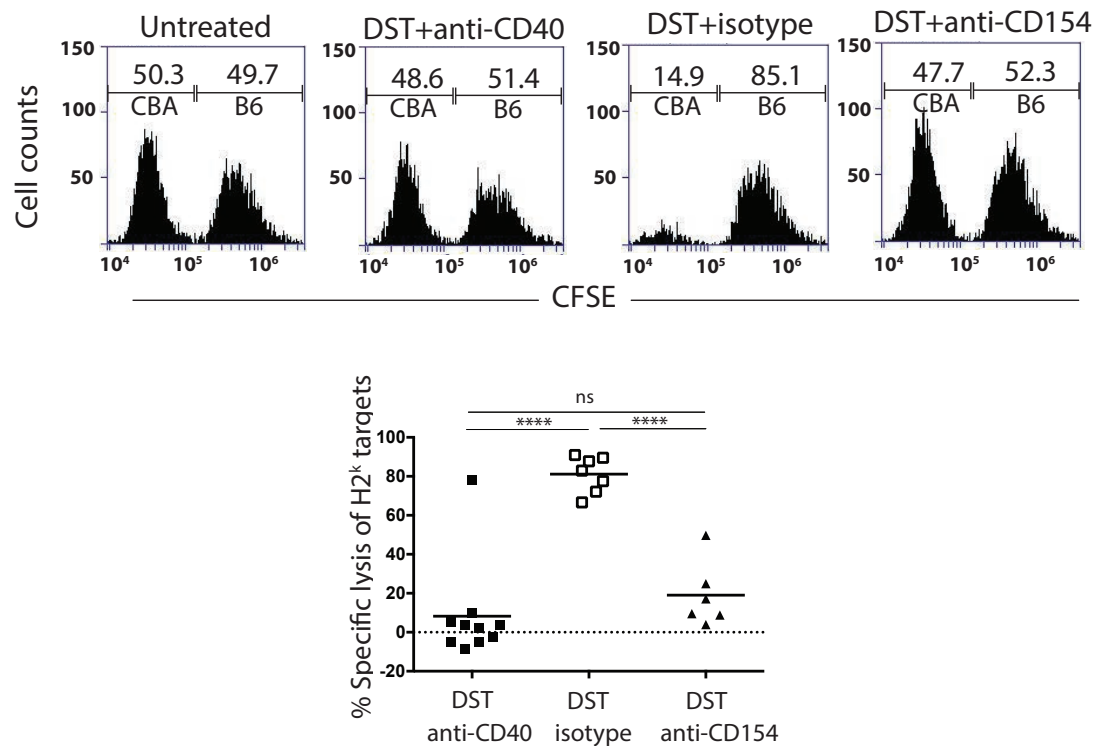


Fig. 2.4. DST/anti-CD40 mAb treatment inhibits cytolysis of allogeneic target cells. C57BL/6 mice were treated on day 0 with a DST of  $10 \times 10^6$  CBA splenocytes i.v. and 0.5 mg of anti-CD154 mAb or 1 mg of anti-CD40 mAb or its isotype control i.p. as indicated. On day 3, CFSE labeled CBA (H2<sup>k</sup>) and C57BL/6 (H2<sup>b</sup>) splenocytes were transferred intravenously at equal proportions into the mice, and the survival of each population was determined 3 hours later. Survival of the CBA splenocytes is shown in the representative histograms and the graph shows percent specific lysis of CBA splenocytes. Data are cumulative of 2 independent experiments. \*p<0.05, \*\*p<0.01, \*\*\*\*p<0.0001.

Chimeras generated from these mice, called 'KB5 chimeras' have 2-12% alloreactive CD8 T cells in the circulation that can be recognized by the clonotypic antibody DES (Iwakoshi et al., 2001). KB5 chimeric mice were injected with a DST from B6 (H-2<sup>b</sup>) mice and a single dose of anti-CD154, anti-CD40 or the isotype control Ab. The frequency of alloreactive DES<sup>+</sup> CD8 T cells was then monitored in the blood. DES<sup>+</sup> CD8 T cells in all mice that received DST underwent an early attrition (Table 2.1). This finding suggests that the DST component of costimulation blockade is responsible for this early death of alloreactive CD8 T cells. This phase of cell death was followed by a rapid expansion in the DST/isotype control group by day 3 suggesting a rapid burst of proliferation. Expansion of DES<sup>+</sup> CD8 T cells was however blunted in the DST/anti-CD40 and DST/anti-CD154 groups and significantly lower numbers were found in the spleens of these mice (Table 2.1, Fig. 2.5A). The inhibition of expansion of alloreactive CD8 T cells observed in the DST/anti-CD40 group between 48 and 72 hours (Table 2.1) suggested these cells are refractory to proliferation. To determine the proliferative potential of alloreactive CD8 T cells with each treatment,  $2 \times 10^6$  VPD450 labeled DES<sup>+</sup> CD8 T cells were adoptively transferred into non-transgenic CBA mice that were then injected with B6 DST and anti-CD154, anti-CD40 or the isotype control Ab after 24 hours. Mice were sacrificed on day 3 post-treatment for proliferation analysis of VPD450 labeled DES<sup>+</sup> CD8 T cells in the spleen.

**Table 2.1. DST/anti-CD40 treatment inhibits expansion of alloreactive CD8 T cells in blood.**

Treatment	n	Day 0	Day 2	Day 3	Day 7
		DES <sup>+</sup> (%)	DES <sup>+</sup> (%)	DES <sup>+</sup> (%)	DES <sup>+</sup> (%)
-	6	6.33±0.86	7.88±1.54 <sup>a</sup>	7.27±1.10 <sup>b</sup>	6.257±1.15
DST/anti-CD40	10	7.05±0.35	0.94±0.06	1.79±0.56	1.62±0.19
DST/isotype control	10	6.06±0.73	0.52±0.07	6.07±1.12 <sup>b</sup>	2.6±0.33
DST/anti-CD154	7	6.71±0.79	0.69±0.34	1.24±0.27	1.26±0.15

KB5 chimeras were administered the indicated treatment and bled at the indicated times. The values indicate the percentage of DES<sup>+</sup> CD8 T cells in the blood (gated on lymphocytes) and are expressed as mean ± SEM. Data are cumulative of two independent experiments. <sup>a</sup> p<0.0001 vs all other groups, <sup>b</sup> p<0.05 vs DST/anti-CD40 and DST/anti-CD154 groups.

Fig. 2.5. DST/anti-CD40 mAb treatment inhibits alloreactive CD8<sup>+</sup> T cell expansion and function.

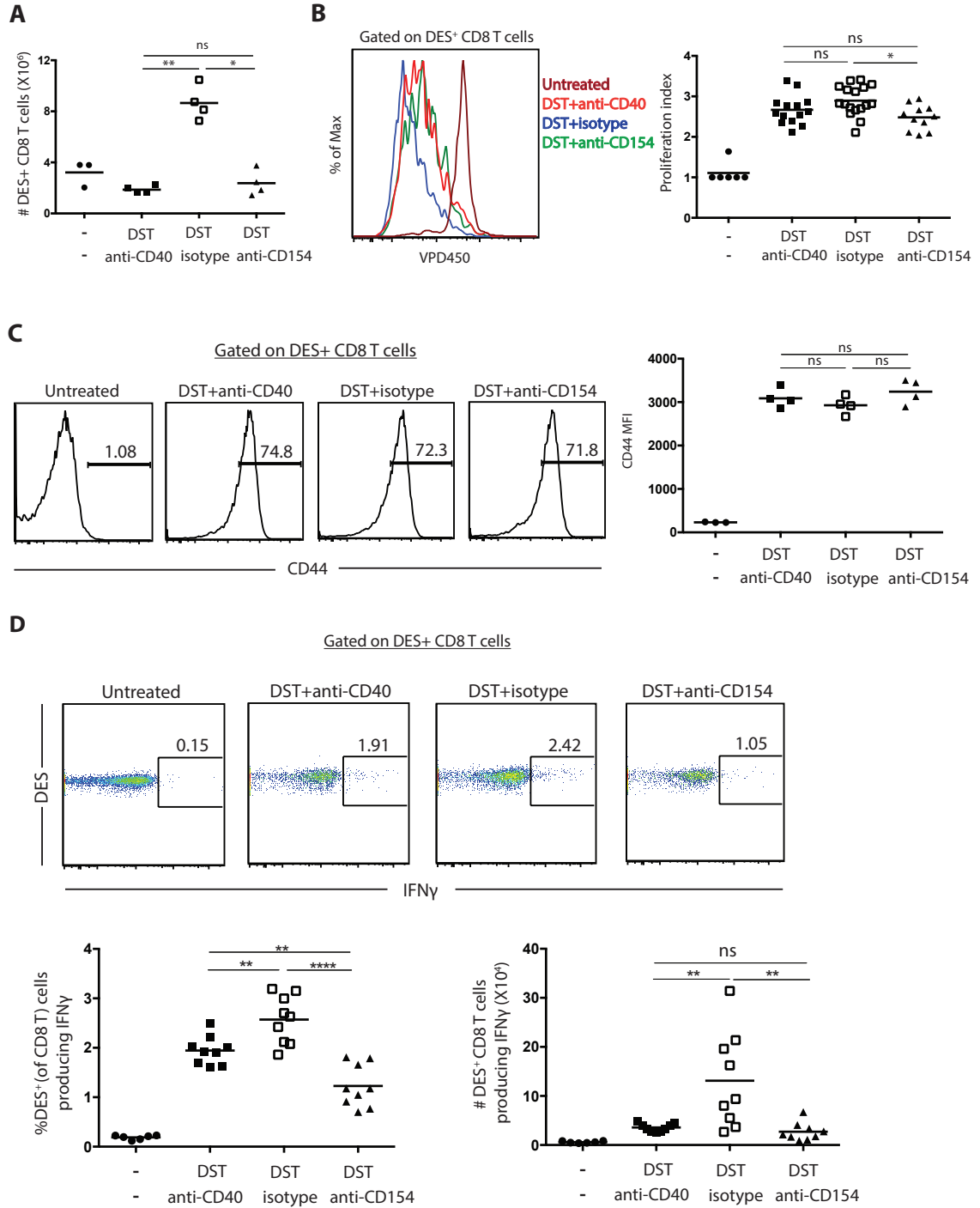


Fig. 2.5. DST/anti-CD40 mAb treatment inhibits alloreactive CD8<sup>+</sup> T cell expansion and function. (A) KB5 chimeric mice were treated on day 0 with a DST of  $10 \times 10^6$  C57BL/6 splenocytes i.v. and 0.5 mg of anti-CD154 mAb or 1 mg of anti-CD40 mAb or its isotype control i.p and sacrificed on day 3. The numbers of DES<sup>+</sup> CD8<sup>+</sup> T cells in the spleen are shown. Data are representative of 2 experiments. (B)  $2 \times 10^6$  VPD450 labeled DES<sup>+</sup> CD8 T cells were transferred intravenously to non-transgenic CBA mice that further received the indicated treatments. Mice were sacrificed on day 3 and DES<sup>+</sup> CD8 T cells from the spleen were assessed for VPD450 dilution. Representative VPD450 profiles and proliferation indices for each treatment group are shown. (C) Mice treated as in (A) were sacrificed on day 3. Representative flow plots for CD44<sup>hi</sup> DES<sup>+</sup> CD8 T cells and CD44 MFI are shown. Data are representative of 2 independent experiments. (D) Mice treated as in (A) were sacrificed on day 3 and splenocytes were stained for intracellular IFN- $\gamma$  upon allogeneic B6 stimulation. The top panel shows representative flow plots of IFN- $\gamma$  staining on DES<sup>+</sup> CD8 T cells. The bottom panel shows percentages and numbers of IFN- $\gamma$  producing DES<sup>+</sup> CD8 T cells. All data are cumulative of 2 independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.0001$ .

Cells in all mice that received DST underwent multiple cycles of divisions; however, anti-CD40 and anti-CD154 treatments resulted in modest delay in cell division (Fig. 2.5B). These data suggest that anti-CD40 mAb induces abortive expansion of DES<sup>+</sup> CD8 T cells to alloantigen challenge, a process where cells proliferate but then undergo cell death impairing the net accumulation of cells.

I next assessed if anti-CD40 mAb reduces activation and function of DES<sup>+</sup> CD8 T cells. I first quantified the expression of the activation marker CD44 on DES<sup>+</sup> CD8 T cells in the spleen on day 3. I found that a majority of DES<sup>+</sup> CD8 T cells in the mice that received DST were CD44<sup>hi</sup> (DST/isotype control group: 79.5 ± 2.4%, DST/anti-CD40 group: 77.1 ± 1.8%, DST/anti-CD154 group: 74.8 ± 1.1%). CD44 expression in the three groups was comparable indicating that anti-CD40 mAb treatment does not inhibit alloreactive CD8 T cell activation (Fig. 2.5C). However, a significantly lower percentage of DES<sup>+</sup> CD8 T cells in the DST/anti-CD40 group produced IFN-γ following ex vivo restimulation with allogeneic C57BL/6 cells relative to DST/isotype control group (Fig. 2.5D). Interestingly, DST/anti-CD154 treated mice showed a significantly lower percentage of IFN-γ producing effector DES<sup>+</sup> CD8 T cells compared to DST/anti-CD40 treatment. The total number of IFN-γ<sup>+</sup> DES<sup>+</sup> CD8 T cells was significantly lower in both DST/anti-CD40 and DST/anti-CD154 groups relative to DST/isotype control (Fig. 2.5D). Together, these data indicate that blocking CD40 in mice limits the magnitude of the alloresponse as well as the function of alloreactive CD8 T cells.



### **Anti-CD40 antibody does not deplete CD40 expressing antigen presenting cells**

Productive activation and function of T cells requires priming by antigen-presenting cells. I tested the hypothesis that the anti-CD40 mAb acts by depleting CD40 expressing antigen-presenting cells thus causing a deficiency of cells required to prime alloreactive T cells and mediate graft rejection. I administered CBA DST and a single dose of anti-CD40, isotype control or anti-CD154 mAb to C57BL/6 mice. Mice were sacrificed 24 hours later and numbers of B cells, dendritic cells and macrophages were determined in the spleen. Spleens of the mice from each treatment group showed comparable numbers of each of the cell types indicating that the anti-CD40 mAb does not deplete CD40 expressing cells (Fig. 2.6).

### **Foxp3<sup>+</sup> Tregs are necessary for the tolerogenic effects of DST/anti-CD40 costimulation blockade**

Regulatory T cells play a vital role in the induction and maintenance of tolerance to allografts (Ferrer et al., 2014; Walsh et al., 2004). Depletion of CD4<sup>+</sup> CD25<sup>+</sup> Tregs by the anti-CD25 mAb PC61 prevented tolerance induction by DST/anti-CD154 (Quezada et al., 2005).

Fig. 2.6. Anti-CD40 mAb does not deplete CD40 expressing cells.

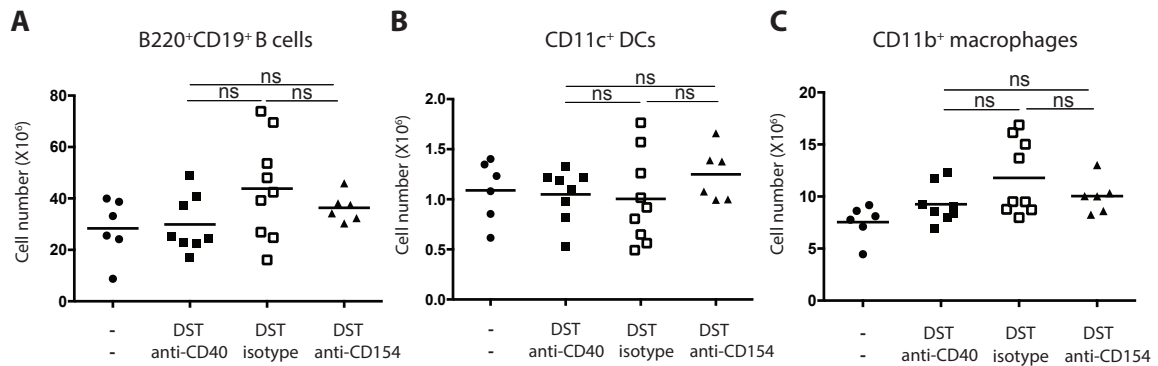
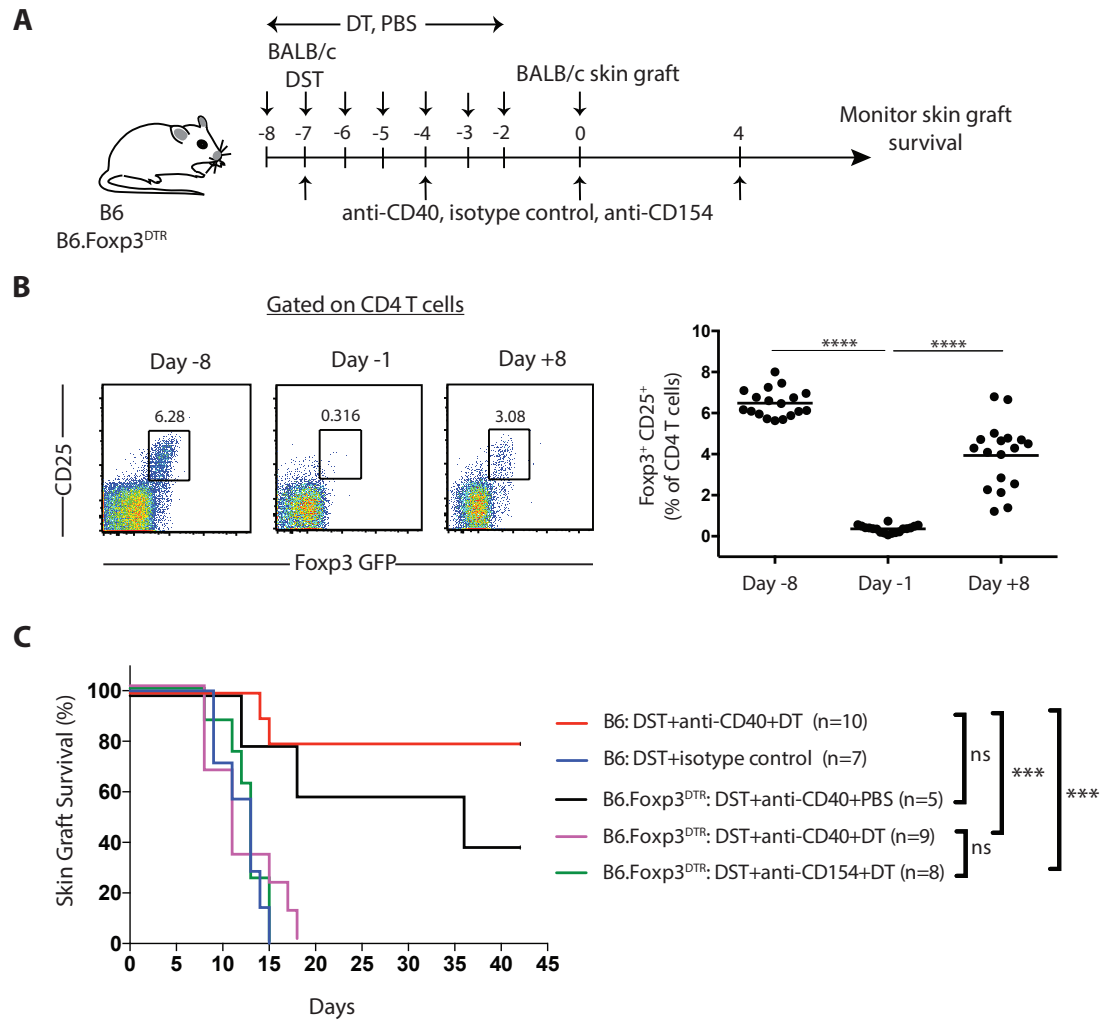


Fig. 2.6. Anti-CD40 mAb does not deplete CD40 expressing cells. C57BL/6 mice were treated with a DST of  $10 \times 10^6$  CBA splenocytes i.v. and 0.5 mg of anti-CD154 mAb or 1 mg of anti-CD40 mAb or its isotype control i.p and sacrificed 24 hours later. The numbers of B220<sup>+</sup> CD19<sup>+</sup> B cells (A), CD11c<sup>+</sup> dendritic cells (B) and CD11b<sup>+</sup> macrophages (C) in the spleen are shown. Data are cumulative of 2 independent experiments.

However, PC61 can potentially deplete activated CD4 and CD8 T cells that have transiently upregulated CD25 confounding interpretations of results obtained with this depletion. To circumvent this problem, I employed B6.Foxp3<sup>DTR</sup> mice in order to test the role of CD4<sup>+</sup> Foxp3<sup>+</sup> Tregs in prolonging allograft survival by the DST/anti-CD40 therapy. These mice express DTR-EGFP genes knocked-in into the *Foxp3* locus and maintain expression of the endogenous *Foxp3* gene intact (Kim et al., 2007). Injection of diphtheria toxin to these mice specifically ablates Foxp3<sup>+</sup> cells. B6.Foxp3<sup>DTR</sup> mice and B6 mice were injected with DT daily from day -8 to day -2 relative to BALB/c skin transplantation on day 0 (Fig. 2.7A). Mice were bled before DT treatment and on day -1 to estimate Treg levels. DT treatment caused over 97% depletion of CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Tregs in the blood of B6.Foxp3<sup>DTR</sup> mice (Fig. 2.7B). Tregs rebounded to 70% of their original levels in blood on day +8. B6.Foxp3<sup>DTR</sup> mice that received DST/anti-CD40/PBS demonstrated prolonged skin allograft survival (MST of 35 days) indicating that they don't have an intrinsic resistance to tolerance induction (Fig. 2.7C). Confirming earlier observations, Treg depletion by DT in DST/anti-CD154 treated B6.Foxp3<sup>DTR</sup> recipients precipitated allograft rejection (MST of 13 days). DT administration induced a similar rejection of skin allografts in B6.Foxp3<sup>DTR</sup> mice treated with DST/anti-CD40 (MST of 11 days). WT C57BL/6 mice treated with DST/anti-CD40/DT exhibited prolonged allograft survival indicating that DT, by itself, does not cause allograft rejection (Fig. 2.7C).

**Fig. 2.7. CD4<sup>+</sup> Foxp3<sup>+</sup> Tregs are necessary for tolerance induction by DST/anti-CD40 mAb treatment.**



**Fig. 2.7. CD4<sup>+</sup> Foxp3<sup>+</sup> Tregs are necessary for tolerance induction by DST/anti-CD40 mAb treatment.** (A) B6 or B6.Foxp3<sup>DTR</sup> mice were grafted with BALB/c skin on day 0 and treated with a DST of  $10 \times 10^6$  BALB/c splenocytes i.v. on day -7 and 0.5 mg of anti-CD154 mAb or 1 mg of anti-CD40 mAb or its isotype control i.p. on days -7, -4, 0 and +4 where indicated. 100 ng of diphtheria toxin or PBS was administered daily from day -8 to day -2 where indicated. (B) B6.Foxp3<sup>DTR</sup> mice that received diphtheria toxin were bled on days -8, -1 and +8. Representative flow plots and graphs showing the frequency of C25<sup>+</sup> Foxp3(GFP)<sup>+</sup> of total CD4<sup>+</sup> T cells. (C) Graft survival curves are shown. Data are cumulative of two independent experiments. \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

These data demonstrate that the tolerogenic properties of DST/anti-CD40 therapy are dependent on Foxp3<sup>+</sup> Tregs.

### **Administration of LPS at the time of DST and anti-CD40 mAb shortens skin allograft survival**

Previous studies have shown that inflammation resulting from viral infections like LCMV and Pichinde virus and bacterial infections like *Listeria monocytogenes* and *Staphylococcus aureus* prevents costimulation blockade mediated tolerance induction (Ahmed et al., 2011; Wang et al., 2008; Welsh et al., 2000). Studies have reported that TLR (Toll like receptor) agonists LPS, poly(I:C), Pam<sub>3</sub>CysK<sub>4</sub> and CpG prevent anti-CD154 mAb mediated long-term allograft acceptance (Chen et al., 2006; Thornley et al., 2006a). I questioned if DST/anti-CD40 induced tolerance is susceptible to inflammatory stimuli. LPS was administered to mice that received DST and anti-CD154 or anti-CD40 mAb on day -7 relative to skin (Fig. 2.8A). Confirming previous observations, LPS treatment resulted in acute allograft rejection in the DST/anti-CD154 group (MST = 14.5 days, Fig. 2.8B). B6 mice in the DST/anti-CD40/LPS group also rejected skin allografts with an MST of 14 days (Fig. 2.8B).

Fig. 2.8. LPS prevents DST/anti-CD40 mediated tolerance induction.

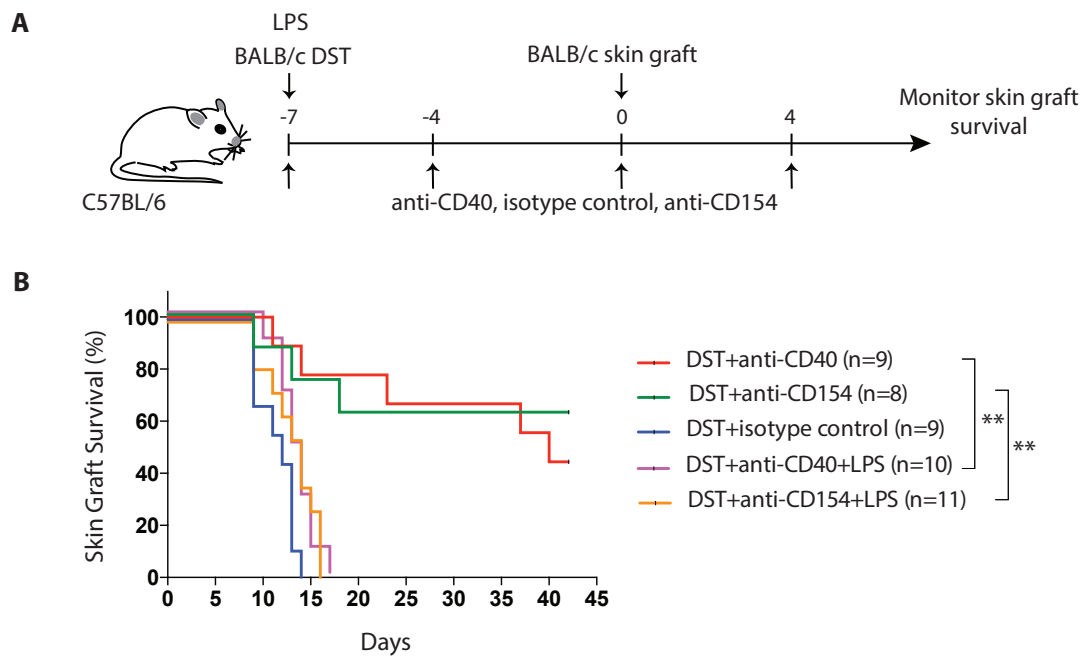


Fig. 2.9. LPS prevents DST/anti-CD40 mediated tolerance induction. (A) C57BL/6 mice were grafted with BALB/c skin on day 0 and treated with a DST of  $10 \times 10^6$  BALB/c splenocytes i.v. on day -7 and 0.5 mg of anti-CD154 mAb or 1 mg of anti-CD40 mAb or its isotype control i.p. on days -7, -4, 0 and +4 where indicated. 50 ug LPS was injected on day -7 relative to transplantation. Graft survival curves are shown in (B). Data are cumulative of two independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$ .

To determine if LPS activated alloreactive B cells during costimulation blockade with anti-CD40 mAb, alloantibodies were measured in treated mice. However, I found that LPS treatment did not increase alloantibody levels compared to DST/anti-CD40 treatment alone (Fig. 2.9A). Previously our lab had shown that CD8 T cells were necessary for LPS to shorten skin allograft survival induced by DST/anti-CD154 (Thornley et al., 2006b). Indeed, the percentage and numbers of CD8 T cells secreting IFN- $\gamma$  in response to allogeneic (BALB/c) stimulation were higher in mice treated with DST/anti-CD154/LPS relative to mice treated with DST/anti-CD154 alone although the difference was not statistically significant (Fig. 2.9B). Similarly higher frequencies of IFN- $\gamma$  producing CD8 T cells were observed in mice treated with DST/anti-CD40/LPS suggesting a prominent role of alloreactive CD8 T cells in LPS mediated allograft rejection despite DST/anti-CD40 treatment.

Fig. 2.9. LPS challenge increases levels of donor-reactive IFN- $\gamma$  producing CD8 T cells, but not alloantibodies in response to DST/anti-CD40.

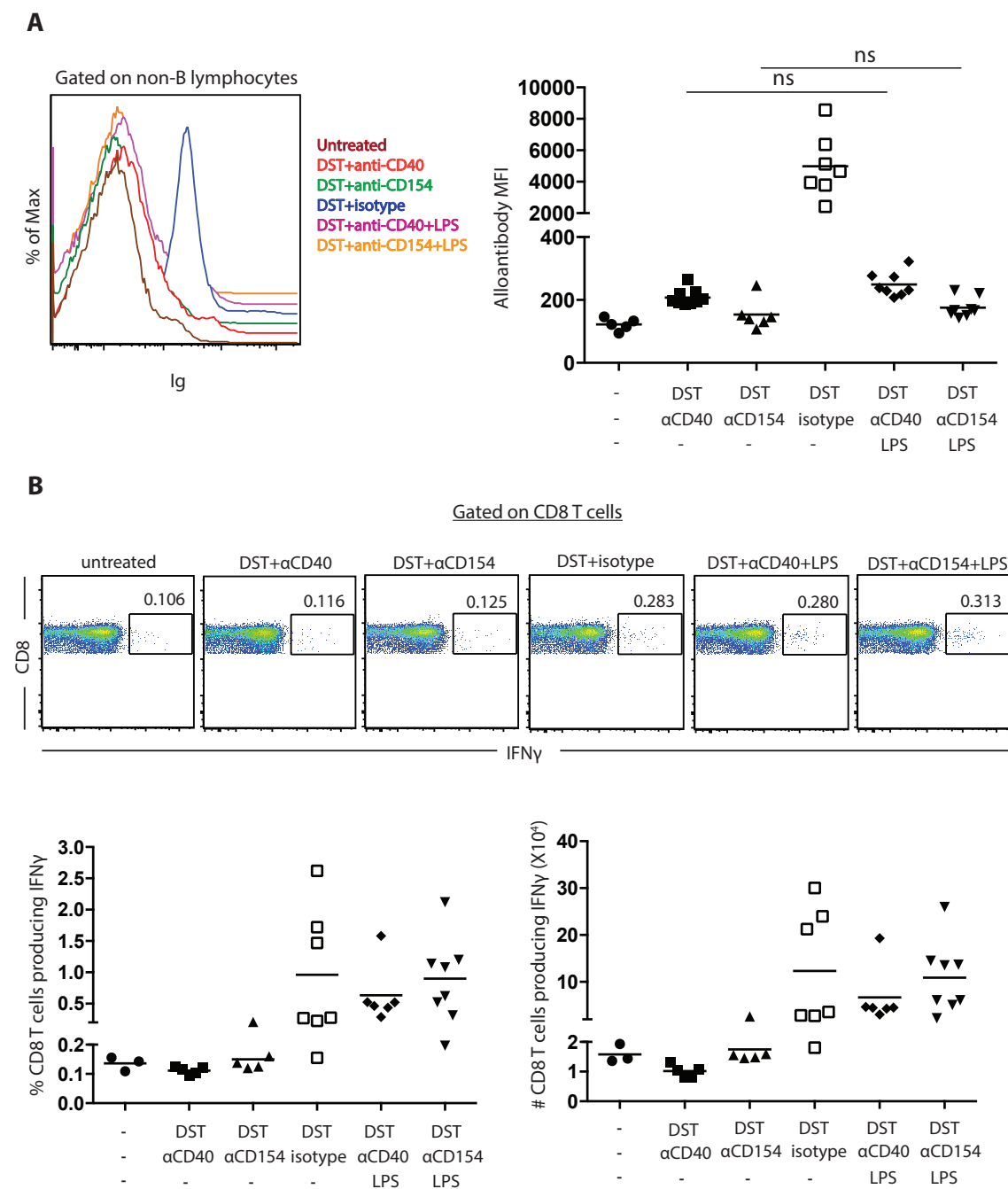




Fig. 2.9. LPS challenge increases levels of donor-reactive IFN- $\gamma$  producing CD8 T cells, but not alloantibodies in response to DST/anti-CD40. C57BL/6 mice were treated as in Fig. 2.8A. (A) Alloantibodies in the plasma of the mice were measured by flow cytometry 2 weeks post-transplantation. Plasma from untreated mice served as negative control for the alloantibody assay. (B) Splenocytes were harvested from the mice 6 weeks post-transplantation and stained for intracellular IFN- $\gamma$  upon allogeneic BALB/c stimulation. The percentages and numbers of IFN- $\gamma$  producing CD8<sup>+</sup> T cells are shown. Data are cumulative of two independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$ .

## Discussion

CD40-CD154 interactions play a critical role in the regulation of the immune response and hence are being actively targeted in transplantation, autoimmunity and cancer. After a brief hiatus following the unexpected development of thromboembolism in patients receiving anti-CD154 reagents in clinical trials for renal transplantation (Kawai et al., 2000), the CD40-CD154 pathway has enjoyed a resurgence with the development and success of anti-CD40 antibodies in experimental models of transplantation. However, the modes of action of these antibodies in vivo at the molecular and cellular level are not clear. CD40 targeting antibodies possessing variable attributes like isotype, target epitope, agonistic potential, serum half-life and cellular depletion have prolonged allograft survival in non-human primates (Adams et al., 2005; Aoyagi et al., 2009; Badell et al., 2012; Cordoba et al., 2015; Haanstra et al., 2003; Lowe et al., 2012; Oura et al., 2012; Page et al., 2012; Pearson et al., 2002). Conceptually, an ideal CD40 antibody candidate for clinical translation in transplantation would 1) be an antagonist 2) have a long serum half-life 3) not deplete CD40 expressing APCs thereby preserving protective immunity to pathogens 4) be non-immunogenic. Knowledge of the mechanisms by which anti-CD40 antibodies prolong graft survival will help investigators design better reagents for clinical translation. In this study, I described a novel murine anti-CD40 mAb and compared its effects on skin allograft survival and donor-reactive T cell responses with the anti-CD154 mAb MR1. My data demonstrate that anti-

CD40 mAb is as effective as anti-CD154 mAb in the induction of transplantation tolerance and suppression of alloimmunity.

CD154 has four known receptors: the classical receptor CD40 and the integrins Mac-1 (Wolf et al., 2011),  $\alpha_{IIb}\beta_3$  (André et al., 2002) and  $\alpha_5\beta_1$  (Leveille et al., 2007). The contribution of each of these interactions to the development of an alloresponse needs to be elucidated to gain a complete understanding of the mechanism of action of anti-CD154 antibodies. The current paradigm is that anti-CD154 antibodies inhibit the binding of CD154 on activated CD4 T cells to CD40 on APCs preventing APC maturation resulting in diminished expansion and differentiation of alloreactive T cells (Ford, 2016).  $\alpha_{IIb}\beta_3$  is expressed only on platelets and its binding to soluble CD154 was shown to be necessary for stability of arterial thrombi (André et al., 2002). The CD154 -  $\alpha_{IIb}\beta_3$  interaction is thus important for thrombosis with no known role in alloimmunity. Mac-1 and  $\alpha_5\beta_1$  are expressed on innate immune cells like monocytes, macrophages and neutrophils and play a role in cellular adhesion and migration. Studies by El-Sawy and colleagues demonstrated that attenuating neutrophil-mediated tissue damage during reperfusion improved the efficacy of costimulation blockade to prevent cardiac allograft rejection suggesting a vital role of innate cells in transplant rejection (El-Sawy et al., 2005). Recently CD154 costimulation blockade was shown to reduce chemokine expression and intra-graft infiltration of neutrophils and CD8 T cells (Ferrer et al., 2012b). It is conceivable that CD154-Mac1/ $\alpha_5\beta_1$  interactions drive the recruitment of immune cells to the

allograft and inhibition of these interactions by anti-CD154 antibody delays graft rejection. Earlier studies attributed the tolerogenic properties of the anti-CD154 mAb (MR1) to Fc-mediated deletion of activated alloreactive T cells (Monk et al., 2003). However, subsequent studies suggested that Fc-dependent functions of anti-CD154 mAb are not critical for induction of tolerance. An aglycosylated form of anti-CD154 exhibiting reduced binding to Fc receptors and complement was able to prolong skin allograft survival with similar efficacy as the glycosylated form (Daley et al., 2008). Moreover, an Fc-silent anti-CD154 domain antibody attenuated donor-reactive effector T cell responses, promoted generation of Foxp3<sup>+</sup> induced Tregs (iTregs) and prolonged skin graft survival comparable to the Fc-intact antibody (Pinelli et al., 2013).

The anti-CD40 mAb used in the studies described here has several point mutations in the mouse IgG2a Fc region to reduce antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) and increase its serum half-life. Mouse IgG2a antibodies have a high activatory : inhibitory FcγR binding ratio and thus naturally possess ADCC and CDC effector functions (Beers et al., 2016). I confirmed that the Fc-modified antibody did not deplete CD40 expressing cells in vivo (Fig. 2.6). Coupled with DST, the anti-CD40 mAb prolonged survival of allogeneic skin in a transplantation model of acute rejection with comparable efficacy to anti-CD154 mAb. In this model, acute rejection is primarily mediated by alloreactive T cells. Deletion, anergy and immunoregulation of alloreactive T cells are important mechanisms of peripheral

tolerance to allografts. Studies have demonstrated a requirement for deletion of alloreactive T cells in order to induce tolerance to allografts (Wells et al., 1999). Using the KB5 synchimera model, I investigated the effects of DST/anti-CD40 treatment on donor-reactive CD8 T cells in detail. I observed that this treatment predominantly impacted the magnitude of the alloresponse with a smaller impact on alloreactive CD8 T cell functionality. It is possible that the skin infiltrating alloreactive CD8 T cells exhibit a more profound reduction in functionality compared to cells in the spleen. I acknowledge that the KB5 model is a model of direct allorecognition and does not fully recapitulate the endogenous alloresponse, which has features of both direct and indirect recognition. A recent study using an indirect model of allorecognition reported that alloreactive CD4 T cells undergo abortive expansion following DST/anti-CD154 treatment, but the residual cells retain the capacity to make IL-2, IFN- $\gamma$  and TNF- $\alpha$  (Chai et al., 2015). The functional impairment of alloreactive CD8 T cells following DST/anti-CD154 or DST/anti-CD40 treatment suggest that CD4 and CD8 T cells might be differentially regulated by costimulation blockade. Further, my observation that DST/anti-CD154 treatment generates a significantly lower percentage of IFN- $\gamma$ <sup>+</sup> DES<sup>+</sup> effector CD8 T cells relative to DST/anti-CD40 treatment raises the possibility of the involvement of other CD154 receptors in regulating IFN- $\gamma$  production by CD8 T cells.

Engagement of CD40 on DCs with CD154 on activated CD4 T cells 'licenses' DCs by inducing upregulation of B7 molecules CD80 and CD86 as well as MHC-I and MHC-II on the DC surface besides promoting secretion of cytokines IL-12, IL-6 and TNF (Pinelli and Ford, 2015). Moreover, CD40 signaling into DCs promotes DC survival by upregulating expression of the anti-apoptotic protein Bcl-xL (Wong et al., 1997). DC-mediated triggering of T cell responses depends on both DC longevity and maturation. I found no significant change in DC numbers after anti-CD40 treatment (Fig. 2.6B) suggesting that the attenuated CD8 T cell responses in mice receiving these antibodies are not a result of reduced alloantigen-presenting DC survival. Ferrer et al. recently demonstrated reduced secretion of inflammatory cytokines IL-6 and IL-12 by DCs from mice treated with anti-CD154 mAb (Ferrer et al., 2012a). It is likely that DST/anti-CD40 treatment has a similar effect of impairing the provision of 'signal three' for development of productive alloreactive T cell responses. An additional interaction likely to be inhibited by the anti-CD40 mAb is that between CD40 expressed on activated CD8 T cells and CD154 on activated CD4 T cells. Using genetic ablation of CD40 on different cell types, Liu et al. demonstrated that loss of CD40 on CD8 T cells was sufficient to prolong allograft rejection and reduce the frequency of IFN- $\gamma$ <sup>+</sup> TNF<sup>+</sup> double-cytokine producing alloreactive CD8 T cells (Liu et al., 2013). The exact contribution of blocking this interaction to prolonging graft survival remains unknown.

The ability of donor-specific antibodies to mediate acute rejection and chronic rejection in clinic is becoming increasingly clear and is considered a major barrier to improving long-term outcomes (Djamali et al., 2014). Therapeutic modalities that control alloantibody production in presensitized transplant recipients and prevent de novo alloantibody generation are of great interest. Interaction of CD40 on activated B cells with CD154 on activated T helper cells is necessary for the generation of T-dependent antibody responses (Foy et al., 1993). CD40 signaling in B cells is required for germinal center formation and progression, antibody isotype switching and affinity maturation (Elgueta et al., 2009). Indeed, I found that inhibition of CD40-CD154 interactions by the anti-CD40 or anti-CD154 mAb coupled with DST led to a drastic decrease in alloantibody levels in vivo (Fig. 2.3A). Thus, CD40 costimulation blockade limits T as well as B cell responses to the graft.

Regulatory T cells are major players in the maintenance of self-tolerance as well as induced tolerance. I demonstrated a requirement for Foxp3<sup>+</sup> Tregs during the induction phase of tolerance by the anti-CD40 mAb. Ferrer et al. showed that DST/anti-CD154 induces peripheral graft-reactive Tregs as early as 7 days post DST and these iTregs infiltrate the graft and mediate immunosuppression. (Ferrer et al., 2011). Using diphtheria toxin to deplete Foxp3<sup>+</sup> cells in B6.Foxp3<sup>DTR</sup> mice, I demonstrated over 95% depletion of CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> cells by day 7 post DST (Fig. 2.7B). I speculate that the DT treatment depleted nTregs as well as prevented the accumulation of a significant

number of iTregs resulting in skin graft rejection. My results demonstrate the dominant role of Foxp3<sup>+</sup> Tregs in tolerance induction by CD40 costimulation blockade and that Foxp3<sup>-</sup> Tregs are not sufficient to induce tolerance.

Certain viral and bacterial infections in the peri-transplant period have been shown to override the tolerizing effects of anti-CD154 mAb by generating CTLs cross-reactive with the alloantigen or by promoting bystander activation of alloreactive T cells (Ahmed et al., 2011; Welsh et al., 2000). Our lab has previously demonstrated that LPS challenge in the pre-transplant period shortened DST/anti-CD154 mediated skin graft survival in a CD8 T cell dependent manner (Thornley et al., 2006b). LPS signaling through TLR4 stimulated type I IFN secretion that prevented the deletion of alloreactive CD8 T cells (Thornley et al., 2007b). My present study reports that skin graft acceptance by DST/anti-CD40 is equally susceptible to LPS (Fig. 2.8B). I observed that LPS treatment did not increase alloantibody levels in the plasma, which is consistent with another report showing that another TLR agonist CpG does not restore alloantibody levels at doses that induce graft rejection (Chen et al., 2006). Higher levels of IFN- $\gamma$  producing CD8 T cells were generated following LPS challenge (Fig. 2.9B) suggesting that LPS bypasses CD40 blockade to promote effector T cell responses. Although this study does not describe the mechanism by which LPS shortens graft survival, I speculate that LPS induces APC maturation resulting in increased costimulatory molecule expression and production of cytokines enhancing T cell responses.



In conclusion, I describe a novel, antagonistic, Fc-mutated non-depleting anti-CD40 mAb that prolongs fully mismatched skin graft survival and attenuates donor-reactive T and B cell responses when administered with DST. This study highlights that anti-CD154 mAb and anti-CD40 mAb has similar effects on skin allograft survival and cell-mediated and humoral responses and thus, these antibodies act predominantly by blocking the CD40-CD154 interaction. The role of CD154 interacting with its integrin receptors in the generation of alloresponses appears to be minimal, if any. In line with other studies, I have shown that depletion of APCs is not necessary to achieve prolonged graft survival by CD40 targeting antibodies (Cordoba et al., 2015; Okimura et al., 2014). Finally, I show that tolerance induction by CD40 blockade is susceptible to inflammatory insults. I propose that therapies targeting multiple costimulatory pathways and the transfer of autologous ex-vivo expanded polyclonal or donor antigen-specific Tregs is needed to achieve robust transplantation tolerance induction in clinic.

## **CHAPTER III**

**Proapoptotic proteins Fas and Bim  
concurrently regulate costimulation  
blockade induced tolerance to allografts**

### Abstract

Apoptosis of CD8 T cells is an essential mechanism that maintains immune system homeostasis, prevents autoimmunity and reduces immunopathology. CD8 T cell death also occurs early during both the response to inflammation and the induction of peripheral tolerance to allografts by costimulation blockade therapy. T cell death is mediated by the extrinsic death receptor pathway and intrinsic or mitochondrial death pathway. Here I studied the effects of a combined deficiency of Fas (extrinsic) and Bim (intrinsic) on peripheral tolerance induction to allografts by costimulation blockade. *Bcl2l11<sup>-/-</sup>Fas<sup>pr/pr</sup>* mice were resistant to tolerance induction by costimulation blockade targeting the CD40-CD154 pathway. This result demonstrates that both extrinsic and intrinsic apoptosis pathways function concurrently to regulate T cell homeostasis during tolerance induction.

### Introduction

Apoptosis is a critical mechanism regulating T cell homeostasis and is essential for T cell development, for suppression of autoreactive T cells, and for the contraction phase of an antigen-specific T cell response. The well described attrition of memory T cells occurring early after both viral infection and peripheral tolerance induction by costimulation blockade therapy also involves apoptosis of T cells. Death of alloreactive T cells is an important component for successful induction of peripheral tolerance to allografts during blockade of costimulation

pathways (Li et al., 2001; Wells et al., 1999). Tolerance induction strategies targeting the CD28-B7 and CD40-CD154 costimulation pathways have been tested extensively in animal models of transplantation (Ford, 2016). Costimulation blockade utilizing the reagents CTLA4-Ig and anti-CD154 mAb extends survival of skin, islets, heart and kidney allografts in mice (Maltzman and Turka, 2013). (CTLA4-Ig + anti-CD154) induced tolerance to allografts in FasL deficient *gld* mice or Fas deficient *lpr* mice suggesting that the Fas-FasL pathway is not necessary for tolerance induction (Li et al., 1999; Trambley et al., 2001; Wagener et al., 2000). Moreover, Bim deficient mice were sensitive to tolerance induction by costimulation blockade (CTLA4-Fc + anti-CD154) indicating that the passive cell death pathway regulated by Bim is dispensable for peripheral tolerance induction (Lehnert et al., 2007).

In the present study, I investigated the effects of a combined deficiency of Fas and Bim on costimulation blockade induced tolerance to skin allografts by generating mice lacking Bim and harboring the inactivating *lpr* mutation in Fas (*Bcl2l11<sup>-/-</sup>Fas<sup>lpr/lpr</sup>*). Mice lacking Bim and bearing the *lpr* mutation in Fas have a block in the contraction of antigen-specific T cells in chronic and certain acute viral infections, dysregulated homeostatic proliferation and develop lymphadenopathy and autoimmunity (Bouillet and O'Reilly, 2009; Fortner et al., 2010; Hughes et al., 2008; Hutcheson et al., 2008; Weant et al., 2008). My studies show that *Bcl2l11<sup>-/-</sup>Fas<sup>lpr/lpr</sup>* mice were resistant to tolerance induction to skin allografts by costimulation blockade therapy consisting of DST/anti-CD154

mAb. These results indicate that Fas and Bim function concurrently to regulate peripheral tolerance induction by costimulation blockade.

## **Materials and Methods**

### *Mice*

C57BL/6J (H2<sup>b</sup>), BALB/c (H2<sup>d</sup>) and CBA/J (H2<sup>k</sup>) mice were obtained from The Jackson Laboratory. Bcl2l11<sup>-/-</sup> and Bcl2l11<sup>-/-</sup>Fas<sup>lpr/lpr</sup> mice were obtained from Dr. Roger Davis's laboratory at University of Massachusetts Medical School, Worcester, MA. All animals were housed in a specific pathogen free facility in microisolator cages and given autoclaved food. All experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

### *Transplantation*

Recipient mice of the specified strain were transplanted with complete MHC mismatched skin and treated with a tolerizing regimen of DST and anti-CD154 mAb as described previously (Iwakoshi et al., 2000). Briefly, full thickness skin grafts were obtained from flanks of donor mice and transplanted onto the dorsal flanks of recipient mice on day 0. A DST of  $1 \times 10^7$  donor splenocytes was injected i.v. on day -7 relative to skin transplantation on day 0. 0.5 mg anti-CD154 mAb, clone MR1 (BioXCell) was given i.p. on days -7, -4, 0 and +4

relative to skin transplantation. Skin grafts were monitored for survival and graft rejection was defined as the first day when the entire graft was necrotic.

#### *Antibodies, flow cytometry and intracellular cytokine staining*

Leukocytes were stained for CD8 $\alpha$  (53-6.7), CD4 (RM4-5), CD44 (IM7), IFN $\gamma$  (XMG1.2). For intracellular cytokine staining,  $2 \times 10^6$  RBC lysed splenocytes from recipient mice were incubated with  $1 \times 10^6$  LPS matured, irradiated syngeneic (H2<sup>b</sup>) or allogeneic (H2<sup>d</sup>) stimulator cells for 4 hrs in complete RPMI and monensin (eBioscience) and assessed for intracellular IFN $\gamma$  production as described previously (Brehm et al., 2007). Samples were analyzed on an LSRII flow cytometer (Becton Dickinson), and data were further analyzed using FlowJo (Tree Star).

#### *Statistics*

Statistical analyses were performed using GraphPad PRISM software. To compare 3 or more means, one-way ANOVA and Tukey's multiple comparisons test were used. All error bars represent the Standard Error of the Mean (SEM). Allograft survival curves were plotted by the Kaplan-Meier method and compared by the log-rank test.

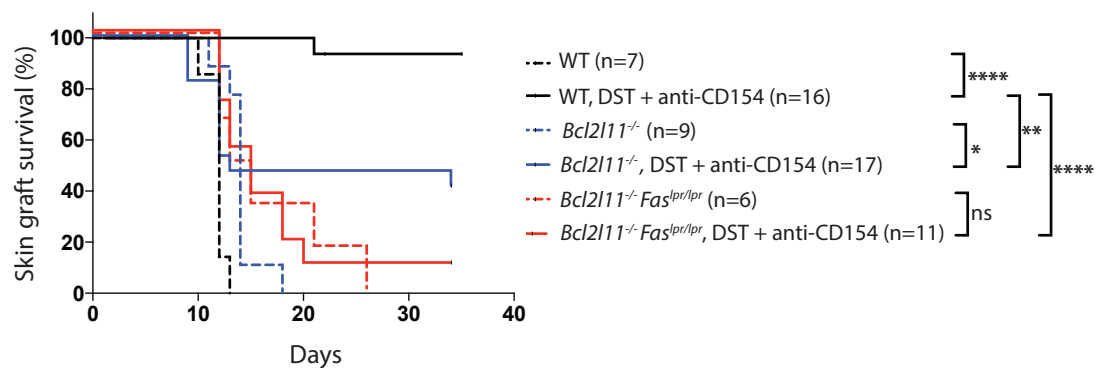
## **Results**

### **Peripheral tolerance induction by costimulation blockade is dependent on Fas and Bim**

To determine the apoptotic pathways involved in allograft survival induced by costimulation blockade, WT C57BL/6J, *Bcl2l11*<sup>-/-</sup> and *Bcl2l11*<sup>-/-</sup>*Fas*<sup>lpr/lpr</sup> mice were transplanted with MHC-mismatched BALB/c skins and treated with DST/anti-CD154 mAb. WT C57BL/6J mice that were given no treatment rejected skin grafts rapidly with a median survival time (MST) of 12 days (Fig. 3.1A). In contrast, WT mice that received DST/anti-CD154 mAb treatment showed prolonged allograft survival, consistent with previous reports (Fig. 3.1A) (Markees et al., 1997). *Bcl2l11*<sup>-/-</sup> mice that received no treatment rejected grafts with kinetics similar to those of WT mice (MST=12 days) (Fig. 3.1A). Interestingly, 10 out of 17 *Bcl2l11*<sup>-/-</sup> mice that received DST/anti-CD154 mAb rejected grafts (MST=13 days) (Fig. 3.1A), suggesting that Bim is partially necessary for tolerance induction. Skin allografts were rejected in *Bcl2l11*<sup>-/-</sup>*Fas*<sup>lpr/lpr</sup> mice that received no treatment, as expected (MST=14 days) (Fig. 3.1A). All *Bcl2l11*<sup>-/-</sup>*Fas*<sup>lpr/lpr</sup> mice that were treated with DST/anti-CD154 mAb rejected their grafts with a MST of 15 days (Fig. 3.1A). These results suggest that loss of Fas function in addition to that of Bim completely prevents the increased survival of skin allografts in mice treated with costimulation blockade.

Fig. 3.1. Costimulation blockade induced tolerance to allografts is dependent on Bim and Fas.

A.



B.

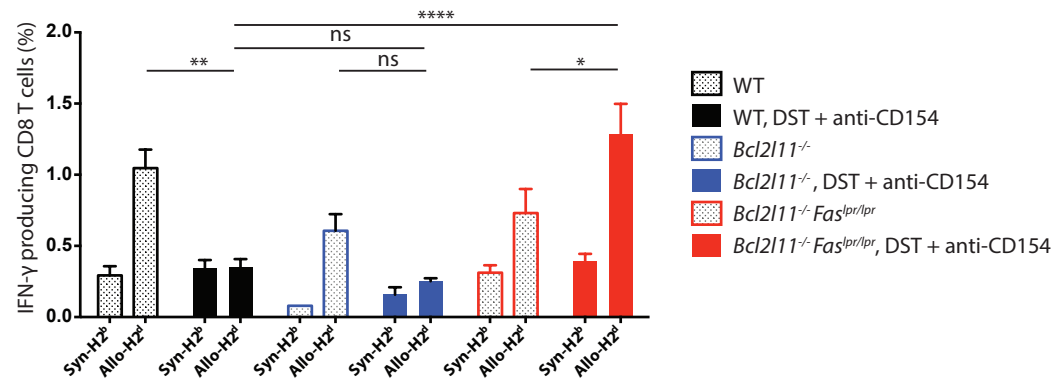


Fig. 3.1. Costimulation blockade induced tolerance to allografts is dependent on Bim and Fas. A) WT C57BL/6, *Bcl2l11*<sup>-/-</sup> and *Bcl2l11*<sup>-/-</sup> *Fas*<sup>*lpr/lpr*</sup> mice were treated with BALB/c DST/anti-CD154 mAb and skin as described in the Materials and Methods. Skin allograft survival was then monitored for all groups. The graph shows skin graft survival curves. B) Splenocytes were harvested from the WT, *Bcl2l11*<sup>-/-</sup> and *Bcl2l11*<sup>-/-</sup> *Fas*<sup>*lpr/lpr*</sup> mice 5 weeks post-transplantation, stimulated with in vitro matured irradiated syngeneic (*H2*<sup>*b*</sup>) or allogeneic (*H2*<sup>*d*</sup>) splenocytes for 4 hours and stained for intracellular IFN-γ by flow cytometry. The bar graphs show percentages of IFN-γ producing CD8 T cells in response to syngeneic and allogeneic stimulation. The data are representative of two independent experiments with 2-5 mice per group. Percentages ± SEM are depicted. \**p*<0.05, \*\**p*<0.01, \*\*\*\**p*<0.0001.



I next investigated the ability of alloreactive CD8<sup>+</sup> T cells in each group of mice to produce IFN- $\gamma$  in response to syngeneic (C57BL/6J) or allogeneic (BALB/c) stimulation. Consistent with our previous observation, DST/anti-CD154 treatment significantly reduced the frequency of donor-reactive IFN- $\gamma$  producing effector/memory CD8<sup>+</sup> T cells in WT mice correlating with increased survival of skin allografts in these mice (Fig. 3.1B) (Brehm et al., 2007). Donor-reactive IFN- $\gamma$  producing CD8<sup>+</sup> T cell frequencies were lowered in *Bcl2l1*<sup>-/-</sup> mice treated with DST/anti-CD154 mAb despite the observation that 10 out of 17 of these mice rejected skin allografts (Fig. 3.1B). *Bcl2l1*<sup>-/-</sup>*Fas*<sup>lpr/lpr</sup> mice were resistant to tolerance induction by DST/anti-CD154 mAb and high frequencies of IFN- $\gamma$  producing CD8<sup>+</sup> T cells were detectable in these mice comparable to those observed in untreated skin-grafted mice (Fig. 3.1B). Together, these data indicate that the deletion of alloreactive T cells and optimal extension of skin allograft survival in mice by costimulation blockade requires both Fas and Bim, with Bim playing a more important role.

## Discussion

T cell deletion is desirable in settings like autoimmunity and organ transplantation. Costimulation blockade is a non-lymphoablative therapy of inducing tolerance to donor tissues by inducing deletion or/and anergy of donor-reactive T cells. Apoptosis of T cells infiltrating into allografts and xenografts was shown to be mediated by the Fas-FasL pathway in some models (Bellgrau et al.,

1995; Lau et al., 1996). However, studies suggest that activation induced cell death (AICD) of alloreactive T cells by Fas is not necessary for tolerance induction by costimulation blockade (Li et al., 1999; Trambley et al., 2001; Wagener et al., 2000). FasL deficient *gld* mice were tolerized to similar extent as WT mice by costimulation blockade consisting of anti-CD154 mAb and CTLA4-Ig in a C3H/HeJ to B6 skin graft model (Trambley et al., 2001). Also, Fas deficient B6.*lpr* mice were tolerized to DBA/2 islet and heart grafts by anti-CD154 mAb/CTLA4-Ig (Li et al., 1999). Moreover, *lpr* mice injected with tumor necrosis factor-alpha (TNF- $\alpha$ ) neutralizing antibody were tolerized to cardiac allografts by CTLA4-Ig suggesting that Fas and TNF receptor (TNFR) independent pathways contribute to alloantigen-driven T cell apoptosis (Wagener et al., 2000). The role of the mitochondrial or passive cell death pathway in the induction of tolerance is less clear. Bcl-xL transgenic mice showed partial resistance to tolerance induction by CTLA4-Ig or anti-CD154 mAb in a BALB/c to B6 model of vascularized cardiac allotransplantation suggesting that the passive cell death pathway is partially necessary for tolerance induction (Wells et al., 1999). However, Bcl-2 transgenic B6 mice were tolerized to BALB/c islet allografts as well as PVG rat islet xenografts by anti-CD154/CTLA4-Fc (Lehnert et al., 2007). Also, this study showed that Bim knockout mice were susceptible to tolerance induction to rat islet xenografts suggesting that the passive cell death pathway is dispensable for tolerance induction (Lehnert et al., 2007). These differences may

be explained by differences in the transplanted tissue, the strains of mice and the costimulation blockade reagents and their dosages.

The two-element costimulation blockade protocol of DST/anti-CD154 mAb results in abortive expansion of alloreactive T cells (Iwakoshi et al., 2000). Tolerance induction by this protocol also requires deletion of mature alloreactive B cells (Li et al., 2007). In this study, I have shown in a stringent BALB/c to B6 skin allograft model, that *Bcl2l11*<sup>-/-</sup> mice are partially resistant whereas *Bcl2l11*<sup>-/-</sup> *Fas*<sup>lpr/lpr</sup> mice are completely resistant to tolerance induction. I propose that the passive cell death pathway is the primary mechanism for tolerance induction with a small contribution by the death receptor pathway. Recent studies have shown that the anti-CD154 mAb inhibits dendritic cell expression of inflammatory cytokines that are required for productive T cell activation and expansion (Ferrer et al., 2012a). In Fas or FasL deficient mice, the passive death pathway activated by the lack of cytokines resulting from CD154 antagonism would mediate apoptosis of alloreactive T cells facilitating tolerance induction. In Bim deficient mice, some cell death would occur by AICD or mitochondrial apoptosis by an alternate BH3 protein such as Puma, Noxa or Bid resulting in partial susceptibility to tolerance induction. However, in mice lacking Fas and Bim, the inhibition of both extrinsic and intrinsic apoptosis completely prevents tolerance induction. These results highlight the concurrent function of multiple apoptotic pathways in regulating immune responses during costimulation blockade.

## **CHAPTER IV**

### **Differential regulation of alloreactive CD8 T cell responses by inflammation**

### **Abstract**

The innate immune system can be activated in patients undergoing solid organ transplantation by inflammation resulting from the release of endogenous danger molecules, infections occurring in peri-transplant period and translocation of microbiota. Inflammatory signals regulate the magnitude and functionality of antigen-specific T cell responses during challenge with viral and bacterial pathogens, but the direct impact of inflammation on the generation of alloreactive T cell responses has not been extensively studied. Understanding the role of inflammation on regulating the activation of alloreactive T cells will aid in the development of novel immune therapies to prolong allograft survival. I used the KB5 chimeras expressing allo-specific TCRs to study the response of alloreactive T cells in the presence of inflammatory signals. My results show that stimuli inducing different inflammatory profiles had significant effects on the size, phenotype and quality of the alloreactive CD8 T cell response as compared to stimulation with alloantigen alone. Inflammation induced by exposure to poly(I:C) increased the number of IFN- $\gamma$  producing and cytolytic alloreactive effector CD8 T cells at the peak of the response. Inflammation induced upon LPS challenge increased the number of alloreactive CD8 T cells at the peak of the response; however their ability to produce IFN- $\gamma$  and cytotoxic activity was decreased. Signaling through recipient TLR4 and IL-6 were necessary for this LPS mediated suppression of CD8 T cell function. LPS slowed the contraction phase of the alloantigen response generating higher levels of memory alloreactive CD8 T cells

relative to poly(I:C). These data indicate that inflammatory signals differentially regulated the kinetics and functionality of alloreactive CD8 T cell responses and may determine susceptibility to immune suppression.

## **Introduction**

Although the rates of acute rejection in clinical transplantation have fallen in recent years with the current immunosuppression regimens, there is an increasing need to develop effective solutions to acute rejection because of the increasing demand for organ grafts (Matas et al., 2014). Identification of the triggers that make grafts susceptible to acute rejection is essential for the development of novel strategies to prolong allograft survival. In addition to the direct and antigen dependent onslaught of the adaptive immune system on the graft, there are also antigen independent factors that predispose the graft to rejection by the immune system (Mori et al., 2014). The role of inflammation in triggering graft rejection is becoming increasingly clear. Broadly, there are three sources of inflammation in the context of transplantation. First, ischemia reperfusion injury results in localized activation of the innate immune system and 'sterile inflammation', a process in which endogenous danger molecules or DAMPs bind to pattern recognition receptors (PRRs) expressed on hematopoietic, endothelial or parenchymal cells to trigger the innate immune system (Mori et al., 2014). Second, infections in transplant recipients can cause activation of PRRs by microbial motifs or PAMPs causing innate immune

activation and inflammation (Alegre et al., 2014). Third, microbiota in the recipient and donor, in the case of colonized organ grafts, can result in inflammation negatively impacting graft survival (Jenq et al., 2012; Lei et al., 2016). Indeed, organs colonized with bacteria such as lungs and intestines are known to have worse outcomes than sterile organs such as heart and kidney (Colvin-Adams et al., 2014; Matas et al., 2014; Smith et al., 2014; Valapour et al., 2014).

Inflammatory stimuli have also been shown to prevent tolerance induction by experimental costimulation blockade therapies in animal models of transplantation. Although some pathogens shorten graft survival by generating effector/memory T cells cross-reactive to alloantigens (heterologous immunity), others do so by activating innate immunity that then activates non-pathogen specific alloreactive T cells, a process termed 'bystander activation' (Miller et al., 2008). PRR ligands such as LPS, poly(I:C), Pam<sub>3</sub>CysK<sub>4</sub> and CpG prevent costimulation blockade mediated long-term graft acceptance by impairing deletion of alloreactive CD8 T cells (Chen et al., 2006; Thornley et al., 2006b).

PRR ligands are used as adjuvants to boost CD8 T cell responses to peptide antigens (Melief et al., 2015). The regulation of cross-presentation of antigens by Toll-like receptor (TLR) ligands in vivo appears to depend on the cellular location of the TLRs. The plasma membrane TLRs TLR2 and TLR4 suppress cross-priming (Brossart and Bevan, 1997; Mandraju et al., 2014) whereas the endosomal TLRs TLR3 and TLR9 enhance it (Jelinek et al., 2011; Mandraju et al., 2014; Schulz et al., 2005; Schwarz et al., 2003). The direct

impact of TLR ligands on CD8 T cell responses has not been studied in the context of alloimmunity. Here I studied the effects of two TLR ligands LPS and poly(I:C) on alloreactive CD8 T cell responses using the KB5 TCR transgenic system. The alloreactive CD8 T cells in this system respond to the foreign MHC on the surface of C57BL/6 cells. This system thus permits us to investigate the effects of inflammatory stimuli on direct recognition by CD8 T cells. Both LPS and poly(I:C) generated higher numbers of effector CD8 T cells at the peak of the primary alloresponse; however only LPS generated higher numbers of memory CD8 T cells by inhibiting cell death during the contraction phase of the response. Although both LPS and poly(I:C) upregulated the early activation markers CD25 and CD69 on exposure to DST, they had opposite effects on alloreactive CD8 T cell function. Poly(I:C) enhanced the frequency of IFN- $\gamma$  producing and granzyme B producing effector CD8 T cells whereas LPS reduced the frequency of IFN- $\gamma$  producers and cytotoxicity of allogeneic targets. Interestingly IL-6 induced by LPS *in vivo* was necessary for LPS mediated suppression of alloreactive CD8 T cell responses. This finding is intriguing given the pro-rejection effects of IL-6 reported in the literature (Ahmed et al., 2011; Booth et al., 2011; Chen et al., 2009b; Shen and Goldstein, 2009; Zhao et al., 2012) and highlights the pleiotropic nature of this cytokine.

## **Materials and Methods**

### *Mice*



C57BL/6J (H-2<sup>b</sup>), BALB/cJ (H-2<sup>d</sup>), CBA/J (H-2<sup>k</sup>), C3H/HeJ (H-2<sup>k</sup>) and B6.B10ScN-*Tlr4*<sup>lps-del</sup>/JthJ (H-2<sup>b</sup>, abbreviated as *Tlr4*<sup>-/-</sup>) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). (CBA/J X KB5.CBA) F1 CD8<sup>+</sup> TCR-transgenic mice were bred at the animal facility of University of Massachusetts Medical School. The TCR transgene is expressed in CBA (H-2<sup>k</sup>) mice by CD8 T cells and specifically recognizes native H-2K<sup>b</sup> (Schonrich et al., 1991). Chimeras were generated as described in Chapter II.

#### *Processing of tissues and leukocyte isolation*

Peripheral blood was collected from mice by tail vein bleeding into tubes containing heparin. Spleen, inguinal and axillary lymph nodes were excised from mice and homogenized using frosted microscope slides. Lungs were perfused with cold PBS and then excised and homogenized to a single cell suspension. For bone marrow extraction, tibia and femurs from mice were excised and marrow was flushed with PBS. Red blood cells from all tissues were lysed with 0.84% ammonium chloride solution. To isolate intraepithelial lymphocytes (IELs), small intestines were removed and the luminal contents were expelled. Peyer's patches were dissected out, the intestines were opened longitudinally and chopped into 5mm pieces. The tissue fragments were digested in a solution of HEPES bicarbonate buffer supplemented with 10% FBS and 0.1mM EDTA for 30 mins at 37°C. Cells were filtered, centrifuged, suspended in Histopaque (Sigma Aldrich), layered onto Histopaque and centrifuged at 400 x g for 30 mins at room

temperature. IELs were recovered from the Histopaque interface. Cells isolated from all tissues were washed, resuspended in flow cytometry buffer (PBS supplemented with 1% FBS and 0.1% sodium azide) and counted before subsequent use for flow cytometry.

### *Reagents*

LPS Ultrapure from *E.coli* 0111:B4 and poly(I:C) (HMW) were obtained from InvivoGen (San Diego, CA) and injected i.p. into mice at 50 or 100 µg dose per mouse. IL-6 neutralizing antibody (MP5-20F3), IL-10R blocking antibody (1B1.3A) and IgG1 isotype control antibody (HRPN) were purchased from BioXCell (West Lebanon, NH) and administered i.p. to mice at a dose of 500 µg per mouse at the specified times. Recombinant mouse IL-6 obtained from BioLegend (San Diego, CA) was injected i.p. at a dose of 3 µg per mouse as indicated. The following antibodies were used for flow cytometry: CD8α (53-6.7), IgG2a (R19-15), active caspase-3 (C92-605), CD25 (PC61), CD69 (H1.2F3), CD44 (IM7), Eomes (Dan11mag), Ly6C (RB6-8C5), IFN-γ (XMG1.2), TNF-α (MP6-XT22), granzyme B (GB11), pY705 STAT3 (4/P-STAT3), purchased from BD Biosciences, EBioscience or BioLegend.

### *Apoptosis assay*

RBC lysed splenocytes were stained for surface markers in flow cytometry buffer followed by fixation and permeabilization using Cytofix/Cytoperm solution (BD

Biosciences) and stained for intracellular active caspase-3 as per the manufacturer's instructions. Freshly stained samples were analyzed on LSRII flow cytometer (BD Biosciences) with the FlowJo software (Ashland, OR).

#### *Proliferation assay*

RBC lysed splenocytes from KB5 transgenic mice were labeled with 1  $\mu$ M VPD450 (BD Biosciences) for 12 mins at 37°C. Cells were washed twice in PBS supplemented with 10% FBS, and splenocytes comprising  $2 \times 10^6$  labeled DES<sup>+</sup> CD8 T cells resuspended in PBS were injected i.v. into non-transgenic CBA mice.

#### *Intracellular cytokine staining*

For intracellular cytokine staining,  $2 \times 10^6$  RBC lysed splenocytes from recipient mice were incubated with  $1 \times 10^6$  LPS matured, irradiated syngeneic (H-2<sup>b</sup>) or allogeneic (H-2<sup>k</sup>) stimulator cells for 4 hrs in complete RPMI and monensin (eBioscience) and assessed for intracellular IFN $\gamma$  production as described previously (Brehm et al., 2007).

#### *In vivo cytotoxicity assay*

The in vivo cytotoxicity assay was performed as described in Chapter II.

#### *Plasma cytokine analysis*

Levels of cytokines IL-6, IL-10, MCP-1, IFN $\gamma$ , TNF and IL-12p70 in the plasma of mice were determined by BD Cytometric Bead Array™ as per the manufacturer's instructions. Levels of IFN- $\beta$  were determined by ELISA. IFN- $\beta$  antibody (7F-D3) (Santa Cruz Biotechnology) was used to coat wells of a flat-bottom 96-well plate overnight at 4°C. The plates were washed with wash buffer (PBS supplemented with 0.05% Tween 20) thrice and blocked with the blocking solution (PBS supplemented with 10% FBS) for 2 hours at 37°C. Plasma samples and IFN- $\beta$  standards (PBL Assay Science) were added to the wells and incubated overnight at 4°C. The plates were washed and incubated with polyclonal rabbit anti-mouse IFN- $\beta$  antibody (PBL Assay Science) for 90 mins at RT, followed by a secondary HRP-linked polyclonal anti-rabbit Ig antibody (Agilent Technologies) for 2 hours at RT. The plates were washed and TMB substrate (Bethyl Laboratories, Montgomery, TX) was added for 15 mins at RT. The reaction was quenched by adding ELISA stop solution containing 0.18M H<sub>2</sub>SO<sub>4</sub> (Bethyl Laboratories) and IFN- $\beta$  concentrations were determined using an EMax Endpoint ELISA Microplate Reader (Molecular Devices).

#### *Phospho-STAT3 (Tyr705) staining*

RBC-lysed splenocytes were resuspended in complete RPMI and plated at  $1 \times 10^6$  cells per well in 96 well round bottom plates. Cells were stimulated with 0.1  $\mu$ g recombinant IL-6 for 30 mins at 37°C and then fixed with BD Cytofix (BD Biosciences) for 12 mins. Cells were washed with flow cytometry buffer, blocked

with Fc block™ (BD Biosciences) and stained for surface markers. After washing, cells were permeabilized with ice-cold BD Perm Buffer III (BD Biosciences) for 30 mins at 4°C in dark. Splenocytes were washed and stained with fluorescently labeled anti-pSTAT3 antibody for 45 mins at RT in dark. Cells were washed, resuspended in flow cytometry buffer and analyzed on LSR II flow cytometer.

### *Statistical analyses*

Statistical analyses were performed using GraphPad PRISM software (San Diego, CA). An unpaired *t*-test was used to compare two means. Three or more means were compared using one-way ANOVA and Tukey's multiple comparisons test. *p* values < 0.05 were considered statistically significant.

## **Results**

### **LPS and poly(I:C) generate distinct cytokine milieus**

To evaluate the cytokine environment generated by the TLR ligands LPS and poly(I:C) in response to alloantigen challenge, KB5 chimeras were administered a DST of C57BL/6 splenocytes (H-2K<sup>b</sup>) with or without LPS or poly(I:C) and levels of cytokines were determined in the plasma. DST did not stimulate significant cytokine production over background levels (Fig. 4.1). Both LPS and DST+LPS treatments induced high levels of IL-6, IL-10, MCP-1, IFN- $\gamma$  and TNF- $\alpha$  (Fig. 4.1A-4.1E) suggesting that DST does not contribute to the pool of cytokines induced by LPS. poly(I:C) and DST+poly(I:C) preferentially induced

MCP-1 and IFN- $\beta$  (Fig. 4.1C, 4.1G) although modest levels of IL-6 and TNF- $\alpha$  were also found in the plasma of these mice (Fig. 4.1A, 4.1E). These results indicate that LPS and poly(I:C) generate qualitatively and quantitatively distinct cytokine milieus independent of the alloantigen.

Fig. 4.1. LPS and poly(I:C) generate distinct cytokine milieus.

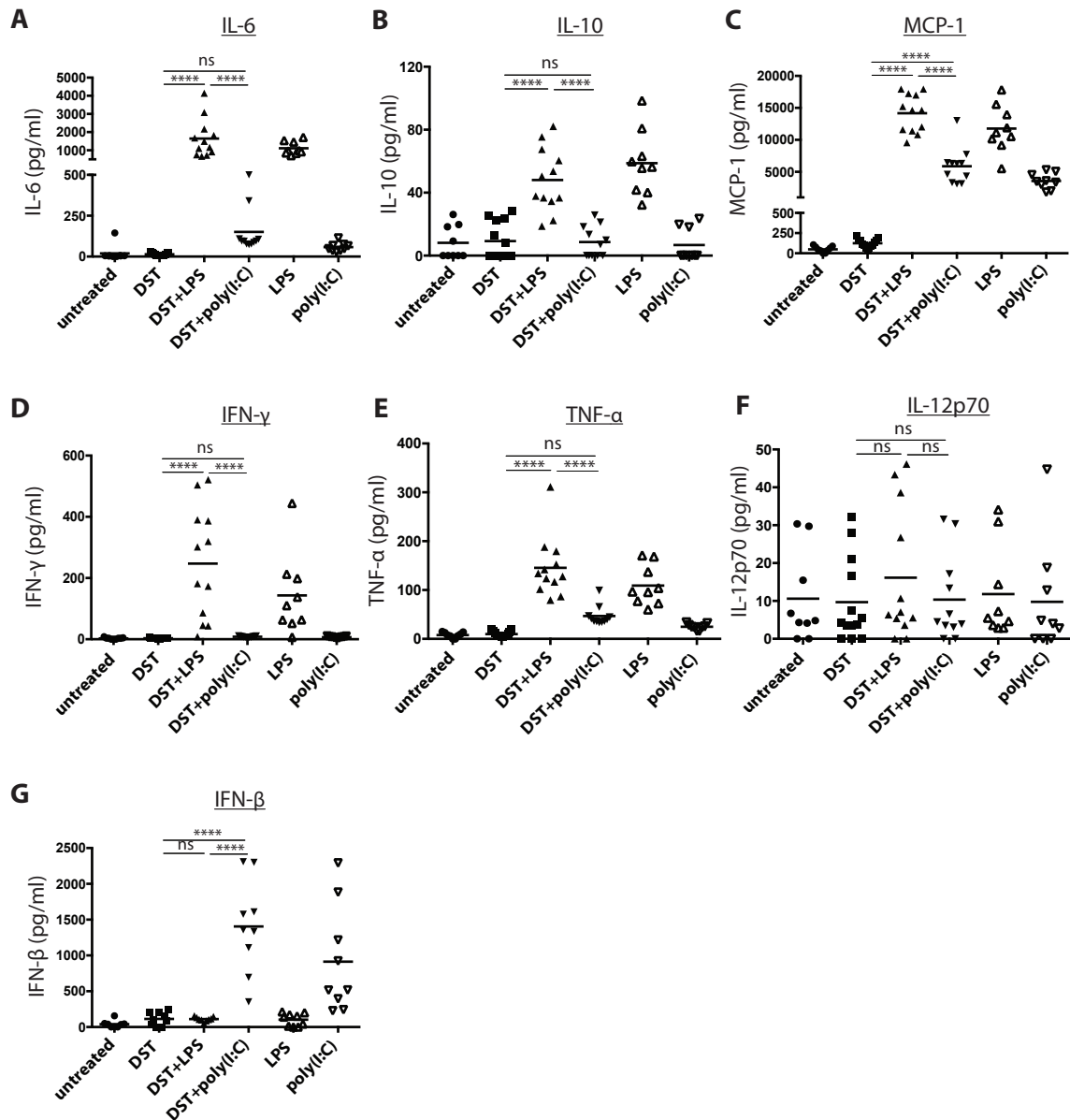


Fig. 4.1. LPS and poly(I:C) generate distinct cytokine milieus. (A-G) KB5 chimeras were administered C57BL/6 DST alone or in combination with LPS or poly(I:C). Mice receiving LPS or poly(I:C) alone served as controls. Plasma samples were collected 9 hours post-treatment and assessed for production of IL-6, IL-10, MCP-1, IFN-γ, TNF-α and IL-12p70 and 6 hours post-treatment for IFN-β. Data are cumulative of three independent experiments. \*\*\*\*p<0.0001.

### **LPS promotes survival of activated alloreactive CD8 T cells**

It is well established that cytokines regulate the differentiation and function of activated CD8 T cells (Joshi et al., 2007). Since LPS and poly(I:C) generated distinct cytokine milieus, I hypothesized that these inflammatory stimuli would differentially regulate alloreactive CD8 T cell responses. To test this hypothesis, KB5 chimeras were injected with a DST of C57BL/6 splenocytes with or without LPS or poly(I:C) and alloreactive DES<sup>+</sup> CD8 T cell population in the blood and spleen was monitored. DES<sup>+</sup> CD8 T cells from all mice that received DST underwent attrition by 24 hours in the blood (Table 4.1). This early attrition occurred in the spleen as well; however it was less pronounced in mice that received LPS (Fig. 4.2A). This reduction in splenic DES<sup>+</sup> CD8 T cell number in response to alloantigen could not be explained by trafficking to other sites such as lymph nodes, lungs, bone marrow and intestinal epithelium (Fig. 4.3). I speculated that this early loss of alloreactive CD8 T cells is a result of cell death. Indeed, I found increased frequencies of DES<sup>+</sup> CD8 T cells staining positively for active caspase-3 in mice that received the alloantigen, relative to untreated controls (Fig. 4.2B). Interestingly, LPS significantly reduced the percentages of active caspase-3<sup>+</sup> DES<sup>+</sup> CD8 T cells (DST: 18.04±0.84%, DST+LPS: 8.82±1.08%, DST+poly(I:C): 15.29±0.74%). No cell division had occurred in DST+LPS treated mice by 24 hours as indicated by the lack of VPD450 dilution (Fig. 4.4) and thus the reduced attrition of alloreactive CD8 T cells in the DST+LPS group is a result of reduced cell death.



**Table 4.1. LPS and poly(I:C) differentially regulate kinetics of CD8 T cells in blood upon alloantigen challenge.**

Treatment	n	Day 0 DES <sup>+</sup> (%)	Day 1 DES <sup>+</sup> (%)	Day 2 DES <sup>+</sup> (%)	Day 3 DES <sup>+</sup> (%)	Day 5 DES <sup>+</sup> (%)
Untreated	6	4.53±0.06	4.67±0.41 <sup>a</sup>	4.27±0.29	4.62±0.22	4.10±0.33
DST	7	6.39±0.22	0.16±0.03	0.51±0.09 <sup>b</sup>	14.64±2.44	4.06±0.46
DST/LPS	7	6.70±0.16	0.44±0.15	9.67±1.19	16.63±1.96	8.19±0.49 <sup>c</sup>
DST/poly(I:C)	9	5.36±0.78	0.58±0.09	12.53±0.58	18.88±2.60	7.13±0.98
LPS	6	5.05±0.65	4.05±.73 <sup>a</sup>	5.17±1.02	5.08±0.87	5.30±1.11
Poly(I:C)	3	2.83±0.18	ND	ND	3.53±0.09	3.70±0.06

KB5 chimeras were administered the indicated treatment and bled at the indicated times. The values indicate the percentage of DES<sup>+</sup> CD8 T cells in the blood (gated on lymphocytes) and are expressed as mean ± SEM. Data are cumulative of two independent experiments. <sup>a</sup>p<0.0001 vs all other groups, <sup>b</sup>p<0.0001 vs DST/LPS and DST/poly(I:C) groups, <sup>c</sup>p<0.0001 vs DST, <sup>d</sup>p<0.01 vs DST.

Fig. 4.2. LPS enhances survival of alloreactive CD8 T cells in response to antigen challenge.

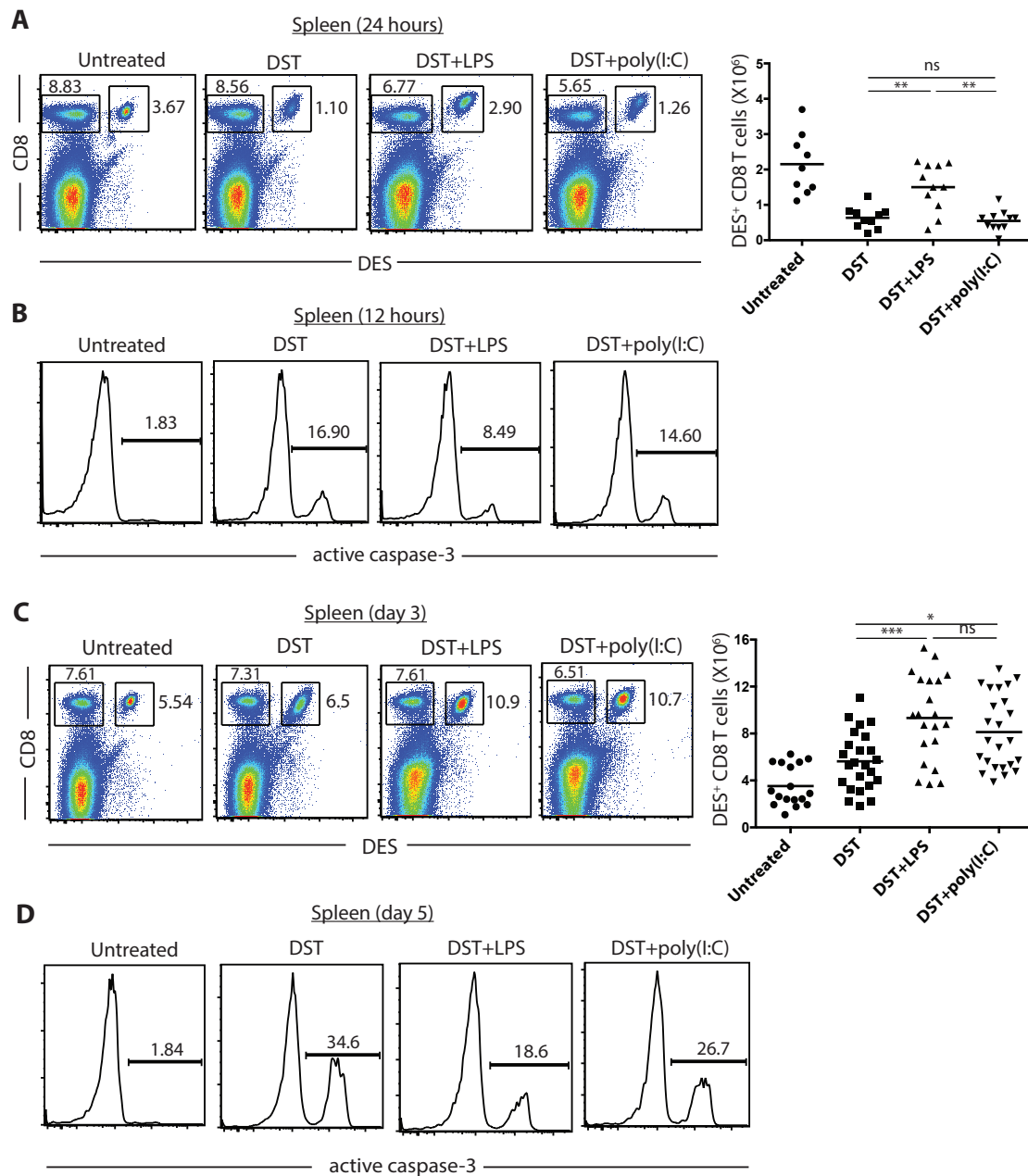


Fig. 4.2. LPS enhances survival of alloreactive CD8 T cells in response to antigen challenge. KB5 chimeras were administered C57BL/6 DST alone or in combination with LPS or poly(I:C). (A) Representative plots of DES<sup>+</sup> CD8<sup>+</sup> T cells from the spleen

recovered 24 hours post-treatment are shown. The graph shows the absolute number of DES<sup>+</sup> CD8 T cells in the spleen. (B) Splenocytes harvested at 12 hours post-treatment were stained for active caspase-3. Representative plots of active caspase-3 staining on DES<sup>+</sup> CD8 T cells are shown. (C) Representative plots of DES<sup>+</sup> CD8<sup>+</sup> T cells from the spleen recovered 3 days post-treatment are shown. The graph shows the absolute numbers of DES<sup>+</sup> CD8 T cells in the spleen. (D) Splenocytes harvested on day 5 post-treatment were stained for active caspase-3. Representative plots of active caspase-3 staining on DES<sup>+</sup> CD8 T cells are shown. Data are cumulative of at least two independent experiments. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.

Fig. 4.3. Trafficking to other compartments does not account for the early loss of alloreactive CD8 T cells from the spleen in response to alloantigen.

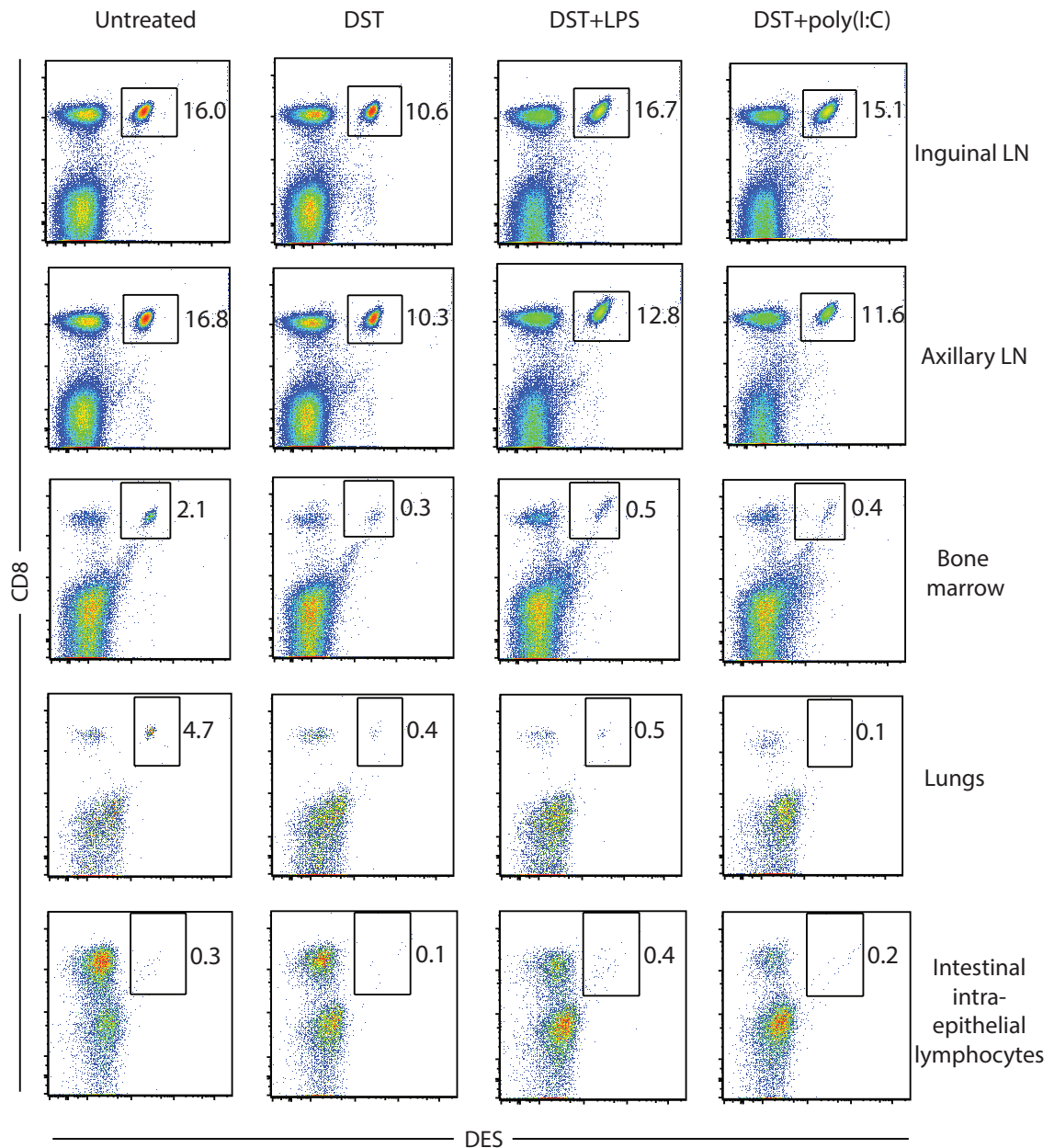


Fig. 4.3. Trafficking to other compartments does not account for the early loss of alloreactive CD8 T cells from the spleen in response to alloantigen. KB5 chimeras were administered C57BL/6 DST alone or in combination with LPS or poly(I:C). Inguinal and axillary lymph nodes, bone marrow, lungs and intestinal intraepithelial lymphocytes were

harvested from the mice 24 hours post-treatment. Representative plots display the percentages of DES<sup>+</sup> CD8 T cells. Data are representative of 3-6 mice per group.

Fig. 4.4. LPS challenge does not induce division of alloreactive CD8 T cells within 24 hours of alloantigen challenge.

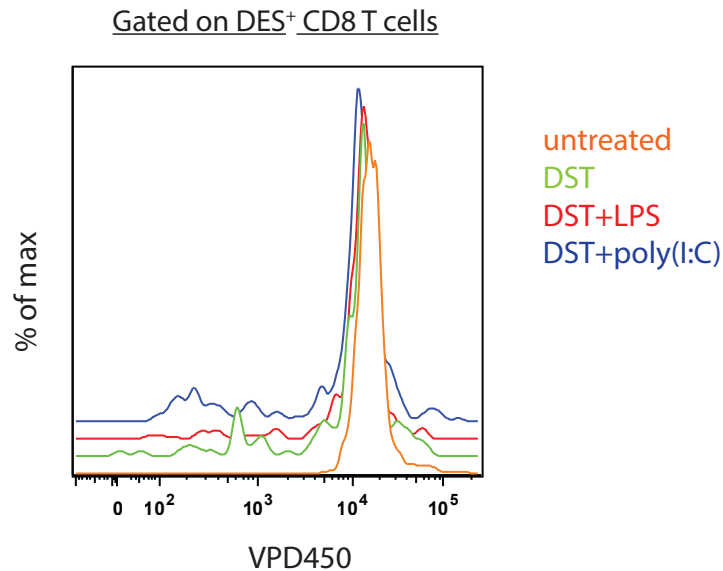


Fig. 4.4. LPS challenge does not induce division of alloreactive CD8 T cells within 24 hours of alloantigen challenge. Splenocytes from KB5 transgenic mice were labeled with VPD450 and  $2 \times 10^6$  VPD450 labeled DES<sup>+</sup> CD8 T cells were adoptively transferred to CBA mice that were then treated with C57BL/6 DST alone or in combination with LPS or poly(I:C). Representative histogram depicts VPD450 profile of DES<sup>+</sup> CD8 T cells in the spleens of CBA mice 24 hours post-treatment. Data are representative of 3-4 mice per group.

Alloantigen in the form of DST triggered expansion of the DES<sup>+</sup> CD8 T cell population following the early death phase; however, co-administration of LPS or poly(I:C) accelerated the expansion and I found significantly higher percentages of DES<sup>+</sup> CD8 T cells in the blood of mice that were exposed to the inflammatory agents on day 2 post-treatment (Table 4.1). The alloresponse peaked on day 3 with LPS and poly(I:C) generating higher numbers of alloreactive CD8 T cells relative to DST alone (Fig. 4.2C). LPS slowed the contraction phase of the alloresponse (Table 4.1) and I found proportionately lower numbers of apoptotic active caspase-3<sup>+</sup> DES<sup>+</sup> CD8 T cells in the spleen of DST+LPS treated mice on day 5 post-treatment compared to mice that received DST with or without poly(I:C) (DST: 26.55±2.89, DST+LPS: 19.64±1.50, DST+poly(I:C): 24.1±1.85) (Fig. 4.2D). Consequently, LPS generated higher number of memory phenotype CD44<sup>hi</sup> DES<sup>+</sup> CD8 T cells (Fig. 4.5). Inflammation alone (LPS or poly(I:C) treatment) did not induce expansion of alloreactive CD8 T cells (Table 4.1) suggesting the requirement for exposure to the alloantigen to initiate the alloresponse. Together, these data suggest that while both LPS and poly(I:C) generate higher numbers of effector alloreactive CD8 T cells at the peak of the primary alloresponse, LPS provides survival signals during contraction to enhance memory formation.

Fig. 4.5. LPS challenge generates higher levels of memory alloreactive CD8 T cells.

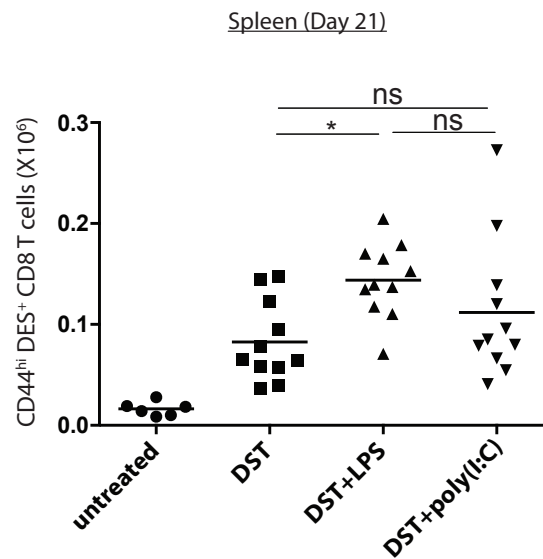


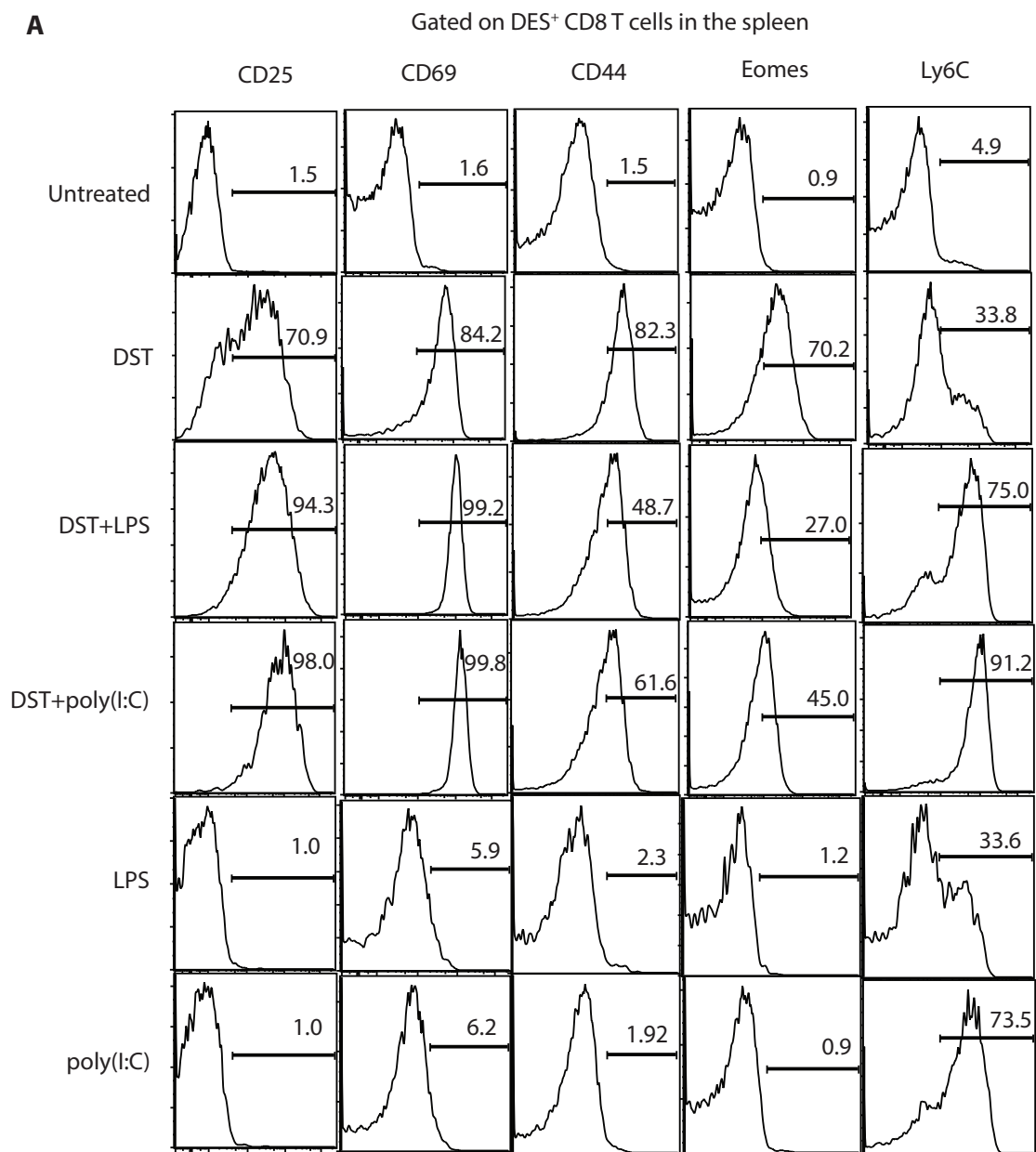
Fig. 4.5. LPS challenge generates higher levels of memory alloreactive CD8 T cells. KB5 chimeras were administered C57BL/6 DST alone or in combination with LPS or poly(I:C). The graph shows the absolute number of CD44<sup>hi</sup> DES<sup>+</sup> CD8 T cells in the spleen 21 days post-treatment. Data are cumulative of two independent experiments. \* $p < 0.05$ , \*\*\*\* $p < 0.0001$ .



### **LPS and poly(I:C) alter the phenotype of alloreactive CD8 T cells in response to the alloantigen**

Since inflammation altered the magnitude of the alloresponse, I hypothesized that inflammation would alter the phenotype or activation profile of alloreactive CD8 T cells. Indeed, LPS and poly(I:C) increased the frequency of DES<sup>+</sup> CD8 T cells expressing the early activation markers CD25 (IL-2R $\alpha$ ) and CD69 in response to DST at 24 hours post-treatment (Fig. 4.6A). Interestingly, poly(I:C) enhanced the expression levels of CD25 and CD69 on DES<sup>+</sup> CD8 T cells compared to LPS (Fig. 4.6B). Inflammation alone was not sufficient to upregulate CD25; however higher levels of CD69 were detected on DES<sup>+</sup> CD8 T cells in response to both LPS and poly(I:C) relative to untreated controls (Fig. 4.6A, 4.6B) in agreement with previous studies (Sun et al., 1998; Tough et al., 1997). Interestingly both LPS and poly(I:C) when administered with DST reduced the percentages of DES<sup>+</sup> CD8 T cells expressing the activation marker CD44 and its expression levels relative to DST alone (Fig. 4.6A, 4.6B). A similar profile was observed for the T-box transcription factor family member Eomes with reduced expression levels upon exposure to inflammation at the peak of the alloresponse (Fig. 4.6A, 4.6B). Inflammation alone did not induce upregulation of CD44 and Eomes expression emphasizing the requirement for antigen encounter to induce the expression of these proteins. Finally, I examined the expression of Ly6C, a protein that was originally shown to have an accessory role in the cytolytic function of CD8 T cells (Johnson et al., 1993).

**Fig. 4.6.** LPS and poly(I:C) alter the phenotype of alloreactive CD8 T cells in response to antigen challenge.



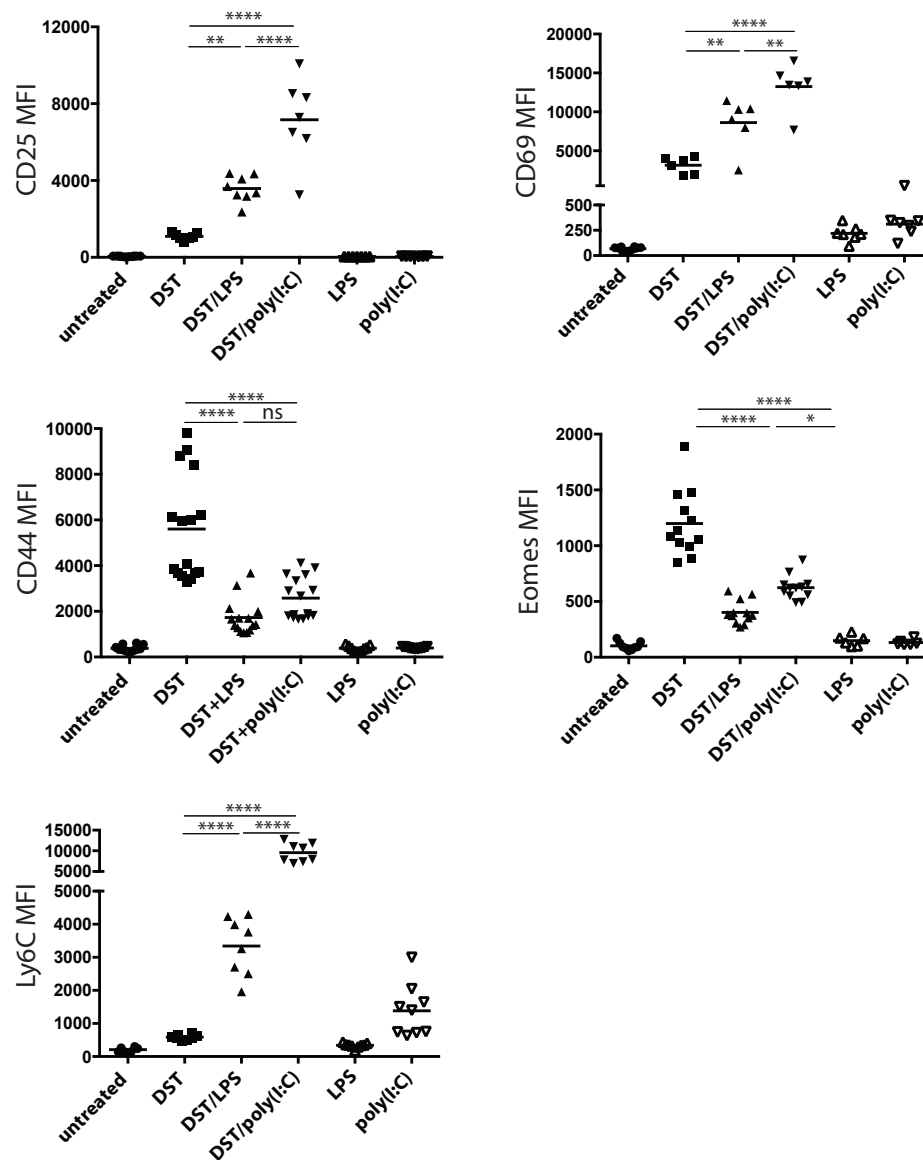
**B**

Fig. 4.6. LPS and poly(I:C) alter the phenotype of alloreactive CD8 T cells in response to antigen challenge. KB5 chimeras were administered C57BL/6 DST alone or in combination with LPS or poly(I:C). Mice were sacrificed one or three days later and splenocytes were stained for T cell activation markers. (A) Representative plots of CD25 and CD69 expression on DES<sup>+</sup> CD8 T cells at 24 hours post-treatment and CD44, Eomes and Ly6C expression at 3 days post-treatment are shown. (B) The graphs show the expression levels (MFIs) of each of the activation markers on DES<sup>+</sup> CD8 T cells. Data are cumulative of at least two independent experiments. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001.

DST alone upregulated Ly6C expression on DES<sup>+</sup> CD8 T cells relative to untreated controls (Fig. 4.6A, 4.6B). Inflammation alone was sufficient to induce Ly6C and coadministration with DST resulted in further increase in Ly6C expression. To summarize, LPS and poly(I:C) alter the phenotype of alloreactive CD8 T cells in response to the alloantigen.

### **LPS suppresses alloreactive CD8 T cell IFN- $\gamma$ production and cytotoxicity**

To investigate the impact of inflammation on alloreactive CD8 T cell function, KB5 chimeras were administered DST with or without LPS or poly(I:C) and the ability of DES<sup>+</sup> CD8 T cells to produce IFN- $\gamma$  upon ex vivo restimulation with allogeneic C57BL/6 cells was assessed. Poly(I:C) enhanced the frequency and numbers of IFN- $\gamma$  producing DES<sup>+</sup> CD8 T cells in response to DST (Fig. 4.7A-4.7C). This observation was consistent with previous studies demonstrating the CD8 T cell enhancing effect of poly(I:C) (Coffman et al., 2010; Schulz et al., 2005). Interestingly, LPS lowered the percentage of IFN- $\gamma$  producing DES<sup>+</sup> CD8 T cells in response to DST (Fig. 4.7A, 4.7B). Although the numbers of IFN- $\gamma$ <sup>+</sup> DES<sup>+</sup> CD8 T cells were lower in the DST+LPS group relative to DST (Fig. 4.7C), the difference was not statistically significant because of LPS generating higher numbers of DES<sup>+</sup> CD8 T cells at the peak of the response (Fig. 4.2C). Inflammation alone did not stimulate significant IFN- $\gamma$  production in DES<sup>+</sup> CD8 T cells over the untreated controls (Fig. 4.7A, 4.7B).

Fig. 4.7. LPS suppresses alloreactive CD8 T cell function.

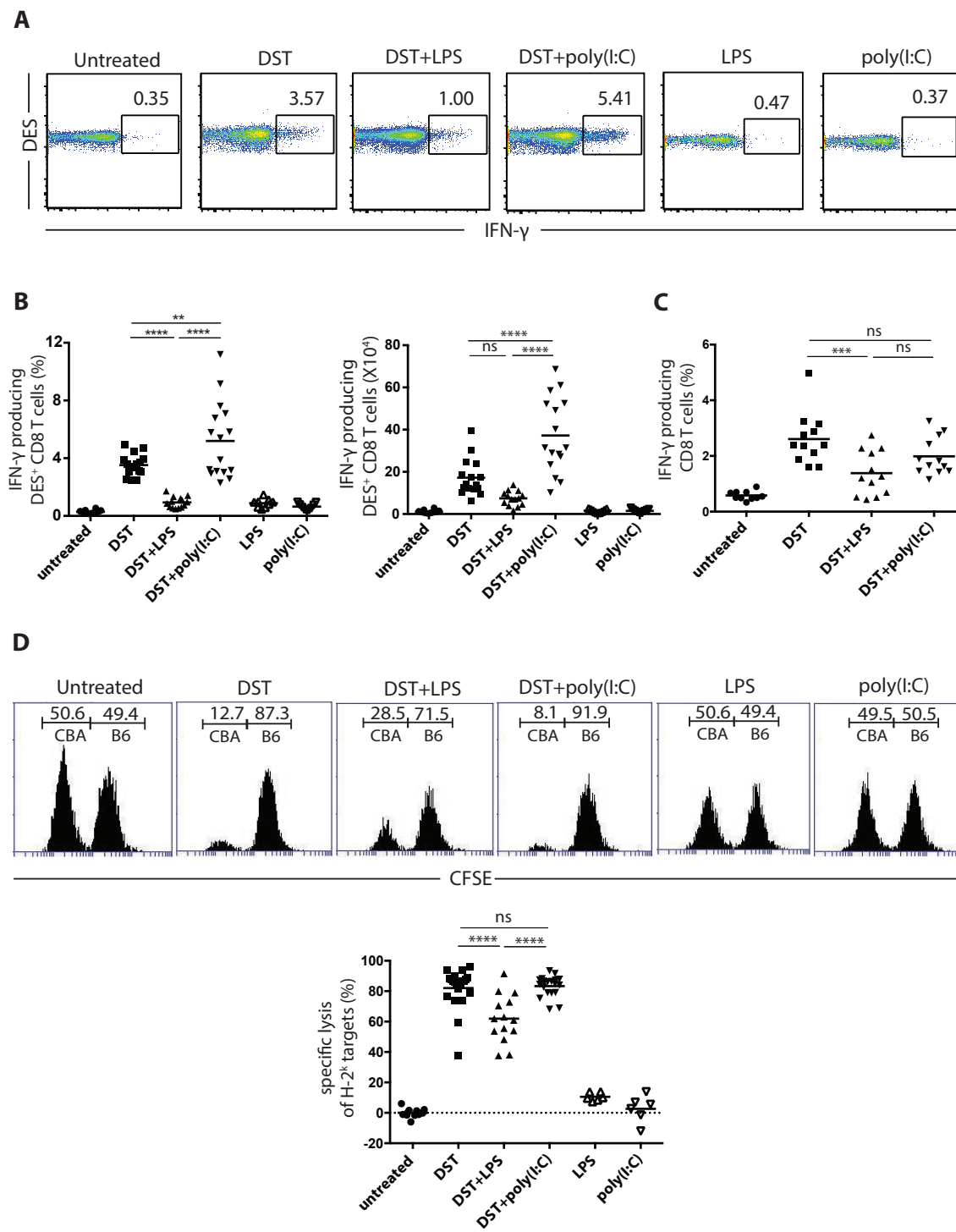


Fig. 4.7. LPS suppresses alloreactive CD8 T cell function. (A) KB5 chimeras were administered C57BL/6 DST alone or in combination with LPS or poly(I:C). Splenocytes were harvested from mice 3 days post-treatment and stained for intracellular IFN- $\gamma$  upon allogeneic (H-2<sup>b</sup>) stimulation. Representative plots of IFN- $\gamma$  staining on DES<sup>+</sup> CD8 T cells are shown. (B) The graphs show the percentages and total numbers of IFN- $\gamma$  producing DES<sup>+</sup> CD8 T cells. (C) C57BL/6 mice were administered CBA (H-2<sup>k</sup>) DST alone or in combination with LPS or poly(I:C). Splenocytes were harvested from mice 3 days post-treatment and stained for intracellular IFN- $\gamma$  upon allogeneic (H-2<sup>k</sup>) stimulation. The graph depicts the percentages of IFN- $\gamma$  producing CD8<sup>+</sup> T cells. (D) C57BL/6 mice were treated on day 0 with CBA DST alone or with LPS or poly(I:C). On day 3, CFSE labeled CBA (H-2<sup>k</sup>) and C57BL/6 (H-2<sup>b</sup>) splenocytes were transferred intravenously at equal proportions into the mice, and the survival of each population was determined 3 hours later. Survival of the CBA splenocytes is shown in the representative histograms and the graph shows percent specific lysis of CBA splenocytes. Data are cumulative of at least 3 independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

To test if the KB5 TCR transgenic system recapitulates the endogenous polyclonal alloresponse, I treated C57BL/6 mice (H-2<sup>b</sup>) with a DST of CBA splenocytes (H-2<sup>k</sup>) and tested the ability of CD8 T cells from the recipient mice to produce IFN- $\gamma$  to ex vivo allogeneic restimulation at various time points post-treatment. Our lab has previously demonstrated that effector alloreactive CD8 T cells produce IFN- $\gamma$  upon antigen restimulation (Brehm et al., 2007). I found that DST priming generated highest frequencies of IFN- $\gamma$  producing CD8 T cells on day 3 (Fig. 4.8), which coincides with the peak of the alloresponse in the KB5 transgenic system. LPS treatment in conjunction with DST significantly reduced the percentage of endogenous IFN- $\gamma$  producers relative to DST alone (Fig. 4.7C). However, I did not find an enhancement of the CD8 T cell response by poly(I:C) in C57BL/6 mice (Fig. 4.7C). Next I investigated the cytotoxic potential of alloreactive CD8 T cells in C57BL/6 mice using an in vivo cytotoxicity assay. Recipient mice were administered CBA (H-2<sup>k</sup>) DST with or without LPS or poly(I:C) and tested for their ability to reject allogeneic CBA splenocytes 3 days later. Mice treated with DST alone exhibited approximately 82% killing of allogeneic CBA targets indicating that alloreactive CD8 T cells in these mice are primed for cytolysis (Fig. 4.7D). LPS treatment significantly reduced the cytolysis of target cells in response to DST.

Fig. 4.8. The endogenous alloreactive CD8 T cell kinetics are similar to those of the KB5 transgenic system.

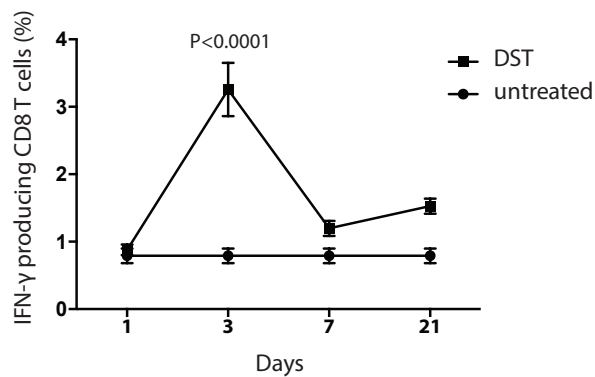


Fig. 4.8. The endogenous alloreactive CD8 T cell kinetics are similar to those of the KB5 transgenic system. C57BL/6 mice were treated with CBA DST and sacrificed on days 1, 3, 7 and 21 post-treatment. Splenocytes were stained for intracellular IFN- $\gamma$  upon allogeneic (H-2<sup>k</sup>) stimulation. The graph shows the percentages of IFN- $\gamma$  producing CD8 T cells at each time point. Data are cumulative of two independent experiments. \*\*\*\*p<0.0001 relative to all other time points.



This result is particularly interesting because the *in vivo* cytotoxicity assay measures the cytotoxic potential of the total CD8 T cell population and the reduction in cytolysis by LPS despite increased generation of effector CD8 T cells (Fig. 4.2C) must mean that LPS has a pronounced effect on cytotoxic potential on a per cell basis. Cytolysis of allogeneic targets was increased by poly(I:C) challenge and was comparable to the DST group, although significantly higher levels of granzyme B producing alloreactive CD8 T cells could be detected upon poly(I:C) challenge at the peak of the alloresponse in KB5 chimeras (Fig. 4.9). Inflammation alone did not result in cytolysis of targets above background levels (Fig. 4.7D). Together, these data indicate that LPS reduces the frequency of IFN- $\gamma$  producing and cytolytic effector alloreactive CD8 T cells whereas poly(I:C) enhances the response when administered with the alloantigen. Also, these inflammatory stimuli fail to confer effector function on CD8 T cells in the absence of the alloantigen.

Fig. 4.9. Poly(I:C) challenge enhances the levels of granzyme B producing effector alloreactive CD8 T cells.

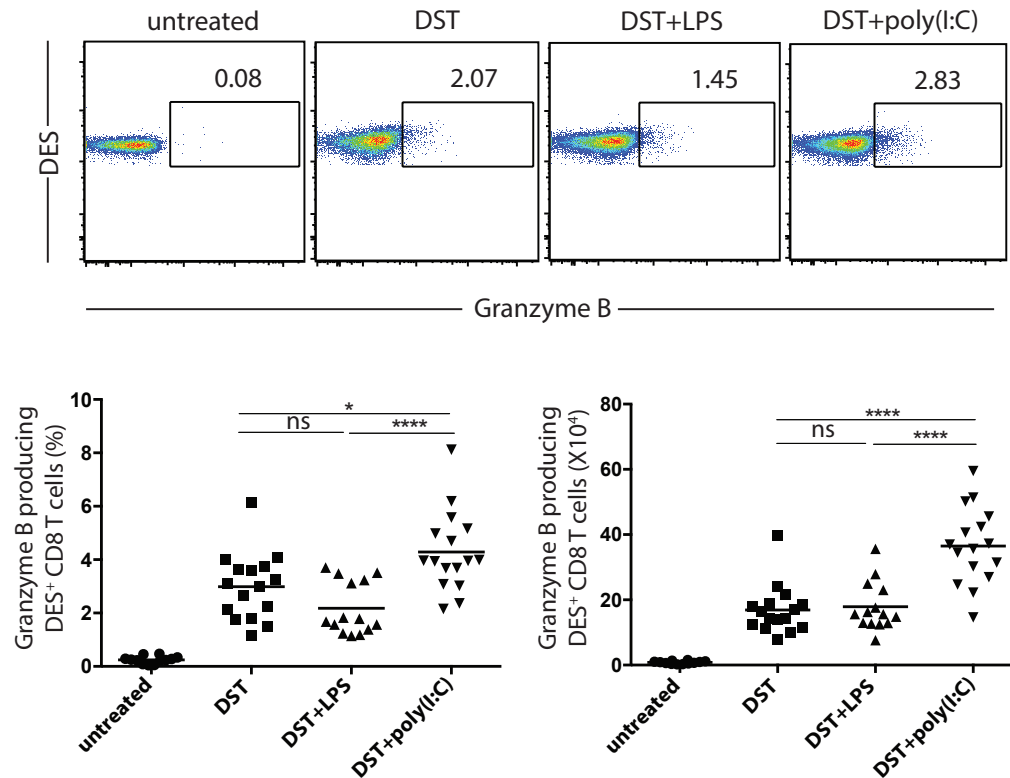


Fig. 4.9. Poly(I:C) challenge enhances the levels of granzyme B producing effector alloreactive CD8 T cells. KB5 chimeras were administered C57BL/6 DST alone or in combination with LPS or poly(I:C). Splenocytes were harvested from mice 3 days post-treatment and stained for intracellular granzyme B. Representative plots of granzyme B staining on DES<sup>+</sup> CD8 T cells are shown. The graph shows the percentages and total numbers of granzyme B producing DES<sup>+</sup> CD8 T cells in the spleen. Data are cumulative of three independent experiments. \*p<0.05.

**LPS signaling through recipient and not donor cells is critical for  
suppression of alloreactive CD8 T cell function**

To test whether LPS signaling through recipient cells or donor cells (DST) is necessary for the suppression of alloreactive CD8 T cell response, I used TLR4 deficient mice as recipients or donors. Co-administration of LPS with wild type (WT) or *Tlr4*<sup>-/-</sup> C57BL/6 DST lowered the percentages of IFN- $\gamma$  producing DES<sup>+</sup> CD8 T cells in the KB5 chimeras to similar extent (Fig. 4.10A) suggesting that LPS signaling through TLR4 on donor cells is not necessary for LPS mediated suppression of the CD8 T cell response. In a converse experiment, WT or *Tlr4*<sup>-/-</sup> C57BL/6 recipients were administered CBA DST along with LPS. While LPS reduced the frequency of IFN- $\gamma$  producing CD8 T cells in WT recipients as demonstrated previously (Fig. 4.7C), the opposite result was observed in *Tlr4*<sup>-/-</sup> recipients (Fig. 4.10B). To confirm the importance of LPS signaling through recipient cells, I employed C3H/HeJ mice (H-2<sup>k</sup>) that have a natural *Tlr4* mutation making them resistant to LPS. C57BL/6 DST stimulated a small percentage of CD8 T cells to produce IFN- $\gamma$  upon ex vivo restimulation (Fig. 4.10C), which could mean that C3H/HeJ cells do not respond well to ex vivo C57BL/6 stimulation or the actual frequency of CD8 T cells reactive to H-2<sup>b</sup> cells in these mice is very low. Interestingly, administration of DST with LPS increased the percentage of IFN- $\gamma$ <sup>+</sup> CD8 T cells to the extent of poly(I:C) treated mice (Fig. 4.10C).

Fig. 4.10. TLR4 expression on recipient cells is necessary for LPS mediated suppression of CD8 T cell function.

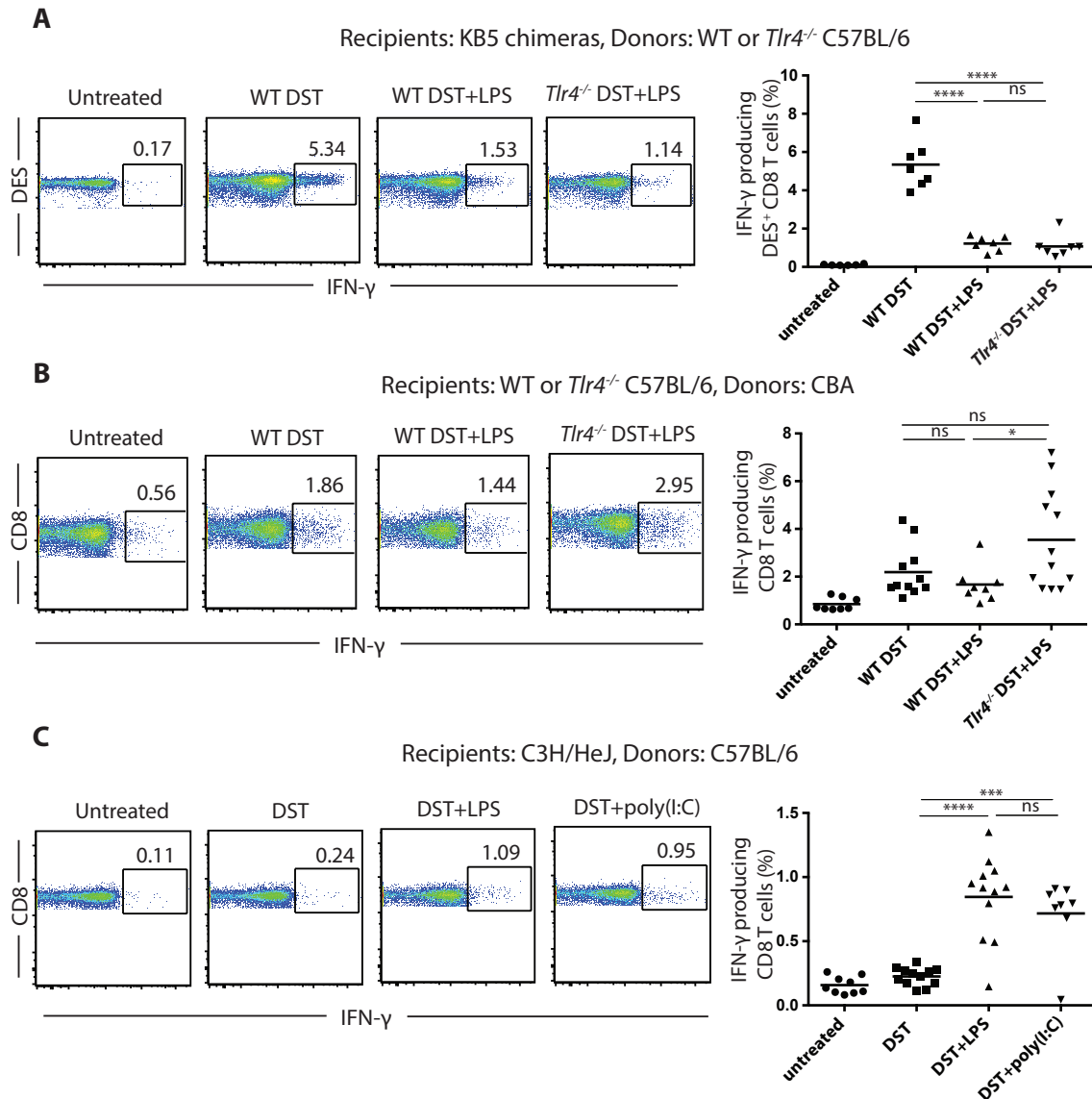


Fig. 4.10. TLR4 expression on recipient cells is necessary for LPS mediated suppression of CD8 T cell function. (A) KB5 chimeras were administered WT or *Tlr4*<sup>-/-</sup> C57BL/6 DST alone or in combination with LPS. Splenocytes were harvested from the mice 3 days post-treatment and stained for intracellular IFN-γ upon allogeneic (H-2<sup>b</sup>) stimulation. Representative plots of IFN-γ staining on DES<sup>+</sup> CD8 T cells are shown. The graph shows the percentages of IFN-γ producing DES<sup>+</sup> CD8 T cells. (B) WT or *Tlr4*<sup>-/-</sup> C57BL/6 mice were treated with CBA DST alone or in combination with LPS. Splenocytes were harvested from the mice 3 days post-treatment and stained for intracellular IFN-γ upon

allogeneic (H-2<sup>k</sup>) stimulation. Representative plots of IFN- $\gamma$  staining on CD8 T cells are shown. The graph shows the percentages of IFN- $\gamma$  producing CD8 T cells. (C) C3H-HeJ mice were treated with C57BL/6 DST alone or in combination with LPS. Splenocytes were harvested from the mice 3 days post-treatment and stained for intracellular IFN- $\gamma$  upon allogeneic (H-2<sup>b</sup>) stimulation. Representative plots of IFN- $\gamma$  staining on CD8 T cells are shown. The graph shows the percentages of IFN- $\gamma$  producing CD8 T cells. Data are cumulative of at least two independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

These data indicate that the presence of functional TLR4 is necessary on recipient cells for LPS to suppress alloreactive CD8 T cell responses.

**IL-6, and not IL-10, is necessary for LPS mediated suppression of alloreactive CD8 T cell function**

Cytokines secreted upon TLR engagement regulate CD8 T cell responses. Several studies have reported that IL-10 can suppress CD8 T cell responses in infection and cancer settings (Bogunovic et al., 2011; den Haan et al., 2007; Sun et al., 2011). Since LPS stimulated IL-10 secretion (Fig. 4.1B), I tested if IL-10 was important for the suppressive effects of LPS. KB5 chimeras were administered C57BL/6 DST with LPS with or without IL-10R blocking antibody and IFN- $\gamma$  production by DES<sup>+</sup> CD8 T cells upon ex vivo restimulation was assessed. Blocking IL-10 signaling did not increase the frequency of IFN- $\gamma$  producers (Fig. 4.11) suggesting that IL-10 is not critical for LPS mediated suppression of alloreactive CD8 T cell responses. TLR ligand induced IL-6 was recently implicated in counter-regulating anti-viral CD8 T cell response during an acute retrovirus infection (Wu et al., 2015). To test the possible role of IL-6 in LPS mediated CD8 T cell suppression in my studies, I administered KB5 chimeras with IL-6 neutralizing antibody or isotype control along with DST+LPS and assessed IFN- $\gamma$  production by DES<sup>+</sup> CD8 T cells to allo-stimulation.

Fig. 4.11. IL-10 signaling is not necessary for LPS to suppress IFN- $\gamma$  production by effector alloreactive CD8 T cells.

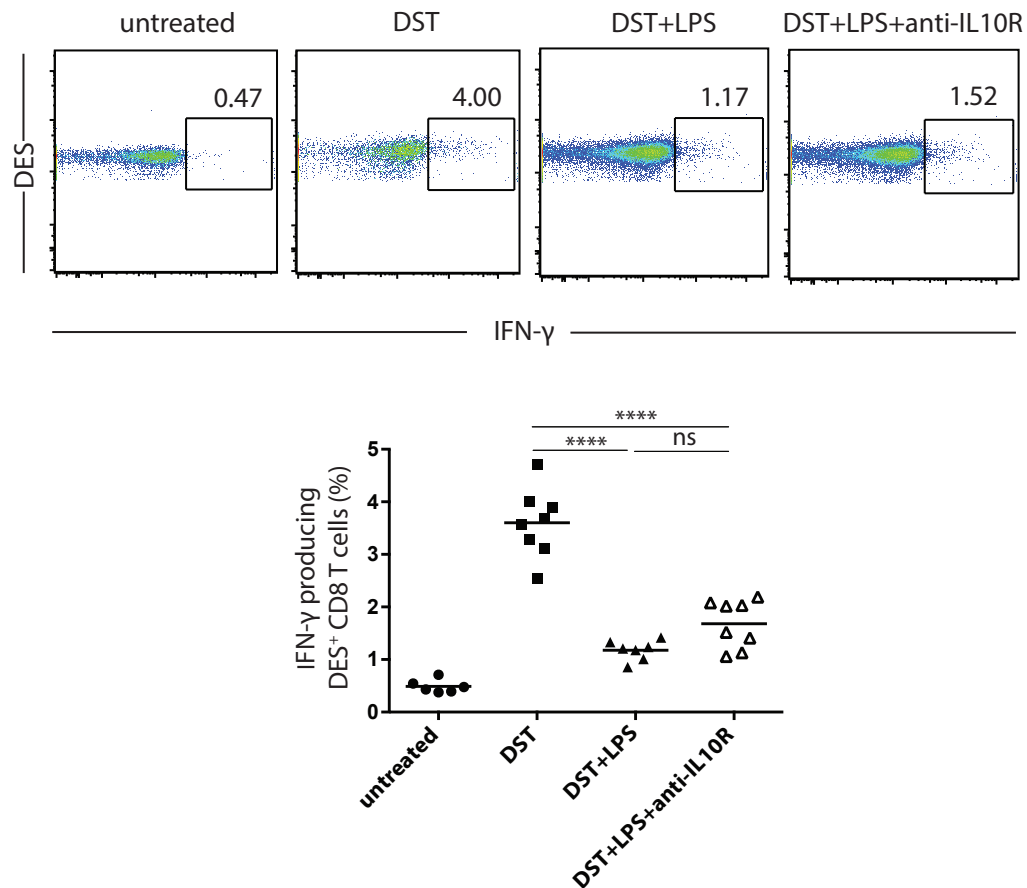
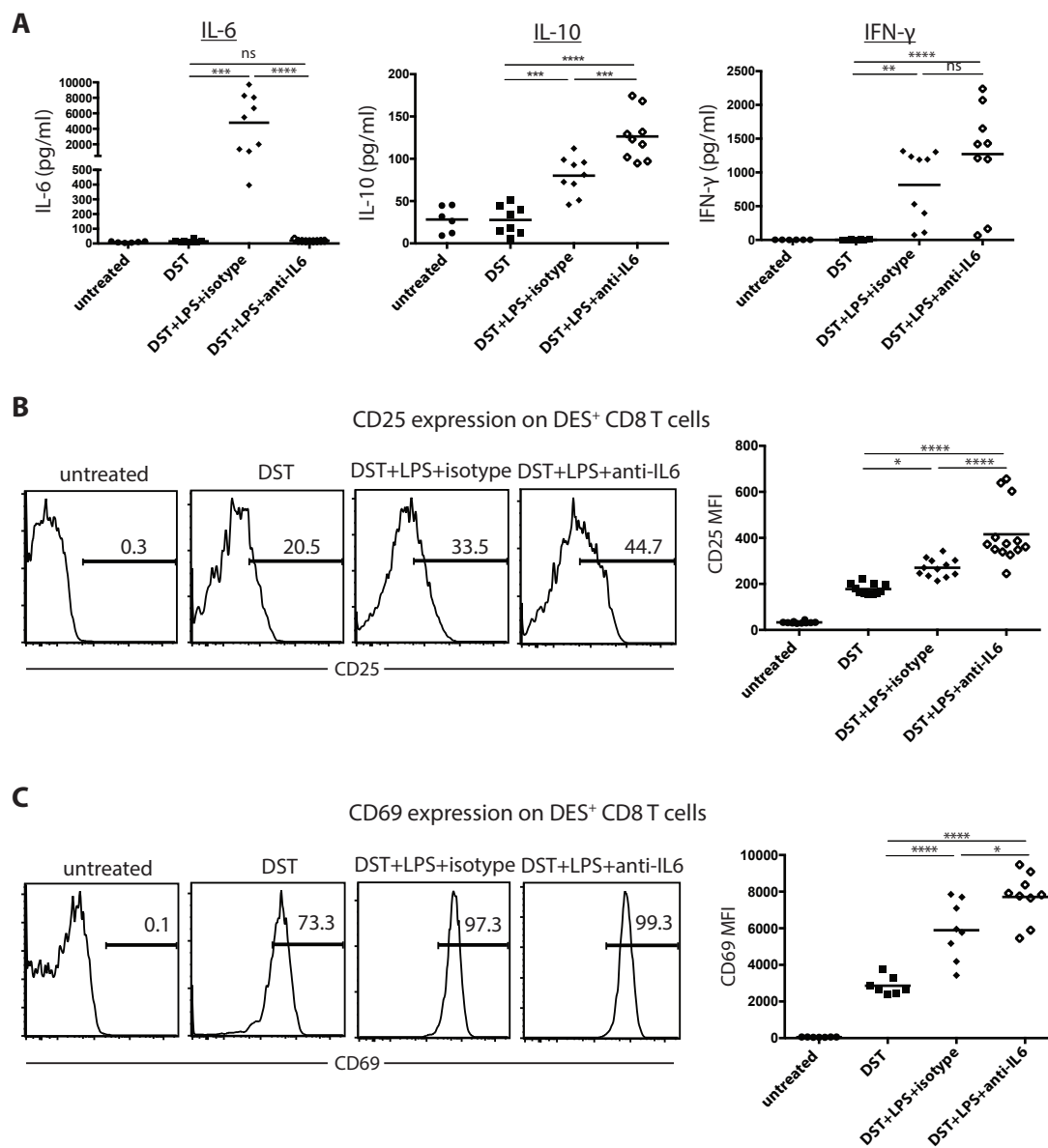


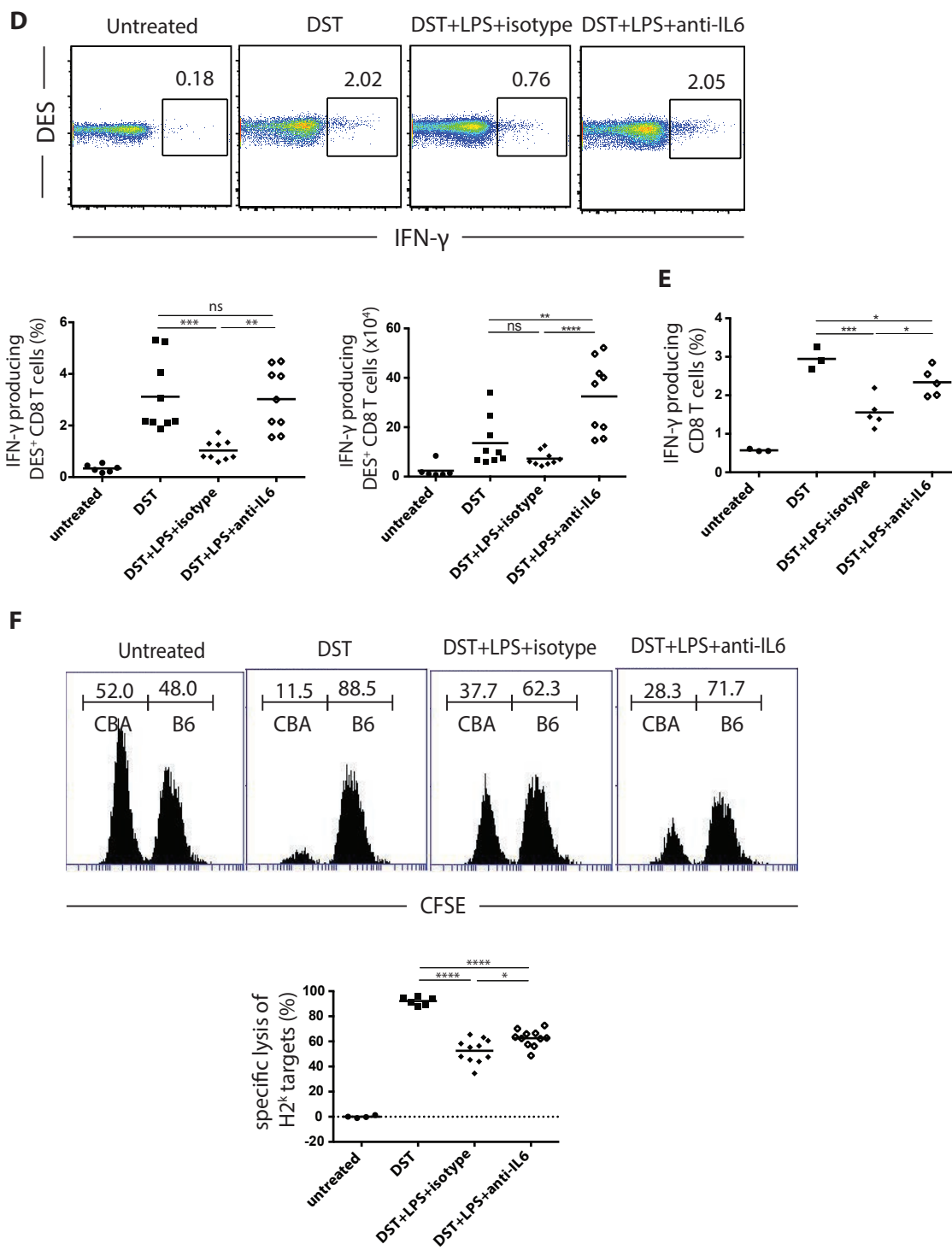
Fig. 4.11. IL-10 signaling is not necessary for LPS to suppress IFN- $\gamma$  production by effector alloreactive CD8 T cells. KB5 chimeras were administered C57BL/6 DST alone or in combination with LPS. Anti-IL10R antibody was injected 2 hrs before and 12 hrs after LPS treatment. Splenocytes were harvested from the mice 3 days post-treatment and stained for intracellular IFN- $\gamma$  upon allogeneic (H-2<sup>b</sup>) stimulation. Representative plots of IFN- $\gamma$  staining on DES<sup>+</sup> CD8 T cells are shown. The graph shows the percentages of IFN- $\gamma$  producing DES<sup>+</sup> CD8 T cells. Data are cumulative of two independent experiments. \*\*\*\* $p$ <0.0001.

Administration of the neutralizing antibody rendered IL-6 in plasma undetectable, suggesting the efficacy of the antibody in vivo (Fig. 4.12A). Neutralizing IL-6 increased expression of the early activation markers CD25 and CD69 on DES<sup>+</sup> CD8 T cells in response to DST+LPS (Fig. 4.12B, 4.12C). Interestingly, neutralizing IL-6 significantly increased the percentages and numbers of IFN- $\gamma$ <sup>+</sup> DES<sup>+</sup> CD8 T cells in response to DST+LPS, relative to mice that received the isotype control (Fig. 4.12D). Similar results were obtained when I compared IFN- $\gamma$  production by endogenous alloreactive CD8 T cells (Fig. 4.12E). IL-6 neutralization also elevated plasma levels of IFN- $\gamma$  and IL-10 (Fig. 4.12A) early in the response suggesting that IL-6 regulates secretion of these cytokines by innate immune cells or non-hematopoietic cells. Moreover, neutralizing IL-6 showed a modest but significant increase in cytotoxicity of target cells in mice treated with DST+LPS compared to mice that received the isotype control (Fig. 4.12F). The cytotoxicity results correlated with the frequency of granzyme B producing DES<sup>+</sup> CD8 T cells since IL-6 neutralization markedly elevated the levels of granzyme B producers (Fig. 4.12G). To test if IL-6 recapitulated the in vivo effects of LPS treatment, I injected KB5 chimeras with DST along with recombinant IL-6 and examined IFN- $\gamma$  production by DES<sup>+</sup> CD8 T cells to allo-stimulation. Exogenous IL-6 did not decrease the frequency of IFN- $\gamma$  producing DES<sup>+</sup> CD8 T cells (Fig. 4.12H) suggesting that IL-6 is not sufficient to inhibit CD8 T cell responses in vivo.



Fig. 4.12. IL-6 is necessary but not sufficient for LPS mediated suppression of CD8 T cell function.





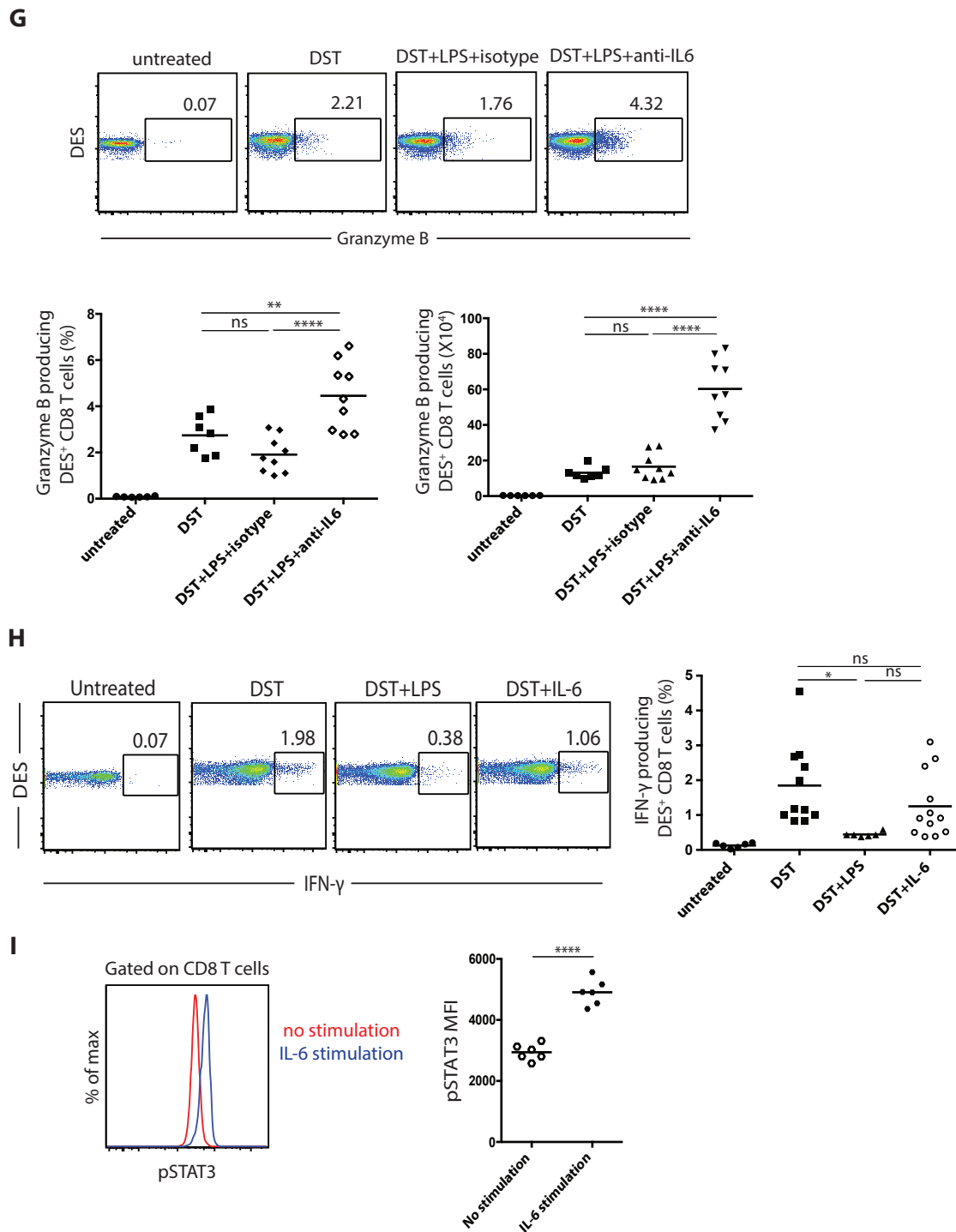


Fig. 4.12. IL-6 is necessary but not sufficient for LPS mediated suppression of CD8 T cell function. (A) KB5 chimeras were administered C57BL/6 DST alone or in combination with LPS. Anti-IL-6 antibody was injected 2 hrs before and 12 hrs after LPS treatment Plasma samples were collected 9 hours post-treatment with DST and analyzed for levels

of IL-6, IL-10 and IFN- $\gamma$ . (B) and (C) Representative plots of CD25 and CD69 expression on DES<sup>+</sup> CD8 T cells at 24 hours post-treatment are shown. The graphs show the expression levels (MFIs) of the activation markers on DES<sup>+</sup> CD8 T cells. (D) Splenocytes were harvested from mice 3 days post-treatment and stained for intracellular IFN- $\gamma$  upon allogeneic (H-2<sup>b</sup>) stimulation. Representative plots of IFN- $\gamma$  staining on DES<sup>+</sup> CD8 T cells are shown. The graphs show the percentages and numbers of IFN- $\gamma$  producing DES<sup>+</sup> CD8 T cells. (E) C57BL/6 mice were treated with CBA DST alone or in combination with LPS and IL-6 neutralizing antibody or its isotype control. Splenocytes were harvested from mice 3 days post-treatment and stained for intracellular IFN- $\gamma$  upon allogeneic (H-2<sup>k</sup>) stimulation. Representative plots of IFN- $\gamma$  staining on CD8 T cells are shown. The graph shows the percentages of IFN- $\gamma$  producing CD8 T cells. (F) C57BL/6 mice were treated as in (E). On day 3, CFSE labeled CBA (H-2<sup>k</sup>) and C57BL/6 (H-2<sup>b</sup>) splenocytes were transferred intravenously at equal proportions into the mice, and the survival of each population was determined 3 hours later. Survival of the CBA splenocytes is shown in the representative histograms and the graph shows percent specific lysis of CBA splenocytes. (G) KB5 chimeras were treated as in (A). Representative plots of granzyme B expression on DES<sup>+</sup> CD8 T cells at 3 days post-treatment are shown. The graphs show the percentages and total numbers of granzyme B producing DES<sup>+</sup> CD8 T cells. (H) KB5 chimeras were administered C57BL/6 DST alone or in combination with LPS or recombinant IL-6. Splenocytes were harvested from the mice 3 days post-treatment and stained for intracellular IFN- $\gamma$  upon allogeneic (H-2<sup>k</sup>) stimulation. Representative plots of IFN- $\gamma$  staining on DES<sup>+</sup> CD8 T cells are shown. The graph shows the percentages of IFN- $\gamma$  producing DES<sup>+</sup> CD8 T cells. (I) Splenocytes were harvested from KB5 chimeras and stimulated with recombinant IL-6. Representative plot of pSTAT3 staining on CD8 T cells is shown. The graph shows the MFI of pSTAT3 on CD8 T cells. Data are cumulative of at least two independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

It is well established that CD8 T cells express the IL-6 receptor (Teague et al., 2000). Binding of IL-6 to its receptor triggers the activation of JAK-STAT pathway involving phosphorylation of STAT3. To test if IL-6 acts directly on CD8 T cells, I examined levels of phosphorylated STAT3 in response to ex vivo IL-6 stimulation. IL-6 stimulation increased pSTAT3 levels in CD8 T cells suggesting that IL-6 acts directly on CD8 T cells (Fig. 4.12I).

## Discussion

The impact of PRR ligands on CD8 T cell responses to soluble protein antigens, peptide immunizations, DC vaccines and pathogens has been extensively studied (Cui et al., 2014; den Haan et al., 2007; Mandraju et al., 2014; Melief et al., 2015). Here I studied the impact of PRR ligands LPS and poly(I:C) on CD8 T cell responses to alloantigens directly presented by DST. I performed a detailed assessment of the kinetics of the alloresponse, phenotype, and functionality of effector CD8 T cells generated upon exposure to these inflammatory stimuli. I found that while poly(I:C) elevated the expression of activation markers on alloreactive CD8 T cells and their primary effector function compared to LPS, LPS generated higher levels of memory CD8 T cells by providing survival signals during contraction phase of the alloresponse.

Antigen-specific CD8 T cell responses have been extensively studied in the context of acute viral infections. The early stage of acute LCMV infection preceding the expansion of antigen-specific T and B cells is characterized by death of both antigen-specific and bystander T cells (Bahl et al., 2006; McNally et

al., 2001). I observed a similar attrition of alloantigen specific DES<sup>+</sup> CD8 T cells as early as 12 hours post DST administration (Fig. 4.2B). Both LPS and poly(I:C) are known to induce upregulation of costimulatory molecules required for T cell activation and survival (Hoebe et al., 2003). My finding that LPS, but not poly(I:C), inhibits this early cell death suggests that cell death at this stage is regulated by factors other than costimulation. I speculate that the distinct cytokine environment generated by LPS contributes to its anti-apoptotic effects. Indeed, TNF- $\alpha$  produced upon LPS challenge has been shown to rescue peripheral T cells from death induced by superantigens (Vella et al., 1995). The early cell death in viral infections was found to be dependent on type I interferons (Bahl et al., 2006; McNally et al., 2001). High amounts of IFN- $\beta$  generated upon poly(I:C) challenge (Fig. 4.1G) might explain increased apoptosis of DES<sup>+</sup> CD8 T cells compared to LPS treatment which did not generate significant levels of interferon. Following this first wave of CD8 T cell death, cells expanded and the response peaked on day 3. Since the DES<sup>+</sup> TCR recognizes the native form of the alloantigen H-2K<sup>b</sup> (Schonrich et al., 1991), my experimental setup tracks the CD8 T cells directly responding to the antigen and hence the kinetics of the CD8 T cell response are faster than those of cells responding to a viral antigen or a minor alloantigen that undergo the processes of antigen processing and presentation by antigen presenting cells. The expansion was accelerated by LPS and poly(I:C) relative to DST alone (Table 4.1). Increased expression of the high affinity IL-2R $\alpha$  chain (CD25) upon LPS and poly(I:C) challenge (Fig. 4.6) may

make the alloreactive CD8 T cells more sensitive to IL-2 signals. IL-2 has been shown to have a prominent role in the primary expansion and accumulation of effector CD8 T cells after antigen challenge (Obar et al., 2010; Starbeck-Miller et al., 2014). It is conceivable that LPS and poly(I:C) by increasing the responsiveness of CD8 T cells to IL-2, accelerate expansion and the generation of higher numbers of DES<sup>+</sup> CD8 T cells in the spleen at the peak of the response (Fig. 4.2C), although I cannot rule out the possible contribution of other cytokines produced upon exposure to these PRR ligands. The relative contribution of costimulation and cytokines to the adjuvant effect of LPS and poly(I:C) needs to be investigated.

LPS was recently shown to accentuate the expression of pro-memory genes in KLRG1<sup>lo</sup> IL-7R<sup>hi</sup> memory precursor effector CD8 T cells and promote their longevity following immunization culminating in larger numbers of memory CD8 T cells compared to other TLR agonists (Cui et al., 2014). Studies suggest that STAT3 dependent cytokines such as IL-6, IL-10 and IL-21 are critical for memory CD8 T cell differentiation (Castellino and Germain, 2007; Cui et al., 2011; Foulds et al., 2006; Laidlaw et al., 2015). IL-10 and IL-21 have been shown to act together to promote memory CD8 T cell development and functional maturation after acute LCMV infection (Cui et al., 2011). These investigators further showed that IL-10 derived from CD4<sup>+</sup> Tregs during the resolution phase of the response was necessary for memory CD8 T cell maturation (Laidlaw et al., 2015). IL-10 was also required for optimal CD8 T cell memory following *Listeria*

*monocytogenes* infection (Foulds et al., 2006). IL-6 was demonstrated to contribute to the survival of activated CD8 T cells in the contraction phase of the immune response in a noninfectious vaccine model (Castellino and Germain, 2007). Since LPS challenge generates higher levels of IL-6 and IL-10 relative to poly(I:C) challenge, it is possible that the combined effect of these two cytokines augments memory alloreactive CD8 T cell formation upon LPS exposure.

Upon recognition by the cell surface receptor TLR4, LPS signals through the adaptor protein MyD88 to trigger signaling cascades culminating in production of pro-inflammatory cytokines IL-6, IL-1 and TNF- $\alpha$ . Besides LPS, TLR4 is known to recognize DAMPs such as high mobility group box 1 (HMGB1), hyaluronan, fibrinogen and heat shock proteins (Mori et al., 2014). In the context of transplantation, TLR4 can potentially be activated by these endogenous DAMPs upon ischemic tissue damage or by LPS from bacterial infections or translocation of microbiota. Studies employing TLR4 deficient mice or local knockdown of TLR4 on the allograft using short-hairpin RNA (shRNA) have demonstrated that inhibition of TLR4 signaling modestly prolongs survival and/or function of certain allografts (Goldberg et al., 2007; Hsieh et al., 2017; Krams et al., 2010; Kwan et al., 2016; Zhang et al., 2010). Moreover, genetic deficiency or gene silencing of MyD88 prolongs the survival of allografts and synergizes with costimulation blockade to promote long-term graft acceptance (Goldstein et al., 2003; Lerret et al., 2015; Tesar et al., 2004; Walker et al., 2006; Zhang et al., 2012). Given these findings, my observation that LPS reduces IFN- $\gamma$  production



and cytotoxicity of alloreactive CD8 T cells, is surprising because CTLs are crucial mediators of acute rejection. I speculate that the dose of LPS used in my studies is higher than the inflammation encountered during organ transplantation. Indeed, studies showed that endotoxin tolerance induced upon repeated injections of LPS or polymicrobial sepsis suppressed antigen-specific CD8 T cell IFN- $\gamma$  production (De Wilde et al., 2009; Delano et al., 2007). The common theme emerging from these studies is that a CD11b<sup>+</sup> Gr-1<sup>+</sup> myeloid derived suppressor cell (MDSC) population expanding in endotoxin tolerant or septic mice suppresses the CD8 T cell response. Interestingly, endotoxin tolerance induced a significant delay in skin graft rejection in a minor antigen mismatch model (De Wilde et al., 2009). However, the rejection kinetics of a full MHC mismatched graft were not affected. Poly(I:C) elevated the levels of IFN- $\gamma$  and granzyme B producing cytotoxic alloreactive CD8 T cells. Although poly(I:C) has not been widely studied in the context of transplantation, its CD8 T cell potentiating effects are known from vaccine, infection and cancer studies (Dowling and Mansell, 2016).

LPS has been shown to suppress cross-presentation of soluble protein antigens to CD8 T cells (Brossart and Bevan, 1997; Mandraju et al., 2014). When the antigen and LPS were administered locally, LPS inhibited cross-priming by altering the composition of DCs in the draining lymph nodes (Mandraju et al., 2014). Upon systemic administration, LPS induced IL-10 producing regulatory Tr1 cells that strongly suppressed the antigen-specific CD8 T cell response in an

IL-10 dependent manner (den Haan et al., 2007). LPS mediated suppression of alloreactive CD8 T cell function was independent of IL-10 in my studies (Fig. 4.11). Interestingly, IL-6 was necessary for this suppression (Fig. 4.12D, E). Originally discovered as a B cell growth factor, IL-6 is a pleiotropic cytokine regulating growth, differentiation, survival and function of immune cells (Hunter and Jones, 2015). Most studies on the impact of IL-6 on T cells have focused on the CD4 T cell subset. IL-6 promotes survival and proliferation of CD4 T cells, promotes differentiation of Th17 cells in conjunction with TGF- $\beta$  and has an important role in memory CD4 T cell formation (Bettelli et al., 2006; Lotz et al., 1988; Nish et al., 2014; Teague et al., 1997; Veldhoen et al., 2006). Moreover, IL-6 releases conventional CD4 T cells from Treg suppression by enhancing their responsiveness to IL-2 (Nish et al., 2014). Recently, it was demonstrated that IL-6 in combination with IL-17 inhibits CTL function in a Theiler's murine encephalomyelitis virus (TMEV) infection model and promotes viral persistence (Hou et al., 2014). Another study reported inhibition of effector CD8 T cell responses by IL-6 in a Friend retrovirus mouse model (Wu et al., 2015). Consistent with these studies, I find that IL-6 is critical for the suppression of effector alloreactive CD8 T cell function. This finding may appear paradoxical since IL-6 induced upon TLR activation or bacterial infection has been shown to play a central role in preventing costimulation blockade mediated long-term allograft acceptance (Ahmed et al., 2011; Chen et al., 2009b; Shen and Goldstein, 2009; Zhao et al., 2012). However, in the absence of costimulation

blockade, systemic IL-6 deficiency or neutralization did not have a significant impact on allograft rejection (Booth et al., 2011; Zhao et al., 2012). Neutralizing IL-6 significantly delayed graft rejection in mice depleted of CD8 T cells suggesting that IL-6 is required for allograft rejection mediated by CD4 T cells (Booth et al., 2011). These mice exhibited a reversal of Th1/Th2 balance in favor of Th2 and a decrease in graft infiltrating cells. Thus, it appears that neutralizing IL-6 has a more dominant effect on CD4 T cells than CD8 T cells in a transplantation setting. Based on these reports, I speculate that an increase in effector CD8 T cell function upon IL-6 neutralization would be offset by a reduction in Th1 activity and infiltration of graft-damaging immune cells. Poly(I:C) challenge also stimulated IL-6 production; however poly(I:C) enhanced CD8 T cell effector function. Type I interferon signaling after or at the time of antigen recognition is known to act as signal 3 for CD8 T cell activation and promote the differentiation of highly cytolytic effector cells (Crouse et al., 2015). Poly(I:C) stimulated high levels of IFN- $\beta$  (Fig. 4.1G). Therefore, it appears that the relative levels of cytokines, rather than absolute amounts, determine their impact on CD8 T cell effector function.

LPS signaling through recipient TLR4 was critical for the inhibition of alloreactive effector CD8 T cell function (Fig. 4.10). I speculate that in *Tlr4*<sup>-/-</sup> recipients that receive *Tlr4*<sup>+/+</sup> DST, LPS signaling through TLR4 on the DST will not stimulate sufficient IL-6 and other cytokines to suppress the CD8 T cell response. IL-6 signals through a receptor comprising the IL-6R $\alpha$  subunit that

binds IL-6 and the gp130 subunit that is involved in signal transduction. While gp130 is ubiquitously expressed on hematopoietic and non-hematopoietic cells, IL-6R $\alpha$  is selectively expressed on leukocytes, megakaryocytes and hepatocytes (Hunter and Jones, 2015). I observed IL-6 signaling in CD8 T cells in vitro (Fig. 4.12I); however it is unclear if IL-6 acts directly on CD8 T cells in vivo to mediate suppression or whether an intermediate cell type is involved. Wu et al. observed that IL-6 directly inhibited effector CD8 T cell activity by inducing the STAT3 signaling pathway (Wu et al., 2015). IL-6 plays a major role in maintaining immature DCs and is required for suppression of LPS mediated DC maturation (Park et al., 2004). Our lab has previously demonstrated that LPS challenge upregulates costimulatory molecules CD80 and CD86 on cells in the DST (Miller et al., 2009). Therefore, IL-6 mediated inhibition of APC maturation resulting in reduced alloreactive CD8 T cell priming is unlikely. Recently TLR2 activation in skin resident cells was shown to trigger IL-6 that induced expansion of MDSCs, which were recruited to the skin and suppressed T cell mediated recall responses such as dermatitis (Skabytska et al., 2014). It is possible that LPS induced IL-6 expands MDSCs in the spleen that suppress alloreactive CD8 T cell function in response to DST. Using conditional knockout mice with cell type specific deletion of IL-6R would shed light on the cell type necessary for IL-6 mediated inhibition of CD8 T cell function.

To summarize, the two PRR ligands LPS and poly(I:C) differentially regulate the phenotype, kinetics and function of alloreactive CD8 T cells

responding to a directly presented alloantigen by generating distinct cytokine profiles (Fig. 4.13). These studies highlight the complexity of regulation of different immune cell populations by cytokines and warrant caution when designing therapeutics targeting cytokine signaling pathways for clinical use.

Fig. 4.13. Summary: LPS and poly(I:C) differentially regulate alloreactive CD8 T cell kinetics and function in response to donor alloantigen (DST).

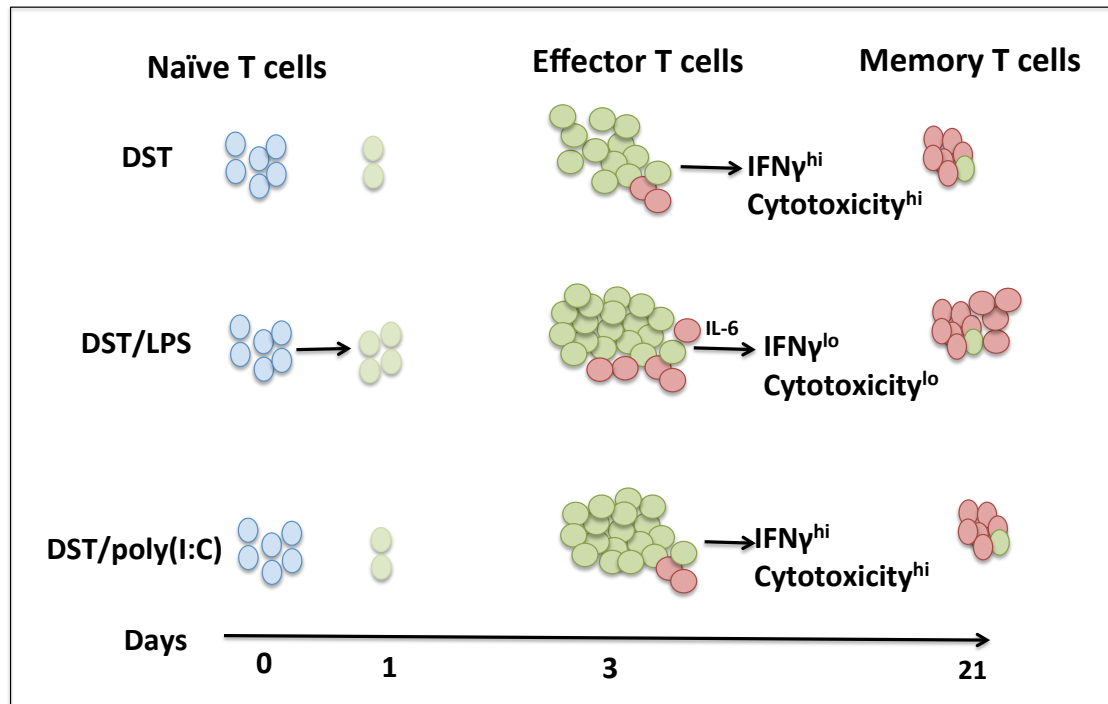


Fig. 4.13. Summary: LPS and poly(I:C) differentially regulate alloreactive CD8 T cell kinetics and function in response to donor alloantigen (DST). LPS inhibits the early deletion of alloreactive CD8 T cells induced by DST challenge. Following the early deletion, cells proliferate in all the groups. Both LPS and poly(I:C) generate higher numbers of alloreactive CD8 T cells at the peak of the response, day 3. LPS reduces the frequency of IFN- $\gamma$  producing and cytolytic effector CD8 T cells, whereas poly(I:C) enhances it. By providing survival signals in the contraction phase of the response, LPS generates higher numbers of memory alloreactive CD8 T cells.

## CHAPTER V

**Improved B cell development in humanized  
NOD-*scid* *IL2R $\gamma$* <sup>null</sup> mice transgenically  
expressing human stem cell factor,  
granulocyte-macrophage colony-stimulating  
factor and interleukin-3**

## Abstract

Immunodeficient mice engrafted with human immune systems support studies of human hematopoiesis and the immune response to human-specific pathogens. A significant limitation of these humanized mouse models is, however, a severely restricted ability of human B cells to undergo class switching and produce antigen-specific IgG after infection or immunization. In this study, I have characterized the development and function of human B cells in NOD-*scid* *IL2R $\gamma$* <sup>null</sup> (NSG) mice transgenically expressing human SCF, GM-CSF and IL-3 (NSG-SGM3) following engraftment with human hematopoietic stem cells, autologous fetal liver and thymic tissues (bone marrow, liver, thymus or BLT model). The NSG-SGM3 BLT mice engraft rapidly with human immune cells and develop T cells, B cells and myeloid cells. A higher proportion of human B cells developing in NSG-SGM3 BLT mice had a mature/naïve phenotype with a corresponding decrease in immature/transitional human B cells as compared to NSG BLT mice. In addition, NSG-SGM3 BLT mice have higher basal levels of human IgM and IgG as compared with NSG BLT mice. Moreover, dengue virus infection of NSG-SGM3 BLT mice generated higher levels of antigen-specific IgM and IgG, a result not observed in NSG BLT mice. These studies suggest that NSG-SGM3 BLT mice show improved human B cell development and permit the generation of antigen-specific antibody responses to viral infection.

## Introduction



Humanized mice are increasingly being utilized to study human immunity as well as infections, autoimmunity, allergies, organ transplantation, vaccine development, and immune regulation (Shultz et al., 2012; Shultz et al., 2007). A key factor for the successful generation of humanized mice is the use of optimal mouse strains that enable the survival of engrafted human cells and tissues. Immunodeficient *scid*, *Rag1<sup>null</sup>* or *Rag2<sup>null</sup>* mice bearing mutations within the IL2 receptor gamma chain (*IL2rg*) gene support the robust engraftment of human immune cells and tissues and the development of functional human immune systems (Brehm et al., 2010; Ito et al., 2002; Shultz et al., 2005; Traggiai et al., 2004).

Current efforts to further enhance immune system development and function in humanized mouse models have focused on improving specific human immune cell populations. One major limitation of the existing humanized mouse models is the severely limited ability of human B cells in these mice to undergo class switching and affinity maturation in response to pathogens or immunization with protein antigens (Seung and Tager, 2013). Antigen-specific antibody responses are generated in these mice but they are largely of the IgM isotype with very low IgG titers, which suggests inefficient class switching (Biswas et al., 2011; Jaiswal et al., 2015). The restricted B cell responses in humanized mice are an obstacle for studies of vaccine development and infectious diseases where humoral responses predominate. The limited ability of human B cells to undergo class switching in humanized mice is attributed to several factors

including impaired T and B cell maturation, lack of secondary lymphoid structures in the peripheral lymphoid organs, poor reconstitution of myeloid antigen-presenting cells (APCs), and insufficiency of human cytokines (Chen et al., 2009c; Covassin et al., 2013; Matsumura et al., 2003; Vuyyuru et al., 2011; Watanabe et al., 2009). Examples of approaches to improve human B cell function include expression of HLA class II and expression or injection of human cytokines that facilitate hematopoiesis and lymphocyte differentiation. While these approaches have enabled improvements in B cell function, generation of high levels of antigen-specific IgG remains problematic.

In this study I evaluated human B cell development and function in humanized NSG mice constitutively expressing SCF, GM-CSF and IL-3, also known as NSG-SGM3 mice. Previous studies have shown that NSG-SGM3 mice have significantly improved engraftment of human acute myeloid leukemia (AML) cells as well as long-term pre-leukemic myeloid cell cultures (Wunderlich et al., 2010). Moreover, NSG-SGM3 mice engrafted with human CD34<sup>+</sup> HSC have elevated levels of neutrophils (Miller et al., 2013) and other granulocytes (Coughlan et al., 2016), myeloid dendritic cells (mDCs) as well as CD4<sup>+</sup> cells with a lineage skewing towards Tregs that were functionally and phenotypically equivalent to human Tregs (Billerbeck et al., 2011). To test the ability of NSG-SGM3 mice to support human B cell development and function, these mice along with NSG mice were transplanted with human fetal thymic and liver tissues and autologous fetal liver derived CD34<sup>+</sup> HSC to generate BLT mice. I found that

NSG-SGM3 BLT mice have enhanced human B cell development, with higher levels of mature naïve B cells and lower levels of immature transitional and transitional B cells as compared with NSG BLT mice. NSG-SGM3 BLT mice also had higher basal levels of human IgM and IgG in the plasma as compared with control NSG BLT mice. Finally, infection of NSG-SGM3 BLT mice with dengue virus stimulated the generation of antigen-specific IgM and IgG responses at levels higher than NSG BLT mice. These results indicate that NSG-SGM3 mice support enhanced development and maturation of human B cells and will be a useful model to study human antigen-specific B cell responses.

## Materials and Methods

### *Mice*

NOD.Cg-*Prkdc*<sup>scid</sup>*Il2rg*<sup>tm1Wjl</sup>/SzJ (NOD-*scid* *IL2γ*<sup>null</sup>, NSG) mice and NOD.Cg-*Prkdc*<sup>scid</sup> *Il2rg*<sup>tm1Wjl</sup> Tg(CMV-IL3,CSF2,KITLG)1Eav/MloySzJ (NSG-SGM3 mice) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). All animals were housed in a specific pathogen free facility in microisolator cages, given autoclaved food and maintained on sulphamethoxazole-trimethoprim medicated water (Goldline Laboratories, Ft Lauderdale, FL, USA) and acidified autoclaved water on alternate weeks. All experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School and the recommendations in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996).

### *Generation of BLT mice*

Male and female NSG and NSG-SGM3 mice at 6-10 weeks of age were irradiated with 100 cGy and implanted with human fetal thymus and liver fragments under the kidney capsule. The fetal tissues (gestational age 16-20 weeks) were obtained from Advanced Bioscience Resources (Alameda, CA, USA). The tissues were washed with RPMI supplemented with penicillin G (100U/ml), streptomycin (100mg/ml), fungizone (0.25ug/ml) and gentamycin (5ug/ml) and 1mm<sup>3</sup> fragments of the fetal thymus and liver were implanted in the renal subcapsular space. Mice were injected subcutaneously with gentamycin (0.2 mg) and cefazolin (0.83 mg) post surgery. To obtain fetal HSC, fetal liver tissue was processed as described previously (Covassin et al., 2013), depleted of CD3<sup>+</sup> T cells and a cell suspension containing 1 to 2x10<sup>5</sup> CD34<sup>+</sup> fetal liver HSC was injected in the tail vein of mice between 4-6 h after irradiation.

### *Antibodies and flow cytometry*

Fluorophore linked primary antibodies used for analysis of hematopoietic cell engraftment were purchased from BD Biosciences, Inc. (San Jose, CA, USA), eBiosciences (San Diego, CA, USA) or BioLegend (San Diego, CA, USA). The following antibodies (clones) were used: mouse CD45 (30-F11), human CD45 (2D1), CD34 (581), CD3 (UCHT1), CD20 (2H7), CD33 (WM53), CD4 (RPA-T4), CD8 (RPA-T8), CD25 (MA-251 and 2A3), CD127 (A019D5), Foxp3 (236A/E7),

CD45RA (HI100), CD27 (M-T271), CD38 (HIT2), CD10 (HI10A), IgD (IAG-2), CD138 (MI15). Single cell suspensions of spleen and bone marrow (recovered from 1 femur) were prepared from mice and whole blood was collected in heparin. Single cell suspensions of  $0.5-1 \times 10^6$  cells or 50-100ul of heparinized whole blood were washed with FACS buffer and incubated with rat anti-mouse CD16/CD32 (clone 2.4G2) for 5-7 mins at 4°C to block Fc binding. Cells were then incubated with antibodies for surface markers for 20 mins at 4°C in the dark. Stained samples were washed with FACS buffer and fixed with 1% paraformaldehyde for cell suspensions or treated with BD FACS lysing solution for whole blood to lyse red blood cells (RBCs) and fix the samples. To detect human Tregs, blood samples were stained for surface markers, lysed and fixed and then incubated with eBioscience fixation/permeabilization buffer for 60 mins. Cells were then stained with antibody against human Foxp3 in eBioscience permeabilization buffer for 60 mins. At least 50000 events were collected on LSRII flow cytometer (BD Biosciences) using the BD FACSDIVA software. FlowJo software (Tree Star, Inc., Ashland, OR, USA) was used to analyze data.

### *Infections and ELISAs*

Total human IgM and IgG concentrations were measured in the plasma of mice by ELISA as per the manufacturer's instructions (Bethyl Laboratories, Inc., Montgomery, TX, USA) using an EMax Endpoint ELISA microplate reader (Molecular Devices, Sunnyvale, CA, USA). To measure dengue virus specific

antibody responses, mice were infected subcutaneously with approximately  $10^6$  plaque forming units (PFUs) of dengue virus serotype-2 strain New Guinea C (DENV-2 NGC). The levels of dengue-specific IgM and IgG were determined using inactivated dengue-2 antigen lysates in ELISAs as described previously (Jaiswal et al., 2015).

### *Statistical analyses*

Statistical analyses were performed using GraphPad PRISM software version 5 (GraphPad, San Diego, CA, USA). An unpaired t-test was performed to determine statistically significant differences between mean values of each data set.

## **Results**

### **NSG-SGM3 BLT mice show accelerated human cell chimerism as compared to NSG BLT mice**

BLT mice were generated on the NSG or NSG-SGM3 background and levels of human CD45<sup>+</sup> hematopoietic cells were examined in the blood at 6, 9 and 12 weeks post implantation and in spleen and bone marrow at week 12 (Fig. 5.1). Levels of human CD45<sup>+</sup> hematopoietic cells were significantly higher in the peripheral blood of NSG-SGM3 mice at 6, 9 and 12 weeks as compared to NSG mice (Fig. 5.1A, 5.1B and 5.1C). The levels of circulating human CD45<sup>+</sup> cells in NSG BLT mice continued to increase over time ( $13.7 \pm 1.6\%$  at 6 weeks,

35.3±3.3% at 9 weeks and 47.3±4.6% at 12 weeks). In contrast, CD45<sup>+</sup> cell levels in the blood of NSG-SGM3 BLT mice reached maximal levels at 6 weeks and did not increase significantly beyond that time point (52.7±2.2% at 6 weeks, 62.5±2.9% at 9 weeks and 64.2±3.3% at 12 weeks). In the spleen, the percentages and total numbers of human CD45<sup>+</sup> cells were similar between NSG and NSG-SGM3 mice at 12 weeks post implantation (Fig. 5.1D and 5.1E). The percentages and total numbers of human CD45<sup>+</sup> cells in the bone marrow were similar between NSG and NSG-SGM3 mice at 12 weeks post implantation (Fig. 5.1F and 5.1G). Together these results indicated that NSG-SGM3 mice support the rapid engraftment of human hematopoietic cells.

Fig. 5.1. Human CD45<sup>+</sup> cell engraftment kinetics in the peripheral blood, spleen and bone marrow of NSG BLT mice and NSG-SGM3 BLT mice.

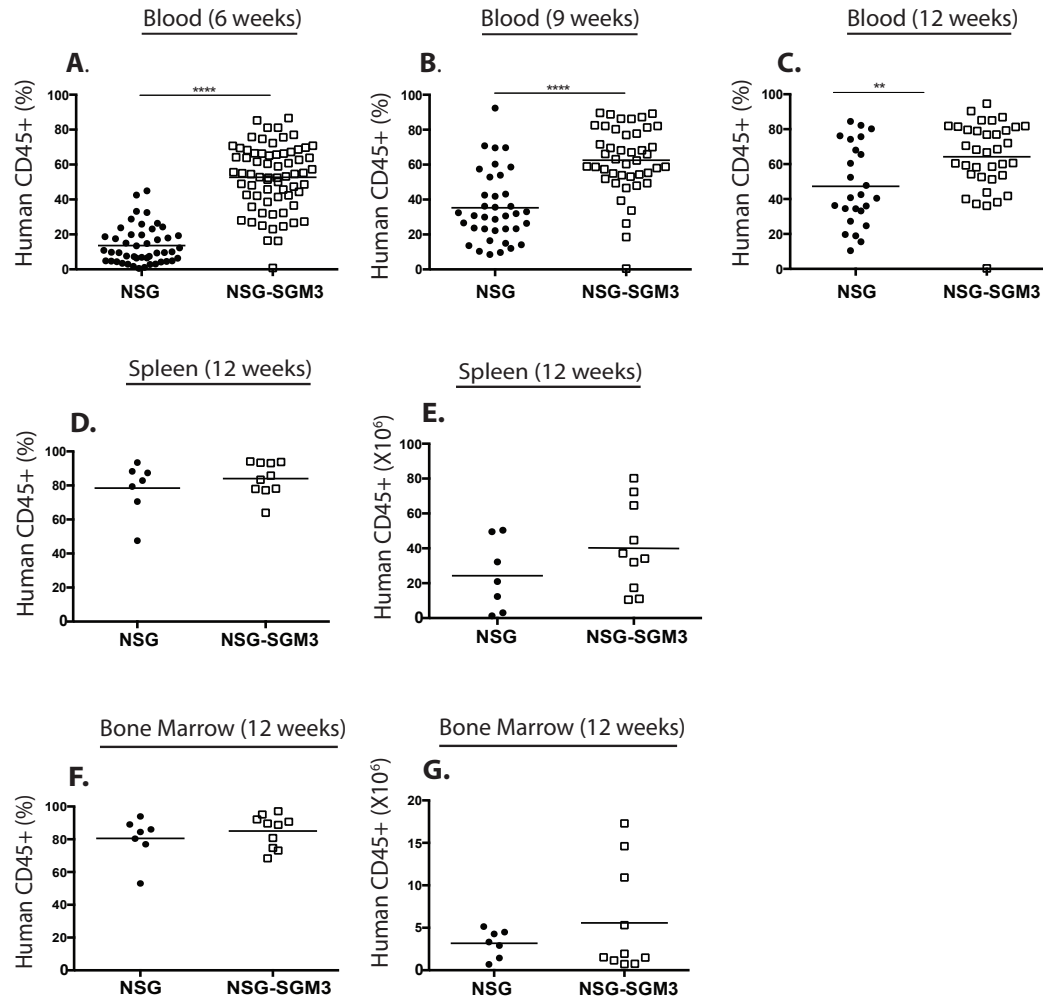


Fig. 5.1. Human CD45<sup>+</sup> cell engraftment kinetics in the peripheral blood, spleen and bone marrow of NSG BLT mice and NSG-SGM3 BLT mice. NSG and NSG-SGM3 mice were irradiated with 100cGy and implanted with 1mm<sup>3</sup> of human fetal thymus and liver in the renal subcapsular space. All mice were injected intravenously with 1x10<sup>5</sup> CD34<sup>+</sup> hematopoietic stem cells (HSC) derived from autologous fetal liver. The peripheral blood of the recipient NSG BLT and NSG-SGM3 BLT mice was screened for total human hematopoietic CD45<sup>+</sup> cell engraftment at the 6-week (A), 9-week (B) and 12-week (C) post-transplantation time points. The spleen (D,E) and bone marrow (F,G) of NSG BLT and NSG-SGM3 BLT mice were screened for total human CD45<sup>+</sup> cell engraftment 12 weeks after transplantation of human fetal tissues. Engraftment results are represented as a percentage of total cells or as total numbers in the spleen (D,E) and in the bone marrow (F,G). \*p<0.05; \*\*p<0.01; \*\*\*\*p<0.0001. The results for peripheral blood are from 4 independent experiments and for spleen and bone marrow are from 2 independent experiments.



### **NSG-SGM3 BLT mice support human T cell development**

A significant advantage of the BLT model is the efficient development of human T cells on autologous human thymic tissues. Human CD3<sup>+</sup> T cell development in NSG-SGM3 BLT and NSG BLT mice was monitored in the blood at 6, 9 and 12 weeks post implantation and in spleen and bone marrow at week 12 (Fig. 5.2). Levels of human T cells were significantly lower in the blood of NSG-SGM3 mice compared to NSG mice and the differences were significant over time (Fig. 5.2A, 5.2B, 5.2C). T cell engraftment improved with the age of mice in both groups. NSG-SGM3 mice had lower percentages of human T cells in the spleen compared to NSG mice at 12 weeks (Fig. 5.2D), but total numbers of T cells were similar (Fig. 5.2E). Human T cell levels were low in the bone marrow of both groups of mice, with significantly higher percentages (Fig. 5.2F) detected in NSG-SGM3 mice compared to NSG mice and similar numbers for each group (Fig. 5.2G).

### **NSG-SGM3 BLT mice support human B cell development**

Human CD20<sup>+</sup> B cell development in NSG-SGM3 BLT and NSG BLT mice was monitored in the blood at 6, 9 and 12 weeks post implantation and in spleen and bone marrow at week 12 (Fig. 5.3).

Fig. 5.2. Human CD3<sup>+</sup> T cell engraftment kinetics in the peripheral blood, spleen and bone marrow of NSG BLT mice and NSG-SGM3 BLT mice.

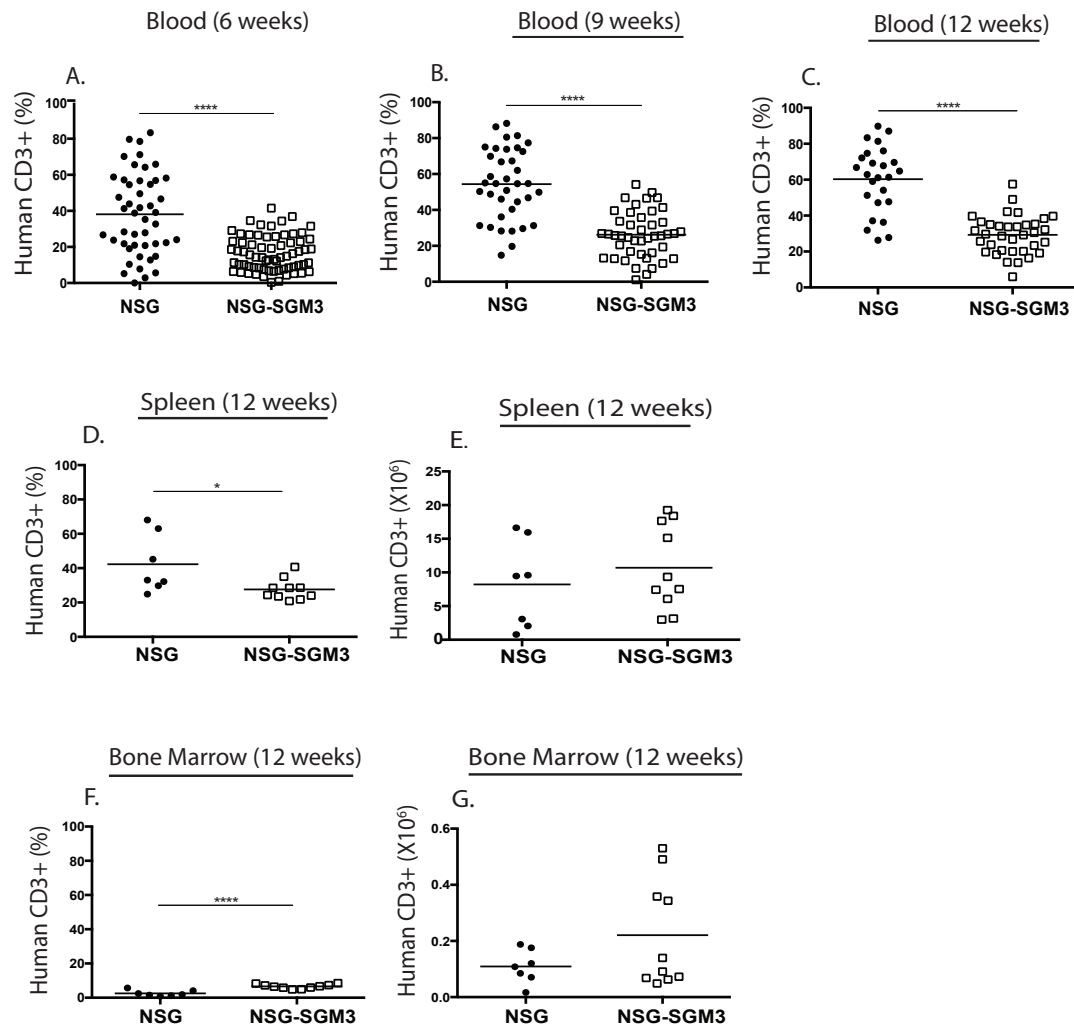


Fig. 5.2. Human CD3<sup>+</sup> T cell engraftment kinetics in the peripheral blood, spleen and bone marrow of NSG BLT mice and NSG-SGM3 BLT mice. The peripheral blood of the NSG BLT and NSG-SGM3 BLT mice was screened for total human CD3<sup>+</sup> T cell engraftment at 6-week (A), 9-week (B) and 12-week (C) post-transplantation of human fetal tissues. The spleen (D,E) and bone marrow (F,G) of NSG BLT and NSG-SGM3 BLT mice were screened for human CD3<sup>+</sup> T cell engraftment 12 weeks after transplantation of human fetal tissues. Engraftment results are represented as a percentage of total human CD45<sup>+</sup> cells or as total numbers in the spleen (D,E) and in the bone marrow (F,G). The results for peripheral blood are from 4 independent experiments and for spleen and bone marrow are from 2 independent experiments.

Levels of human B cells were significantly lower in the blood of NSG-SGM3 mice at week 6 compared to NSG mice but the levels were comparable at weeks 9 and 12 (Fig. 5.3A, 5.3B, 5.3C). NSG-SGM3 and NSG mice had similar percentages and total numbers of human B cells in the spleen (Fig. 5.3D and 5.3E) and bone marrow (Fig. 5.3F and 5.3G) at 12 weeks.

### **NSG-SGM3 BLT mice support enhanced myeloid cell development compared to NSG BLT mice**

Previous studies have shown that NSG-SGM3 mice engrafted with human HSC have significantly improved myeloid cell development (Billerbeck et al., 2011; Coughlan et al., 2016; Miller et al., 2013; Wunderlich et al., 2010). Human CD33<sup>+</sup> myeloid cell development in NSG-SGM3 BLT and NSG BLT mice was monitored in the blood at 6, 9 and 12 weeks post implantation and in spleen and bone marrow at week 12 (Fig. 5.4). At all time points tested significantly higher levels of human CD33<sup>+</sup> cells were detected in the blood of NSG-SGM3 BLT mice as compared to NSG BLT mice (Fig. 5.4A, 5.4B, 5.4C). In the spleen, the percentages and total numbers of human CD33<sup>+</sup> cells were significantly higher in NSG-SGM3 mice at 12 weeks post implantation compared to NSG mice (Fig. 5.4D and 5.4E). The percentages and total numbers of human CD45<sup>+</sup> cells in the bone marrow were similar between NSG and NSG-SGM3 mice at 12 weeks (Fig. 5.4F and 5.4G).

Fig. 5.3. Human CD20<sup>+</sup> B cell engraftment kinetics in the peripheral blood, spleen and bone marrow of NSG BLT mice and NSG-SGM3 BLT mice.

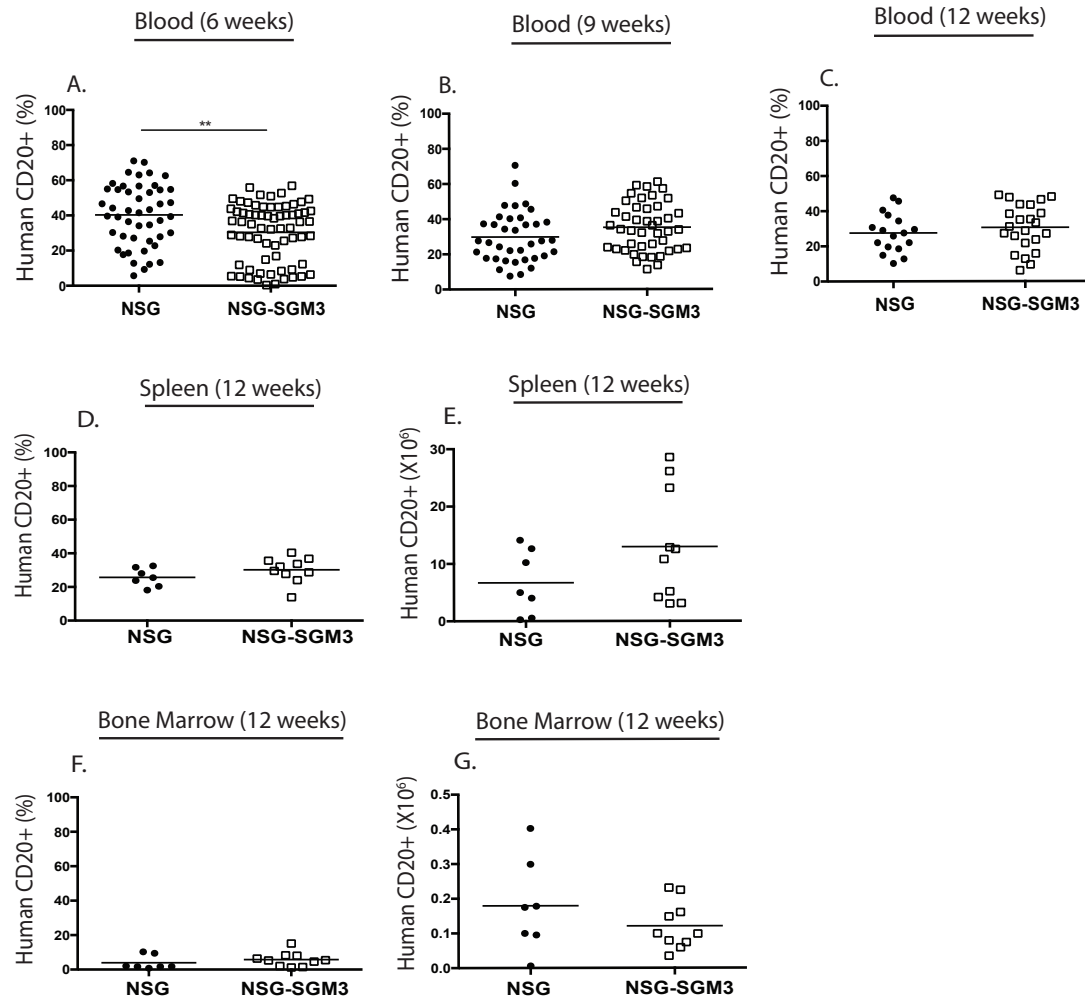


Figure 3. Human CD20<sup>+</sup> B cell engraftment kinetics in the peripheral blood, spleen and bone marrow of NSG BLT mice and NSG-SGM3 BLT mice. The peripheral blood of the NSG BLT and NSG-SGM3 BLT mice was screened for total human CD20<sup>+</sup> B cell engraftment at 6-week (A), 9-week (B) and 12-week (C) post-transplantation of human fetal tissues. The spleen (D,E) and bone marrow (F,G) of NSG BLT and NSG-SGM3 BLT mice were screened for human CD20<sup>+</sup> B cell engraftment 12 weeks after transplantation of human fetal tissues. Engraftment results are represented as a percentage of total human CD45<sup>+</sup> cells or as total numbers in the spleen (D,E) and in the bone marrow (F,G). \*p<0.05; \*\*p<0.01; \*\*\*p<0.001; \*\*\*\*p<0.0001. The results for peripheral blood are from 4 independent experiments and for spleen and bone marrow are from 2 independent experiments.

**Fig. 5.4. Human CD33<sup>+</sup> myeloid cell engraftment kinetics in the peripheral blood, spleen and bone marrow of NSG BLT mice and NSG-SGM3 BLT mice**

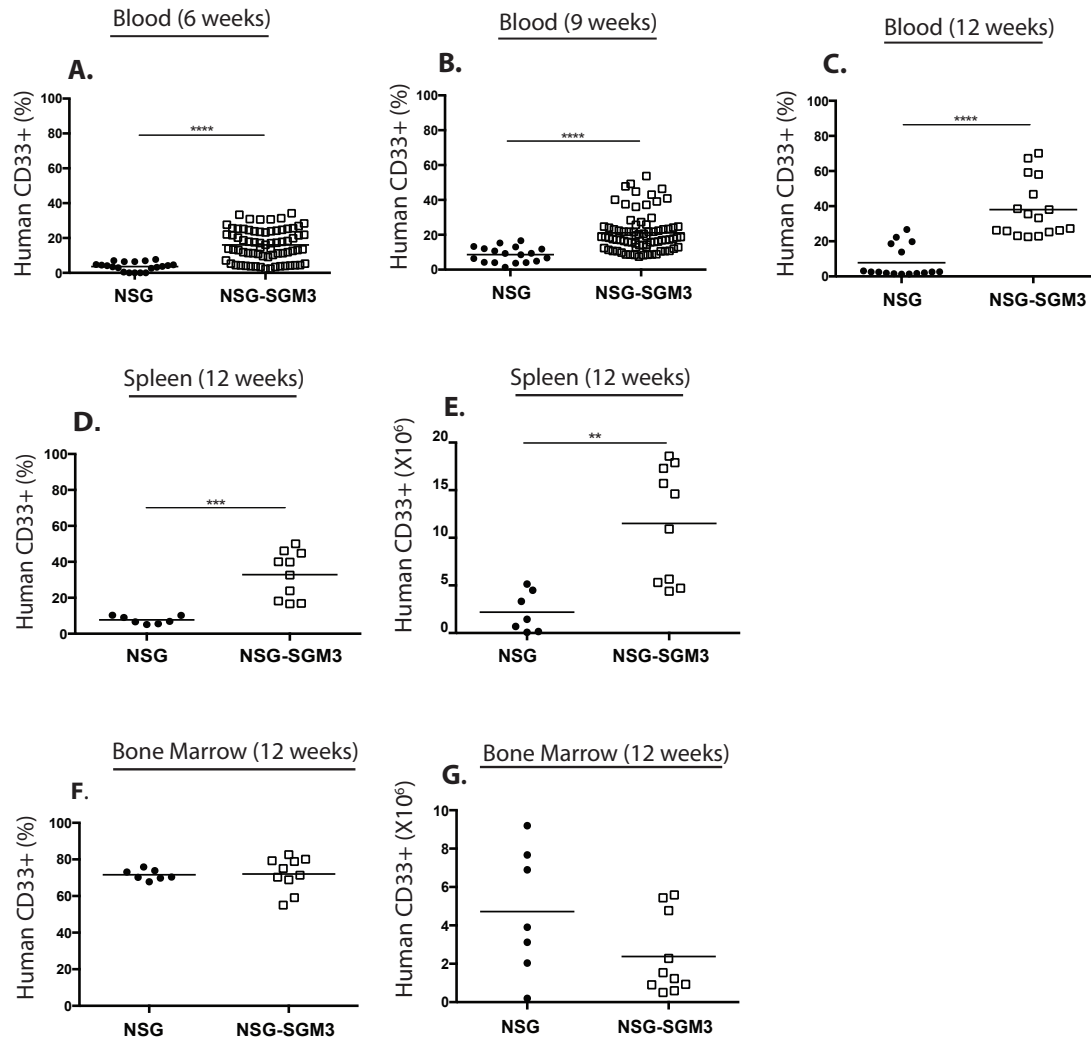


Fig. 5.4. Human CD33<sup>+</sup> myeloid cell engraftment kinetics in the peripheral blood, spleen and bone marrow of NSG BLT mice and NSG-SGM3 BLT mice. The peripheral blood of the NSG BLT and NSG-SGM3 BLT mice was screened for total human CD33<sup>+</sup> myeloid cell engraftment at 6-week (A), 9-week (B) and 12-week (C) post-transplantation of human fetal tissues. The spleen (D,E) and bone marrow (F,G) of NSG BLT and NSG-SGM3 BLT mice were screened for human CD33<sup>+</sup> myeloid cell engraftment 12 weeks after transplantation of human fetal tissues. Engraftment results are represented as a percentage of total human CD45<sup>+</sup> cells or as total numbers in the spleen (D,E) and in the bone marrow (F,G). In Fig. B, data points shown for NSG-SGM3 BLT are a combination of 8 and 9 week time points. \*p<0.05; \*\*p<0.01; \*\*\*p<0.001; \*\*\*\*p<0.0001. The results for peripheral blood are from 4 independent experiments and for spleen and bone marrow are from 2 independent experiments.

Together, these data show that NSG-SGM3 BLT mice show a heightened development of human myeloid development as has been found in HSC-engrafted NSG-SGM3 mice (Billerbeck et al., 2011).

**NSG-SGM3 BLT mice show improved engraftment of CD4<sup>+</sup> regulatory T cells as compared to NSG BLT mice**

NSG-SGM3 mice engrafted with human HSC have previously been shown to have enhanced development of CD4<sup>+</sup> human Tregs (Billerbeck et al., 2011). T-cell subsets were characterized in NSG-SGM3 BLT mice, by comparing CD4<sup>+</sup> to CD8<sup>+</sup> T cell ratios, Treg levels and T cell phenotype in the blood of both groups of mice at 12 weeks post tissue implantation (Fig. 5.5). The CD4<sup>+</sup> to CD8<sup>+</sup> ratio was approximately 5:1 in both strains of mice (Fig. 5.5A). Significantly higher levels of human CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup>Foxp3<sup>+</sup> T regulatory cells were detected in the blood of NSG-SGM3 mice compared to NSG mice (Fig. 5.5B). Analysis of CD45RA expression levels by human CD4 and CD8 T cells showed a lower proportion of T cells expressing CD45RA in the blood of NSG-SGM3 mice compared to NSG mice (Fig. 5.5C and 5.5D). These data indicate that NSG-SGM3 BLT mice have higher levels of CD4<sup>+</sup> regulatory T cells, and lower levels of naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cells.

Fig. 5.5. Characterization of human CD3<sup>+</sup> T cells in the peripheral blood of NSG BLT and NSG-SGM3 BLT mice at 12 week-post transplantation

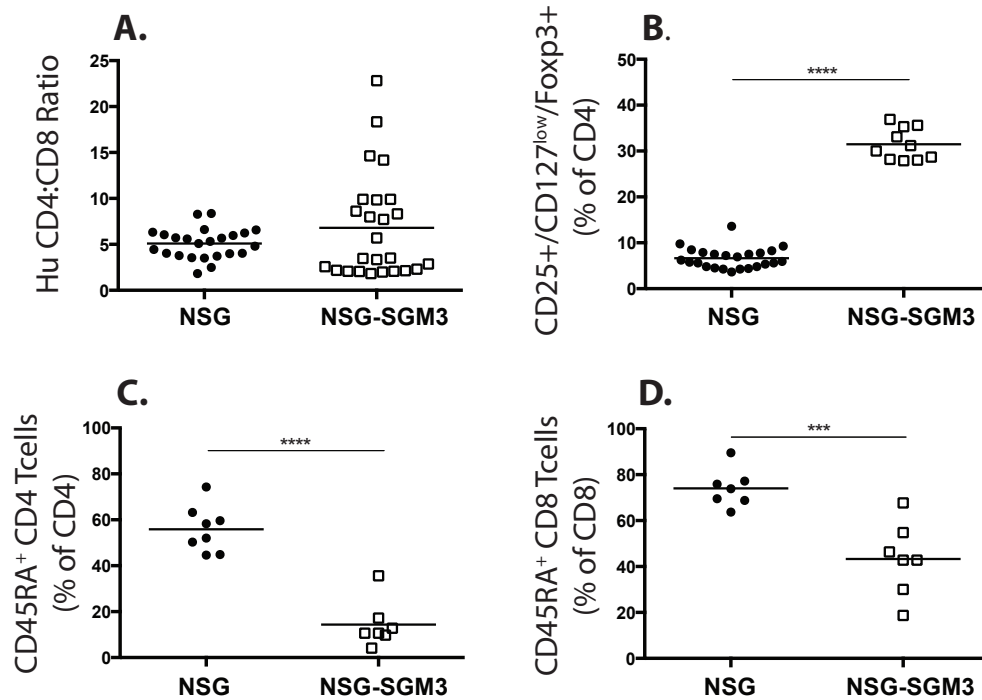


Fig. 5.5. Characterization of human CD3<sup>+</sup> T cells in the peripheral blood of NSG BLT and NSG-SGM3 BLT mice at 12 week-post transplantation. The ratio of human CD4:CD8 T cells gated on CD3<sup>+</sup> T cells is shown in (A). The proportion of CD25<sup>+</sup>CD127<sup>low</sup>Foxp3<sup>+</sup> regulatory T cells (Treg) gated on CD4<sup>+</sup> T cells is shown in (B). The percentages of CD4<sup>+</sup> and CD8<sup>+</sup> T cells expressing naïve T cell marker CD45RA are shown in (C) and (D) respectively. \*\*\*p<0.001; \*\*\*\*p<0.0001. The results for peripheral blood are from 2 independent experiments.

### **NSG-SGM3 BLT mice develop higher levels of mature naïve B cells compared to NSG BLT mice**

Previous studies have described a predominance of immature B cells in humanized mice (Biswas et al., 2011; Covassin et al., 2013; Matsumura et al., 2003). To evaluate B cell development in NSG-SGM3 BLT mice, the phenotype of B cells in the periphery was examined (Fig. 5.6). B cells were categorized into 5 groups based on their phenotypic markers; immature/transitional B cells ( $CD27^-CD10^+$ ), transitional B cells ( $CD27^-CD10^-CD38^+$ ), mature naïve B cells ( $CD27^-CD10^-IgD^+$ ), memory B cells ( $CD27^+CD10^-$ ) and plasma cells ( $CD138^+$ ). Immature/transitional B cells were present at comparable levels in the peripheral blood of NSG-SGM3 and NSG mice (Fig. 5.6A). NSG-SGM3 mice had lower levels of immature/transitional B cells in the spleen (Fig. 5.6B) and bone marrow (Fig. 5.6C). Transitional human B cells were significantly lower in blood (Fig. 5.6D) and spleen (Fig. 5.6E) of NSG-SGM3 mice compared to NSG mice and levels were similar in the bone marrow (Fig. 5.6F). Mature/naïve B cells were significantly higher in the blood (Fig. 5.6G), spleen (Fig. 5.6H) and bone marrow (Fig. 5.6I) of NSG-SGM3 mice compared to control NSG mice. Healthy human adults have between 60-70% mature naïve B cells in the peripheral blood (Perez-Andres et al., 2010). Memory B cells were present at significantly higher levels in the blood of NSG-SGM3 mice compared to NSG mice (Fig. 5.6J) and similar in spleen and bone marrow (Fig. 5.6K and 5.6L).



**Fig. 5.6. Characterization of human CD20<sup>+</sup> B cells in the peripheral blood, spleen and bone marrow of NSG BLT and NSG-SGM3 BLT mice at 12 weeks post-transplantation**

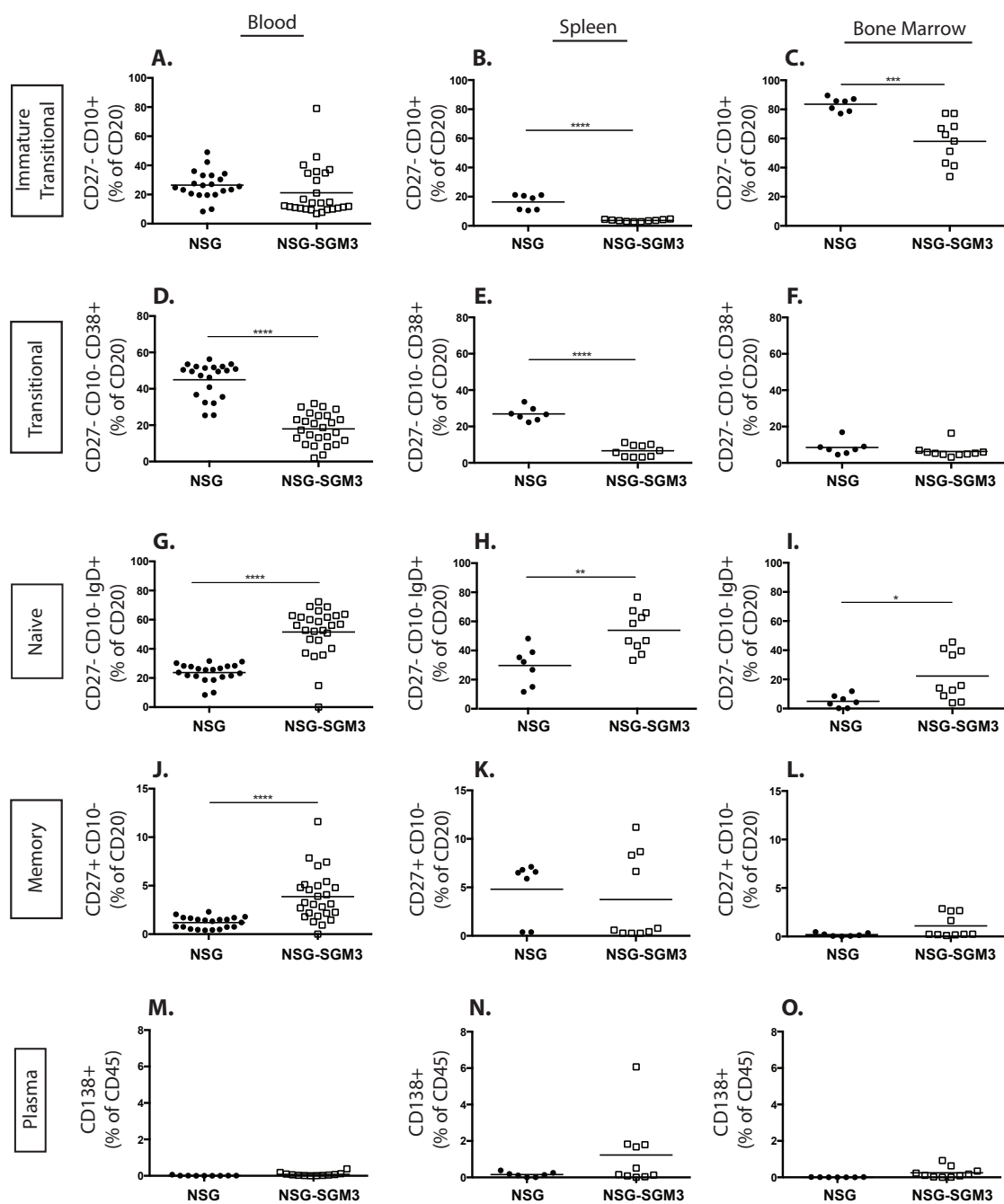


Fig. 5.6. Characterization of human CD20<sup>+</sup> B cells in the peripheral blood, spleen and bone marrow of NSG BLT and NSG-SGM3 BLT mice at 12 weeks post-transplantation. Human B cells were divided into 5 categories and expressed as a percentage of total human CD20<sup>+</sup> B cells: CD20<sup>+</sup>CD27<sup>-</sup>CD10<sup>+</sup> immature transitional B cells in blood (A), spleen (B), bone marrow (C); CD20<sup>+</sup>CD27<sup>-</sup>CD10<sup>-</sup>CD38<sup>+</sup> transitional B cells in blood (D), spleen (E), bone marrow (F); CD20<sup>+</sup>CD27<sup>-</sup>CD10<sup>+</sup>IgD<sup>+</sup> mature naïve B cells in blood (G), spleen (H), bone marrow (I); CD20<sup>+</sup>CD27<sup>+</sup>CD10<sup>-</sup> memory B cells in blood (J), spleen (K), bone marrow (L) and CD20<sup>+</sup>CD138<sup>+</sup> plasma cells in blood (M), spleen (N), bone marrow (O). \*p<0.05; \*\*p<0.01; \*\*\*p<0.001; \*\*\*\*p<0.0001. The results for peripheral blood are from 3 independent experiments and for spleen and bone marrow are from 2 independent experiments.

Healthy human adults are reported to have 10-30% memory B cells circulating in blood (Perez-Andres et al., 2010). Plasma cells were present at very low levels in both groups of mice in all tissues (Fig. 5.6M, 5.6N, 5.6O). Together these data indicate that NSG-SGM3 BLT mice have lower levels of immature and transitional B cells and higher levels of mature naïve B cells relative to NSG BLT mice, suggesting that the NSG-SGM3 BLT mice have improved human B cell maturation.

### **NSG-SGM3 BLT mice show an improved ability to generate IgG antibodies**

A major limitation of humanized mice is their reduced ability to generate human IgG responses. The enhanced human B cell maturation observed in the NSG-SGM3 BLT mouse model (Fig. 5.6) suggested that these mice may have an increased ability to undergo Ig class switching. The basal levels of human IgM and IgG in the plasma of resting BLT mice were therefore evaluated at 12 weeks post tissue implant. NSG-SGM3 mice had 5.6 fold higher levels of human IgM compared to NSG mice (Fig. 5.7A). Human IgG levels were 4.5 fold higher in NSG-SGM3 mice compared to NSG mice (Fig. 5.7B). Next the mice were infected with DENV-2 (Dengue virus serotype-2) and the generation of DENV-2 specific antibodies was assessed by sandwich ELISA 4 weeks post infection. The generation of IgM responses to the inactivated lysates of dengue antigen and the DENV-2 E (envelope) protein in NSG BLT mice but limited antigen-specific IgG responses has been previously demonstrated (Jaiswal et al., 2012).

Fig. 5.7. Evaluation of total antibody titers and dengue virus specific antibody responses in NSG BLT and NSG-SGM3 BLT mice

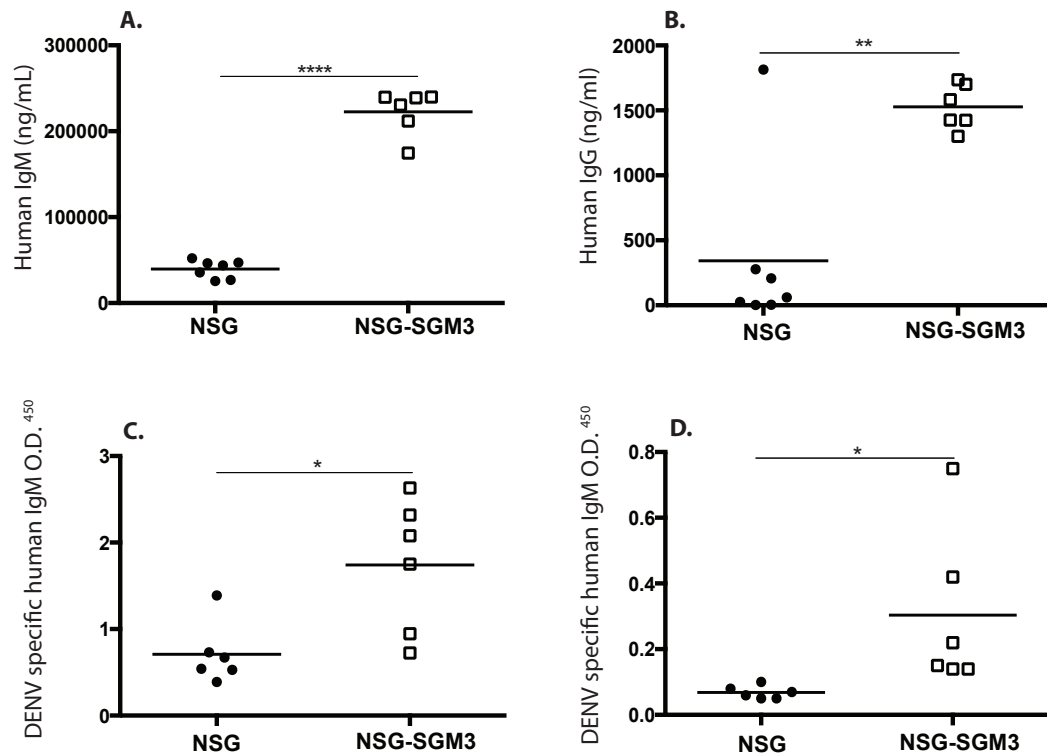


Fig. 5.7. Evaluation of total antibody titers and dengue virus specific antibody responses in NSG BLT and NSG-SGM3 BLT mice. Mice were bled at 12 weeks post implantation of human tissues and total human IgM (A) and human IgG (B) levels (ng/ml) were determined in the plasma of these mice by ELISA. Mice were infected with DENV-2 and plasma samples were tested to determine DENV-2 specific IgM (C) and IgG (D) by sandwich ELISA. Each symbol indicates an individual BLT mouse. The results are from 2 independent experiments.

NSG-SGM3 BLT mice infected with DENV-2 had significantly higher levels of DENV-2 specific IgM (Fig. 5.7C) and increased levels of DENV-2 specific IgG (Fig. 5.7D). These data indicate that transgenic expression of SCF, GM-CSF and IL-3 generates higher levels of total human IgM and IgG suggesting improved class switching and induces improved viral antigen-specific antibody responses.

### **Discussion**

The BLT model enables the generation of humanized mice having a complete human immune system, including the development of both conventional and regulatory HLA-restricted T cells. Although B cells develop in BLT mice, the circulating antibody levels are significantly lower compared to adult humans. Moreover, the generation of antigen-specific antibody responses in BLT mice, specifically IgG responses, are weak, limiting their use in studying humoral responses to pathogens and testing candidate vaccines (Seung and Tager, 2013). Two studies have demonstrated that the immunoglobulin gene repertoires of human B cells in humanized (HSC-engrafted) mice are similar to those of normal human peripheral B cells suggesting that humanized mice have the genetic potential to mount broad and high affinity antibody responses to diverse pathogens (Becker et al., 2010; Ippolito et al., 2012). The poor antigen-specific antibody responses are unlikely to be attributed to a genetic defect in the immunoglobulin repertoire. Studies have suggested that the contributing factors are defects in T and B cell maturation, disorganized secondary lymphoid

structures and an absence of human cytokines (Chen et al., 2009c; Covassin et al., 2013; Matsumura et al., 2003; Vuyyuru et al., 2011; Watanabe et al., 2009).

Mature B cells that encounter their cognate antigen in lymphoid follicles and receive T cell help enter a germinal center where they undergo the processes of class switching, that generates different antibody isotypes, and somatic hypermutation, that diversifies their antibody repertoire (LeBien and Tedder, 2008). Activated B cells can then differentiate into plasmocytes and become antibody-secreting plasma cells or become long-lived memory B cells. Human B cells develop in NSG BLT mice and generate human immunoglobulins although at lower levels than in adult humans (Covassin et al., 2013). Human B cells in humanized mice have been shown to generate antigen-specific antibody responses to human immunodeficiency virus 1 (HIV-1) (Biswas et al., 2011; Brainard et al., 2009), West Nile virus (Biswas et al., 2011), dengue virus (Jaiswal et al., 2012) and Epstein-Barr virus (EBV) (Yajima et al., 2008). These antibody responses are predominantly of the IgM isotype with very little to no IgG responses. A few studies have demonstrated antigen-specific IgG responses in a small subset of mice infected with HIV-1 (Sato et al., 2010; Sun et al., 2007), albeit at low titers. The inability to efficiently generate antibody response to most antigens has necessitated the development of humanized mouse models with improved class-switching.

The expression of human transgenes and inactivation of specific mouse genes has improved humoral responses in some models. Transgenic expression

of human signal regulatory protein alpha (SIRP alpha) in BALB/c-*Rag2*<sup>-/-</sup> *IL2rg*<sup>null</sup> mice that were injected with human HSC significantly elevated the total levels of human IgG in the plasma, but could not elicit a strong IgG response to the protein antigen ovalbumin (OVA) (Strowig et al., 2011). However, transgenic expression of HLA-DR4 in NOD-*Rag1*<sup>-/-</sup> *IL2rg*<sup>null</sup> mice engrafted with HLA-DR4<sup>+</sup> HSC elicited an IgG response to tetanus toxoid vaccine as well as class switching to IgA, IgE and all subtypes of IgG (Danner et al., 2011). In addition HLA-DR4 expression in NOD *Shi-scid* *IL2rg*<sup>null</sup> mice induced an anti-OVA IgG response (Suzuki et al., 2012). Expression of specific human cytokines has also improved the engraftment of human hematopoietic cells and enhanced the development and function of human immune cells, including B cells (Chen et al., 2009c). Administration of the recombinant human cytokine BAFF (B cell activating factor, also called BLyS) to NOD-*Rag1*<sup>-/-</sup> *Prf1*<sup>-/-</sup> mice injected with human PBLs improved the engraftment of B cells, elevated serum immunoglobulin levels and generated an antibody response to thymus-independent antigens in pneumovax vaccine (Schmidt et al., 2008). Hydrodynamic injection of human GM-CSF and IL-4 in HSC-engrafted NSG mice allowed the induction of tetanus toxoid specific IgG as well as neutralizing antibodies against H5N1 influenza virus upon immunization (Chen et al., 2012). While the approaches described above have improved human B cell functionality in humanized mice, further advancements are necessary to make these models applicable to study human B cell immunity.

In this study I have shown that NSG-SGM3 BLT mice that transgenically express human SCF (also called c-kit ligand or Steel factor), GM-CSF and IL-3 show heightened human B cell engraftment consistent with the important role of these human hematopoietic growth factors in hematopoiesis as well as in proliferation and survival of HSC in vitro (Broudy, 1997). Three studies have described HSC-engrafted NSG mice transgenically expressing membrane bound SCF (Brehm et al., 2012; Coughlan et al., 2016; Takagi et al., 2012). In one study, transgenic expression of membrane bound SCF circumvented the need for irradiation and permitted high levels of engraftment of human CD45<sup>+</sup> cells in HSC engrafted NSG mice (Brehm et al., 2012). Another study reported these mice to have an improved human myeloid cell compartment, specifically cells of the granulocytic lineage (Takagi et al., 2012). A recent study showed that the human myeloid cells in the SCF NSG mice migrated to the renal tissue to become resident dendritic cells and some of these mice could be used as a source of human bone marrow-derived macrophages (Coughlan et al., 2016). GM-CSF and IL-3 are important for the development and function of myeloid cells. Human IL-3/GM-CSF knock-in mice engrafted with human HSC were shown to have improved myeloid cell reconstitution in the lung and the engrafted human alveolar macrophages mounted an innate immune response to influenza virus showing that the myeloid cells were functionally responsive (Willinger et al., 2011). NSG-SGM3 mice engrafted with human HSC (the Hu-SRC-SCID model)



have been described previously (Billerbeck et al., 2011). These mice were shown to have elevated myeloid cell frequencies, specifically myeloid DCs, and CD4<sup>+</sup> Foxp3<sup>+</sup> regulatory T cells. NSG-SGM3 BLT mice have a similar increase in myeloid cell levels (Fig. 5.4) and Tregs (Fig. 5.5B).

Interestingly, NSG-SGM3 BLT mice had significantly higher frequencies of mature naïve B cells and proportionately reduced frequencies of immature and transitional B cells compared to control NSG BLT mice (Fig. 5.6). Immature B cells develop from the lymphoid progenitors in the bone marrow by passing through the pro-B and pre-B cell stages. These immature B cells expressing surface IgM (sIgM) exit the bone marrow and enter into the periphery. The early bone marrow emigrants are called immature transitional B cells and they express the markers CD10 and CD38 (Kaminski et al., 2012). In the periphery, they develop into transitional B cells that can gain access to lymphoid follicles in the spleen and become more sensitive to T cell help. Upon receiving appropriate cytokine signals and positive signals through the B-cell receptor, they become mature B cells that repopulate the periphery (Chung et al., 2003). Successful differentiation from the transitional to mature B cell stage is governed by the cytokine BAFF (Mackay et al., 2003). BAFF is a member of the TNF (tumor necrosis factor) family and is secreted mainly by myeloid cells such as neutrophils, monocytes, macrophages and dendritic cells (Mackay and Schneider, 2009). BAFF is also shown to be produced by activated T cells to some extent (Mackay and Leung, 2006). I propose two explanations for improved

B cell maturation observed in NSG-SGM3 BLT mice. Firstly, the increased frequencies of activated T cells characterized as CD45RA<sup>+</sup> (Fig. 5.5C, 5.5D) could facilitate B cell maturation in NSG-SGM3 mice. Recently, Lang et al. demonstrated a requirement of T cells for human B cell maturation in BALB/c-*Rag2*<sup>-/-</sup> *IL2rg*<sup>null</sup> mice engrafted with CD34<sup>+</sup> HSC (Lang et al., 2013). The study showed that adoptive transfer of autologous T cells elevated mature B cell frequencies, whereas T cell depletion diminished mature B cell levels. T cell activation, characterized by the expression of CD45RO, HLA-DR and CD49d, correlated with B cell maturation suggesting T cell activation might be important for B cell maturation. I did not find increased numbers of T cells in NSG-SGM3 BLT mice relative to NSG BLT mice (Fig. 5.2). However, the increased frequencies of CD45RA<sup>+</sup> activated T cells (Fig. 5.5C, 5.5D) in NSG-SGM3 BLT mice could possibly explain the improved B cell maturation in these mice. Secondly, the well engrafted human myeloid cell compartment in the NSG-SGM3 mice (Fig. 5.4) could secrete increasing amounts of human BAFF that binds the BAFF-R on transitional B cells rescuing them from death and easing them into the mature B cell stage. Also consistent with its role of a 'survival cytokine', BAFF would bind BAFF-R on mature B cells mediating their longevity and thus elevating the mature B cell frequencies.

Since improved B cell maturation contributes to enhanced B cell functionality in terms of antigen-specific antibody responses, I tested the hypothesis that improved B cell maturation in NSG-SGM3 BLT mice would

generate antigen-specific antibody responses. Previously our lab has demonstrated that NSG BLT mice can elicit DENV-2 antigen-specific antibody responses that are predominantly of the IgM class (Jaiswal et al., 2012). NSG-SGM3 BLT mice elicited significantly stronger IgM as well as IgG responses to inactivated DENV-2 lysates relative to NSG BLT mice. I propose that improved B cell maturation in these mice contributes to the enhanced levels of human immunoglobulins and antigen-specific antibody responses. The histology sections of spleens of NSG and NSG-SGM3 BLT mice upon dengue infection showed similarly disorganized architecture ruling out the formation of organized secondary lymphoid structures in NSG-SGM3 mice. However, I cannot rule out other mechanisms for enhanced antibody responses in these mice.

To summarize, transgenic expression of SCF, GM-CSF and IL-3 in NSG BLT mice resulted in improved human myeloid cell reconstitution, enhanced B cell maturation and antigen-specific antibody responses to dengue virus infection. Thus, NSG-SGM3 BLT mice prove to be a useful tool to study antibody responses to viral infections. Further improvements in B cell development, such as the development of secondary lymphoid structures to support class switching, engraftment of human follicular dendritic cells and enhanced B and T cell maturation, are necessary to achieve strong humoral responses.

## **CHAPTER VI**

### **Discussion**

In this thesis, I examined the role of costimulation and inflammation on the generation of alloreactive CD8 T cell responses. In Chapter II, I demonstrated that blocking CD40-CD154 interactions using an antagonistic CD40 antibody prolonged skin allograft survival and suppressed donor-specific CD8 T cell responses predominantly by impairing expansion of the CD8 T cell population. The pro-apoptotic proteins Fas and Bim functioned together to promote graft acceptance by CD154 costimulation blockade (Chapter III). In Chapter IV, I examined the impact of inflammatory stimuli LPS and poly(I:C) on CD8 T cell responses to the donor alloantigen DST and found that LPS and poly(I:C) differentially regulated the kinetics of the response, phenotype and functionality of alloreactive CD8 T cells. In Chapter V, I showed that transgenic expression of human cytokines SCF, GM-CSF and IL-3 in humanized NSG mice promoted B cell maturation and development of antibody responses.

Achieving transplantation tolerance clinically has been the 'Holy Grail' for transplantation immunologists for over two decades. Costimulation blockade has emerged to be a promising strategy to achieve this goal. Considering the central role of the CD40-CD154 pathway in the generation of adaptive immune responses, targeting this pathway is highly desirable. Using a stringent skin transplantation model of complete MHC mismatch, I showed that CD40 targeting antibodies can replace CD154 targeting antibodies to promote graft acceptance. This study supports the need to develop CD40 blocking reagents for clinical application. Besides transplantation, blocking CD154 has shown therapeutic

benefits in models of several autoimmune diseases (Peters et al., 2009). CD40 antagonists will potentially be efficacious in halting progression of autoimmunity. The absence of thromboembolism or other serious adverse events in phase 1 clinical trials of the CD40 antagonist ASKP1240 provides a ray of hope that targeting the promising CD40-CD154 pathway could become a reality in clinical transplantation and autoimmunity (Goldwater et al., 2013).

Although I showed that CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs were necessary for DST/anti-CD40 mediated long-term allograft acceptance, I did not examine whether Tregs were induced by this treatment. Peripheral induction of Tregs is a feature of DST/anti-CD154 therapy (Ferrer et al., 2011). Although the exact mechanism of Treg induction by blocking CD154 is not known, the favored hypothesis is that DST/anti-CD154 inhibits production of cytokines such as IL-6 (Ferrer et al., 2012a) that are critical inhibitors of Treg differentiation. To test if DST/anti-CD40 induces Tregs, OT-II T cells can be adoptively transferred into congenic recipient mice receiving DST/anti-CD40 treatment and a skin graft expressing ovalbumin (OVA) followed by counting CD25<sup>+</sup>Foxp3<sup>+</sup> OT-II T cells in the draining lymph nodes a week after transplantation. I hypothesize that DST/anti-CD40 treatment would promote iTreg differentiation. Also I expect a higher Treg: Teff ratio within the graft after DST/anti-CD40 treatment relative to DST/isotype control treatment. A combination therapy of anti-CD154 mAb and CTLA4-Ig induces modest prolongation of allograft survival in the BALB/c -> B6 skin graft model (Gilson et al., 2009). The BALB/c -> B6 skin graft model is

resistant to CD28/CD154 blockade (Trambley et al., 1999); hence testing DST/anti-CD40 in a less stringent model such as BALB/c → C3H will reveal if the anti-CD40 mAb can synergize with CTLA4-Ig to prolong allograft survival. Combination therapy of CD40 antagonist and belatacept in clinical transplantation might provide greater therapeutic benefit compared to belatacept monotherapy due to the Treg-promoting effects of blocking CD40 signaling.

DST/anti-CD40 treatment reduced IFN- $\gamma$  production by alloreactive DES<sup>+</sup> CD8 T cells at the peak of the primary response (day 3) and by endogenous donor-reactive CD8 T cells in the skin-grafted mice at a late time-point (day 42) suggesting that CD8 T cells that survive the deletion might be anergic. However, I did not pursue the 'anergy' aspect in my project. A recent study reported anergy in CD8 T cells that survived CD3 antibody mediated depletion (Baas et al., 2016). In this study, the graft-infiltrating CD8 T cells co-expressed PD-1, PD-L1 and produced TGF- $\beta$  and neutralizing either of these molecules abrogated anti-CD3 antibody-induced tolerance. It would be interesting to examine if similar mechanisms exist to maintain tolerance by DST/anti-CD40 costimulation blockade. Moreover, to test if the alloreactive CD8 T cells surviving DST/anti-CD40 mediated deletion are functionally competent, these cells can be sorted from tolerant mice and tested for their ability to reject established allografts in Rag<sup>-/-</sup> mice. Another interesting avenue would be to test the requirement of myeloid suppressor cells in DST/anti-CD40 mediated tolerance induction,

although the lack of specific depleting agents for these populations will confound interpretations of data from these experiments.

Memory T cells present a formidable barrier to long-term graft survival in clinical transplantation. Since memory T cells are resistant to tolerization by DST/anti-CD154 treatment, it is reasonable to expect that they will be resistant to DST/anti-CD40 treatment. Combining OX40 blockade with DST/anti-CD40 might overcome the barrier imposed by memory T cells generated by homeostatic proliferation or previous priming (Vu et al., 2006).

In Chapter III, I found that loss of Bim made mice partially resistant to tolerance induction by DST/anti-CD154. Combined deficiency of Fas and Bim completely abrogated tolerance induction. Donor-reactive CD4 and CD8 T cells and B cells are deleted in the context of DST/anti-CD154 costimulation blockade. TUNEL or annexin V staining on cells in the skin draining lymph nodes should reveal which of the cell subsets was rescued from death by Fas/Bim deficiency. Fas and Bim double deficient mice have elevated numbers of T and B cells as well as macrophages and dendritic cells (Hutcheson et al., 2008). Hence the precursor frequency of T and B lymphocytes capable of mediating graft rejection would be higher in these mice than in normal mice. Since the feasibility of tolerizing cells is inversely proportional to their frequency, *Bcl2l11<sup>-/-</sup>Fas<sup>lpr/lpr</sup>* mice have an inherent limitation to tolerization. Downregulating Fas and Bim mediated apoptotic signaling in normal mice with Fas ligand decoy receptors (Connolly et al., 2001) or small molecule inhibitors or micro-RNA (Qian et al., 2011) at the



time of transplantation would be an approach to overcome the limitation of abnormally high lymphocyte counts in *Bcl2l11<sup>-/-</sup>Fas<sup>lpr/lpr</sup>* mice. My data suggest that in the absence of Bim, apoptotic signaling through Fas (or other apoptotic proteins) is not sufficient to delete alloreactive cells and promote long-term graft acceptance by DST/anti-CD154 costimulation blockade. It would be interesting to test the requirement of Bim in a minor antigen mismatch model such as transplanting male (H-Y expressing) skin on female mice. In this case, the minor antigen will be indirectly presented by host APCs. Alloreactive T cells with indirect specificity are present at significantly lower frequencies than direct specificity alloreactive T cells. It is possible that Fas-mediated apoptosis can effectively delete this smaller pool of alloreactive T cells in the absence of Bim and hence Bim deficient female mice exhibit prolonged survival of male grafts in response to DST/anti-CD154 costimulation blockade.

In Chapter IV, I found that TLR agonists LPS and poly(I:C), that signal through different pathways, differentially regulate CD8 T cell responses to MHC mismatched splenocytes (DST) (Fig. 6.1).

Fig. 6.1. Model

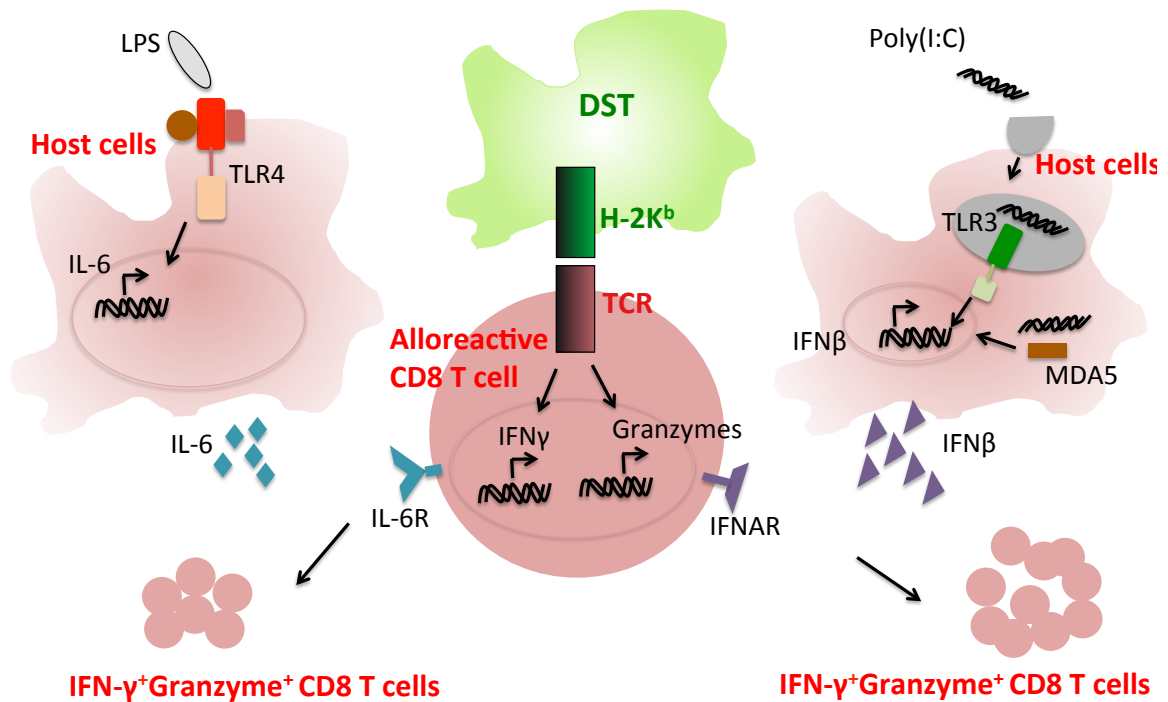


Fig. 6.1. Model

LPS signals through TLR4 on recipient cells and stimulates production of IL-6. IL-6 inhibits the generation of IFN- $\gamma$  and granzyme producing alloreactive CD8 T cells in response to DST. Poly(I:C) signals through TLR3/MDA5 and promotes generation of cytokine producing cytotoxic effector CD8 T cells in a type I interferon dependent manner.

My skin-grafting experiments in Chapter II revealed that DST in the absence of CD40/CD154 blockade was not tolerogenic and the grafts were rapidly rejected. Moreover, my studies in Chapter IV illustrated that DST could induce an antigen-specific CD8 T cell response characterized by proliferation of cells and IFN- $\gamma$  production and cytotoxicity of targets. However, CD8 T cell responses to a skin graft will vary from CD8 T cell responses to DST since DST is systemically administered whereas skin transplantation is local (Chai et al., 2015). I found that LPS reduced the frequency of IFN- $\gamma$  producing alloreactive CD8 T cells and their cytotoxic ability. Future studies should test if LPS-mediated inhibition of alloreactive CD8 T cell function translates to delaying skin graft rejection if LPS is administered at the time of transplantation. It is possible that LPS delays graft rejection when the mismatch is in a minor, but not a major histocompatibility antigen.

I focused my studies on primary effector responses of alloreactive CD8 T cells. LPS challenge generated higher levels of CD44<sup>hi</sup> memory-like alloreactive CD8 T cells relative to poly(I:C). This finding poses several questions: Do LPS and poly(I:C) differentially regulate the proportion of KLRG1<sup>hi</sup>IL-7R $\alpha$ <sup>lo</sup> short-lived effector cells (SLECs) and KLRG1<sup>lo</sup>IL-7R $\alpha$ <sup>hi</sup> memory precursor effector cells (MPECs)? Are the gene expression profiles of fate determining transcription factors such as T-bet, Blimp-1, Bcl-6, Id2, Id3 different in CD8 T cells from mice treated with the two TLR agonists? Understanding how LPS and poly(I:C) regulate recall responses would be of interest and relevant in the context of

inflammation in the transplant recipient resulting from infectious or non-infectious sources prior to receiving the graft. To test memory responses, KB5 chimeric mice primed with DST and LPS or poly(I:C) can be challenged with a second dose of DST 4 weeks after the initial treatment and expansion of DES<sup>+</sup> CD8 T cells monitored in response to the second challenge.

Poly(I:C) challenge has been shown to promote the generation of highly cytotoxic CD8 T cells. I observed higher frequencies of granzyme B producing and IFN- $\gamma$  producing effector alloreactive CD8 T cells in poly(I:C) treated mice. Is type I interferon generated upon poly(I:C) challenge necessary and sufficient to potentiate the CD8 T cell response? If it is so, then is direct type I interferon signaling into CD8 T cells sufficient to enhance the CD8 T cell effector function? Our lab has previously demonstrated that poly(I:C) can abrogate DST/anti-CD154 mediated tolerance induction by promoting alloreactive effector CD8 T cell responses in a type I interferon dependent and TLR3 independent manner (Thornley et al., 2007a). Does poly(I:C) then require the cytosolic MDA5 receptor to enhance the alloreactive CD8 T cell response? Mice with genetic deficiency of type I interferon receptor, MDA5, TLR3 would be required to answer these questions. The CBA background of KB5 mice is not amenable to generating knockouts limiting their usefulness. Instead 2C TCR transgenic mice on the C57BL/6 background with allospecificity for H-2L<sup>d</sup> would facilitate conducting these studies.

Inhibition of alloreactive CD8 T cell function by LPS is an interesting finding. Future experiments should test if TLR4 activation by infection with Gram negative bacteria such as *Salmonella typhimurium* induces a similar response as LPS challenge. My experiments demonstrated a requirement for IL-6 in LPS mediated suppression of CD8 T cell function. TLR2 activation is known to generate high levels of IL-6 (Skabytska et al., 2014) and no type I interferon. I hypothesize that the relative amounts of IL-6 and type I interferons determine the impact of TLR stimulation on CD8 T cell function. I would thus speculate that challenge with TLR2 agonists would suppress CD8 T cell function, perhaps more than LPS. Also, neutralizing IL-6 in poly(I:C) treated mice should further enhance CD8 T cell function.

IL-6 was necessary, but not sufficient to inhibit CD8 T cell function. Future studies should address the involvement of other cytokines that might be acting in concert with IL-6 to regulate alloreactive effector CD8 T cell function. Multiplex cytokine assays would reveal differences in cytokines not investigated in this study. The finding that IL-6 can suppress CD8 T cell function brings to the forefront the pleiotropic nature of this cytokine. 'Classical' IL-6 signaling occurs in cells that express membrane-bound IL-6R subunit along with gp130. IL-6 can bind soluble IL-6R $\alpha$  and signal into gp130 expressing cells in a process termed 'trans signaling'. The signaling subunit gp130 is shared with membrane-bound receptors for other cytokines such as IL-11, IL-27, LIF (leukemia inhibitory factor), CNTF (ciliary neurotrophic factor), CLC (cardiotrophin-like cytokine), CT-

1 (cardiotrophin-1) and OSM (oncostatin M) (Jones et al., 2011). The anti-IL-6R antibody tocilizumab for rheumatoid arthritis inhibits both modes of signaling (Kim et al., 2015). The general consensus in the IL-6 field appears to be that classical signaling regulates homeostatic or regenerative properties of this cytokine whereas trans-signaling drives its pro-inflammatory activities (Hunter and Jones, 2015). A recent study showed that a subset of naïve CD8 T cells expresses membrane-bound IL-6R $\alpha$  and in vitro TCR activation in the presence of exogenous IL-6 differentiates CD8 T cells to effector cells that produce less IFN- $\gamma$  and more IL-21 (Yang et al., 2016). This in vitro study suggests that IL-6 mediated suppression of IFN- $\gamma$  production by CD8 T cells is a result of classical IL-6 signaling. However, both modes of IL-6 signaling will occur in vivo in naïve CD8 T cells because of the presence of soluble IL-6R $\alpha$  (sIL-6R $\alpha$ ) in addition to the membrane-bound form. To determine which of the modes of IL-6 signaling is responsible for suppressing CD8 T cell function in vivo, reagents that selectively block classical vs trans-signaling should be used. A natural soluble form of gp130 (sgp130) binds the IL-6/sIL-6R $\alpha$  complex and selectively inhibits trans-signaling. Recombinant sgp130-Fc fusion protein has been used in several studies to delineate the role of trans-signaling in inflammation (Jones et al., 2011). Likewise, antibodies selectively targeting sIL-6R $\alpha$  have been generated (Lissilaa et al., 2010). Another interesting finding by Yang et al. is that TCR activation downregulates IL-6R $\alpha$  expression on CD8 T cell surface within 24 hours. In an in vivo context this would mean that CD8 T cells would lose their responsiveness to

classical signaling. This raises an interesting question: if IL-6 is encountered during the effector phase of the CD8 T cell response, will it affect CD8 T cell function and in what manner?

The finding that TLR4 induced IL-6 can suppress CD8 T cell function has vast implications. TLR4 is expressed on tumors from a wide variety of tissues (Huang et al., 2005). Endogenous TLR4 ligands released by tumor cells (Huang et al., 2008) may activate TLR4 signaling triggering secretion of IL-6 by tumors. Indeed, IL-6 is a component of microenvironments of many tumors. Also STAT3 is found to be constitutively activated in tumors and tumor infiltrating immune cells and contributes to immunosuppression (Yu et al., 2007). It is possible that IL-6 signaling into tumor infiltrating CD8 T cells inhibits their function contributing to immune evasion by the tumor. TLR4 signaling is thus a double-edged sword. While TLR4 agonists such as MPLA (monophosphoryl lipid A) are used as vaccine adjuvants because of their ability to enhance costimulation and cytokine production, my data adds to the body of literature showing that TLR4 signaling can be immunosuppressive.

In Chapter V, I found that transgenic expression of human SCF, GM-CSF and IL-3 in humanized NSG mice improved B cell maturation and facilitated generation of dengue antigen-specific antibody responses. Future studies should investigate the mechanisms by which these cytokines promote B cell maturation. Plasma levels of the B cell maturation factor BAFF in these mice should be evaluated. NSG-SGM3 BLT mice can serve as a tool to study humoral

responses to human-specific viruses. In the future, supplementation of humanized mice with human 'survival cytokines' like BAFF and IL-7 by hydrodynamic injection or transgene expression might be a positive step in resolving the issue of impaired B cell development and function in humanized mice.



## **CHAPTER VII**

### **References**

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