

THY-1 SIGNALING IN T CELLS IS WEAKER AND HAS DELAYED SIGNALING
KINETICS, PROMOTES DELAYED ACQUISITION AND TRIGGERING OF
CYTOTOXIC EFFECTOR FUNCTION, AND PREFERENTIALLY PROMOTES IL-17A
AND IL-4 PRODUCTION IN COMPARISON TO TCR SIGNALING

by

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Submitted in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
April 2011

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DALHOUSIE UNIVERSITY

DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY

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DALHOUSIE UNIVERSITY

DATE: April 25, 2011

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TITLE: THY-1 SIGNALING IN T CELLS IS WEAKER AND HAS DELAYED SIGNALING KINETICS, PROMOTES DELAYED ACQUISITION AND TRIGGERING OF CYTOTOXIC EFFECTOR FUNCTION, AND PREFERENTIALLY PROMOTES IL-17A AND IL-4 PRODUCTION IN COMPARISON TO TCR SIGNALING

DEPARTMENT OR SCHOOL: Department of Microbiology and Immunology

DEGREE: PhD CONVOCATION: October YEAR: 2011

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ABSTRACT

Thy-1 is a glycosylphosphatidylinositol-anchored protein that is expressed on murine T lymphocytes and is involved in T cell-mediated immune responses. In the presence of costimulatory signals, monoclonal antibody (mAb)-induced signaling through Thy-1 is associated with hallmarks of T cell activation, including IL-2 production and T cell proliferation. Thy-1-induced signaling promotes cytotoxic effector molecule expression, but is unable to trigger delivery of the lethal hit to target cells, suggesting that Thy-1 provides an incomplete T cell receptor (TcR)-like signal. However, the effect of Thy-1 signaling on cytokine production and the development of T helper (Th) cell phenotypes (Th1, Th2, Th17) remains unclear. The purpose of this work was to further our understanding of Thy-1-mediated signal transduction and the role that Thy-1 plays in the development of effector T cell responses. I found that, in the context of costimulatory signals, anti-Thy-1 mAb induced significantly less IL-2 production, CD25 expression and T cell proliferation than anti-TcR β mAb. Several key signaling molecules, including protein tyrosine kinases, zeta chain-associated protein-70 and extracellular signal-regulated kinase were activated with delayed kinetics during Thy-1-mediated T cell activation. The delayed signaling kinetics resulted in the delayed acquisition of cytotoxic effector function and also delayed delivery of the lethal hit to target cells. Interestingly, Thy-1-mediated signaling induced significantly more IL-17 and IL-4 synthesis and less IFN- γ synthesis in comparison to TcR-mediated signaling. Moreover, Thy-1-activated CD4⁺ T cells produced high levels of IL-17 and IL-4 but minimal IFN γ when restimulated with anti-Thy-1 mAb or anti-TcR β mAb with or without costimulatory signals. The unique ability of Thy-1 signaling to induce IL-17 production correlated with the expression of the Th17 lineage-specific transcription factor, retinoic orphan receptor gamma t. These observations show that Thy-1 signaling differs from TcR signaling in its ability to induce Th cell cytokines. Taken together, my findings show that Thy-1 signaling can provide the full TcR-like signal required for both the differentiation and triggering of Th cells and cytotoxic T lymphocytes, albeit with delayed kinetics in comparison to TcR signaling. They also suggest that Thy-1 signaling may be important in the development of Th2 and Th17 responses.

LIST OF ABBREVIATIONS AND SYMBOLS USED

α	Alpha
ACT	A daptor Protein NF- B Activator
Ab	A ntibody
ADAP	A dhesion and D egranulation-promoting A daptor P rotein
ADCC	A ntibody- D ependent C ell-Mediated C ytotoxicity
Ag	A ntigen
AICD	A ctivation-Induced C ell D eath
AP-1	A ctivator P rotein-1
APC	A ntigen P resenting C ell
APS	A mmonium P ersulfate
Asn	A sparagine
ATCC	A merican T ype C ulture C ollection
β	Beta
Bid	B cl-2 interacting d omain death agonist
BM	B one M arrow
BMDC	B one M arrow-derived D endritic C ells
BSA	B ovine S erum A lbumin
Ca ²⁺	C alcium
cAMP	C yclic A denosine M onophosphate
CBP	C sk B inding P rotein
CD	C luster of D ifferentiation
cDC	C onventional D endritic C ell

CDK	Cyclin-Dependent Kinase
CFSE	Carboxyfluorescein Diacetate, Succinimidyl Ester
CIA	Collagen-Induced Arthritis
CLR	C-type Lectin Receptors
ConA	Concanavalin A
cSMAC	Central Supramolecular Activation Complex
cRPMI	Complete Roswell Park Memorial Institute 1640 Medium
CTL	Cytotoxic T Lymphocyte
CTLA	Cytotoxic T Lymphocyte Antigen
δ	Delta
DAG	Diacylglycerol
DC	Dendritic Cell
DD	Death Domain
DEPC	Diethylpyrocarbonate
DISC	Death-Inducing Signaling Complex
DN	Double Negative
dNTP	Deoxynucleotide Triphosphate
DP	Double Positive
DR	Death Receptor
DRM	Detergent-resistant Membrane
dSMAC	Distal Supramolecular Activation Cluster
DTH	Delayed-type Hypersensitivity
DTT	Dithiothreitol
ε	Epsilon

EAE	Experimental Autoimmune Encephalomyelitis
EAM	Experimental Autoimmune Myocarditis
EAU	Experimental Autoimmune Uvitis
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene Glycol Tetraacetic Acid
ELISA	Enzyme-Linked Immunosorbant Assay
ER	Endoplasmic Reticulum
ERK	Extracellular Signal-Regulated Kinase
ETP	Early Thymic Progenitors
FACS	Fluorescence-Activated Cell Sorting
FADD	Fas-associated Death Domain
Fas	FS-7 Cell-associated Cell Surface Antigen
FasL	Fas Ligand
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate
γ	Gamma
Gads	Grb-2-related adaptor downstream of Shc
GATA-3	GATA-binding protein-3
G-CSF	Granulocyte Colony Stimulating Factor
GEF	Guanine Exchange Factor
GI	Gastrointestinal
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
GPI	Glycosylphosphatidylinositol

GPI-AP	GPI-anchored Protein
GRAIL	Gene-related to Anergy in Lymphocytes
Grb-2	Growth Factor Receptor-bound Protein 2
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish Peroxidase
HSC	Hematopoietic Stem Cell
IAP	Inhibitor of Apoptosis
IBD	Inflammatory Bowel Disease
ICAM	Intercellular Adhesion Molecule
ICOS	Inducible Costimulatory Molecules
IDDM	Insulin-Dependent Diabetes Mellitus
IFN	Interferon
IFN γ R	IFNγ Receptor
Ig	Immunoglobulin
IGD	Inheritable GPI Deficiency
IgSF	Ig Gene Superfamily
I κ B	Inhibitor of NFκB
IL	Interleukin
IL-12R	IL-12 Receptor
IL-17R	IL-17 Receptor
IL-18R	IL-18 Receptor
IL-23R	IL-23 Receptor
IL-4R	IL-4 Receptor
IP ₃	Inositol Triphosphate

IP-10	IFN-inducible Protein 10
IPEX	Immune Dysregulation, Polyendocrinopathy, Entropathy X-linked Syndrome
IRF	IFN Regulatory Factor
ITAM	Immunoreceptor Tyrosine-based Activation Motif
iTreg	Inducible Treg Cell
Itk	IL-2 induced tyr kinase
Jak	Janus Kinase
JNK	c- Jun Kinase
κ	Kappa
LAT	Linker for Activation of T cells
Lck	Leukocyte-Specific Tyrosine Kinase
LFA-1	Leukocyte Function Associated Antigen-1
LN	Lymph Node
lpr	Lymphoproliferation
LPS	Lipopolysaccharide
μ	Micro
mAb	Monoclonal Antibody
MAPK	Mitogen-Activated Protein Kinase
MCF	Mean Channel Fluorescence
MCP-1	Monocyte Chemoattractant Protein-1
Met	Methionine
MHC	Major Histocompatibility Complex
MHC I	MHC Class I

MHC II	MHC Class II
MIG	Monokine Induced by IFN γ
MIP	Macrophage Inflammatory P rotein
M-MLV	Moloney-Murine Leukemia V irus
MMP	Matrix Metallo proteinases
mRNA	messenger R ibonucleic A cid
MS	Multiple Sclerosis
MTOC	Microtubule Organizing C omplex
NaCl	Sodium Chloride
NFAT	Nuclear Factor of A ctivated T cells
NF κ B	Nuclear Factor κ B
nTreg	Natural Treg Cell
PAGE	Polyacrylamide G el E lectrophoresis
PAMPS	Pathogen-Associated M olecluar P atterns
pAPC	Professional A ntigen P resenting C ell
PBS	Phosphate B uffered S aline
pDC	Plasmacytoid D endritic C ell
PDK	Phosphoinositide-dependent Protein K inase 1
PE	Phycoer thrin
PH	Pleckstrin H omology
PI	Phosphatidylinositol
PI-3K	Phosphatidylinositol-3 K inase
PIG-A	Phosphatidylinositol G lycan Anchor Biosynthesis, class A
PIP ₂	Phosphatidylinositol 4, 5-bis p hosphate

PI(4,5)P ₂	Phosphatidylinositol 4,5-bisphosphate
PI(3,4,5)P ₃	Phosphatidylinositol 3,4,5-bisphosphate
PI(3,4)P ₂	Phosphatidylinositol 3,4-bisphosphate
PI-PLC	Phosphatidylinositol-specific Phospholipase C
PKC	Protein Kinase C
PLC	Phospholipase C
PM	Plasma Membrane
PMA	Phorbol 12-myristate 13-acetate
pMHC	Peptide-Major Histocompatibility Complex
PNH	Paroxysmal Nocturnal Hemoglobinuria
PRR	Pattern Recognition Receptors
PrPc	Cellular Prion Protein
PS	Phosphatidylserine
pSMAC	Peripheral Supramolecular Activation Cluster
PTEN	Phosphatase and Tensin Homolog Deleted on Chromosome 10
PTK	Protein Tyrosine Kinase
RA	Rheumatoid Arthritis
RANKL	Receptor Activator of NFκB Ligand
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
RAMB	Rabbit Anti-mouse Brain
rh	Recombinant Human
RNA	Ribonucleic Acid
RNASE	Ribonuclease
RIG-I	Retinoic Acid-inducible Gene I

RIPA	R adioimmunoprecipitation
RLH	R IG-I-like H elicases
RNI	R eactive N itrogen I ntermediates
ROR	R etinoid-related O rphan R eceptor
RPMI	R oswell P ark M emorial I nstitute
rm	R ecombinant M ouse
ROS	R eactive O xygen S pecies
RT	R everse T ranscriptase
Runx	R unt-related T ranscription F actor
SCF	S tem C ell F actor
SDS	S odium D odecyl S ulfate
SEFIR	S EF/ I L-17 R
sh	S hort- h airpin
SH2	S rc H omology 2
SLP-76	S H2-domain C ontaining L eukocyte P rotein of 76 kDa
SOS	S on of S evenless
STAT	S ignal T ransducer and A ctivator of T ranscription
θ	T heta
TAE	T ris- A cetate- E DTA
TAK	T GF- β -activated K inase
T-bet	T -box E xpressed in T C ells
tBID	t runcated B cl-2 interacting d omain death agonist
TBS	T ris- B uffered S aline
TBST	T ris- B uffered S aline- T ween 20

TcR	T Cell Receptor
TEC	Thymic Epithelial Cell
TEMED	N,N,N',N'-tetramethylethylenediamine
Tfh	T follicular Helper Cells
TGF	Transforming Growth Factor
Th	T helper
TIM	T cell Ig and Mucin
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
TRAF	TNF Receptor-associated Factor
TNFR	TNF Receptor
Treg	T regulatory
TSP	Thymic Settling Precursor
TSLP	Thymic Stromal Lymphopoietin
TSLPR	TSLP Receptor
Tyr	Tyrosine
VCAM	Vascular Cellular Adhesion Molecule
WASp	Wiskott-Aldrich Syndrome protein
ζ	Zeta
ZAP-70	Zeta Chain-associated Protein 70 kDa

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor, Dr. David Hoskin for his endless patience, encouragement, mentorship and support for the past seven years since I started in his lab as an honors student. I greatly appreciate the fact that you are easily approachable and always available to answer questions, but also that you provide a great deal of freedom for independent thinking. I also would like to express my sincere gratitude to the members of my advisory committee, Dr. Brent Johnston, Dr. Robert Liwski and Dr. Jean F Legare for providing invaluable guidance and support. I would like to thank Julie Long, Derek Minney, Matt Tunis and Trina Racine for your invaluable technical assistance. My research has greatly benefited from the use of reagents and equipment from the labs of Dr. Jean Marshall, Dr. Kenneth West, Dr. Rob Liwski, Dr. Brent Johnston and Dr. Craig McCormick.

I would like to express my sincere thanks to the wonderful group of people that I have worked with in the Hoskin lab over the many years. Each and every one of you made working in the Hoskin lab an interesting and very enjoyable experience. In particular, I would like to thank Dr. David Conrad for not only your endless advice and support, but also your mad editing skills, great humor and friendship. I would also like to thank Dr. Melanie Power-Combs also for taking the time to read and edit my thesis.

Finally, I would like to thank my family and for their endless support through the many trials and tribulations, tears and celebrations that have brought me to this stage in my life. Daniel, I could not have gotten through these past several years without your support and encouragement.

Chapter 1. Introduction

1.1 Overview of T cells

The vertebrate immune system is a complex defense system that surveys for and protects against invading pathogens and cancer. It is classically divided into two distinct arms, innate and adaptive immunity, which are elegantly intertwined (1). The innate immune system is the first line of defense and involves physical and physiological barriers (e.g. skin, mucosal barriers, temperature and pH), recruitment and activation of phagocytic cells (e.g. neutrophils and macrophages) and the production of soluble mediators of inflammation (e.g. complement, acute-phase proteins, cytokines and chemokines) (2). Innate immune responses are rapidly invoked upon recognition of conserved molecular patterns that are common to different groups of pathogenic organisms, known as pathogen-associated molecular patterns (PAMPs), which distinguish microorganisms from the host. In contrast, the adaptive immune system is highly specific, targeting specific pathogens that activate it via PAMPs (3). Upon primary challenge with a pathogen adaptive immune responses are slow to react. Unlike the innate immune system, however, the adaptive immune system provides long-lasting specific immunity against a particular pathogen due to the induction of immunological memory. Each subsequent encounter with that same pathogen activates the adaptive immune response more rapidly and robustly.

The innate immune system is responsible for both activating and modulating the nature of adaptive immune responses. Dendritic cells (DCs) are professional antigen presenting cells (pAPCs) of the innate immune system that play a central role in the recognition of pathogens and the activation of adaptive immune responses (4). DCs express numerous cell surface receptors, known as pattern recognition receptors (PRRs), which distinguish between different types of pathogens through the recognition of PAMPs. Upon recognition of a pathogen, DCs phagocytose the microorganism and process the protein into antigenic peptides. They then migrate to lymphoid tissues and present the antigenic peptides to T cells of the adaptive immune system, resulting in T cell activation.

Depending upon the nature of the pathogen, activated DCs produce distinct cytokines and chemokines that influence the nature of the ensuing adaptive immune response.

T cells are at the heart of adaptive immune responses. T cells are lymphocytes that develop in the thymus and express antigen (Ag) receptors on their surface that enable them to recognize specific Ags. Each T cell is unique in that it expresses only one type of T cell receptor (TcR) that recognizes a distinct portion of antigen, known as an epitope. Individual groups of T cells that express a TcR with the exact same epitope specificities are known as clones. Somatic recombination of the genes encoding the TcR during maturation results in an enormously diverse TcR repertoire, with any individual expressing at least 10^9 different T cell clones (5). This not only enables high specificity of adaptive immune responses, but also allows responsiveness to a large variety of foreign Ags.

The majority of T cells express a TcR that is a heterodimer composed of two transmembrane polypeptide chains designated alpha (α) and beta (β). $\alpha\beta$ T cells typically recognize antigenic peptide displayed by pAPCs in the cleft of major histocompatibility complex (MHC) molecules. A small subset of T cells, however, express a TcR composed of gamma (γ) and delta (δ) chains. While the key role of $\alpha\beta$ T cells in adaptive immune responses is well established, the role that $\gamma\delta$ T cells play in immune responses is just starting to be elucidated. The observations that $\gamma\delta$ T cells express a limited repertoire of TcRs that typically recognize conserved non-peptide antigen in the absence of classical MHC presentation, are highly localized to epithelial surfaces and acquire a pre-activated memory cell-like status early in development (6), suggests that $\gamma\delta$ T cells are poised to provide a first line of defense. $\gamma\delta$ T cells are therefore considered to be innate immune cells.

Conventional $\alpha\beta$ T cells can be divided into three major subsets based on their effector functions. Cytotoxic T lymphocytes (CTLs) typically express CD8 and recognize antigenic peptide displayed in MHC class I (MHC I) molecules. CTLs directly target and destroy host cells that are either infected with

intracellular pathogens or that are cancerous. In contrast, T helper (Th) cells express CD4 and recognize antigenic peptide displayed in MHC class II (MHC II) molecules. Th cells regulate the nature of the immune response by expressing distinct cytokine profiles that shape both the innate and adaptive response.

Although the immune system is responsible for the elimination of harmful pathogens and protection against disease, the immune response can have detrimental consequences to the host. An immune response that is too robust, erroneously activated or directed toward self-antigen can cause extensive damage to host tissue and autoimmune disease. Consequently, the immune system has evolved control mechanisms that maintain the fine balance between effective and detrimental immune responses. In recent years, a third type of effector T cell, designated the T regulatory (Treg) cell, has emerged as a key player in controlling immune responses. Treg cells typically express CD4 and are crucial to controlling the strength of immune responses to invading pathogens and suppressing autoimmune reactions (7).

1.2 T Cell Activation

T cell activation is an important immune process that is highly regulated to ensure that T cells only mediate their destructive effector functions against pathogens and cancer cells. Mature T cells that leave the thymus are considered to be naïve as they have not yet interacted with foreign Ag and do not express effector molecules. Naïve T cells become activated and differentiate into effector T cells, which are armed with the molecules necessary to elicit T cell-mediated immune responses, only upon receiving at least two distinct signals resulting from the interaction with a pAPC within secondary lymphoid tissues.

1.2.1 Signal 1

In order for a T cell to be activated, the TcR must first recognize cognate antigenic peptide presented in self-MHC (pMHC) by a pAPC. Since thymic education eliminates T cells that recognize self-Ag with high affinity, T cells effectively distinguish between non-self and self and should respond only to non-self. The signal transduction pathway that is triggered by TcR ligation, designated

signal 1, involves the activation of a complex signaling cascade that modulates the expression of genes necessary for clonal expansion and effector function.

1.2.1.1 Membrane Proximal Events

The cytoplasmic tails of α and β TcR chains are very short and have no intrinsic enzymatic activity. Consequently, the TcR forms a multi-subunit signaling complex through non-covalent interactions with TcR zeta (ζ) chain homodimers and CD3 epsilon (ϵ) γ and $\epsilon\delta$ heterodimers (8) (Figure 1.1). The cytoplasmic tails of the TcR ζ and CD3 chains contain immunoreceptor tyrosine-based activation motifs (ITAMs), which become phosphorylated at tyrosine (Tyr) residues during TcR signaling (9). Recognition of agonist pMHC by the TcR results in the activation of the Src family protein tyrosine kinase (PTK) leukocyte-specific tyrosine kinase (Lck), which phosphorylates three ITAMs on each TcR ζ chain (10). Lck is thought to be activated upon removal of an inhibitory phosphate group from Tyr₅₀₅ by the CD45 phosphatase followed by autophosphorylation of activating Tyr₃₉₄ (11, 12). Constitutive association of Lck with the cytoplasmic portion of the co-receptors CD4 and CD8 enables Lck to be brought into close proximity of the TcR ζ chain ITAMs upon binding of these coreceptors to extracellular invariant motifs on the MHC molecules during TcR signaling (13). The phosphorylation of ITAMs by Lck is a crucial event during T cell activation since the phosphorylated Tyr residues provide docking sites for the PTK ζ chain-associated protein 70 kDa (ZAP-70) (10). Upon docking to TcR ζ chain ITAMs, ZAP-70 is phosphorylated on Tyr residues by Lck, resulting in the activation of ZAP-70 catalytic activity (14). ZAP-70 is further activated by the autophosphorylation of additional Tyr residues within the activation loop of the ZAP-70 kinase domain, which further enhances ZAP-70 activity. Active ZAP-70 initiates multiple downstream signaling pathways that are important in T cell activation.

Although currently an active area of research, the exact mechanism by which engagement of TcRs by pMHC on the outside of the cell triggers TcR signaling events inside the cell remains unclear. It has long been believed that TcR crosslinking by multimeric pMHC, resulting in TcR aggregation, was the

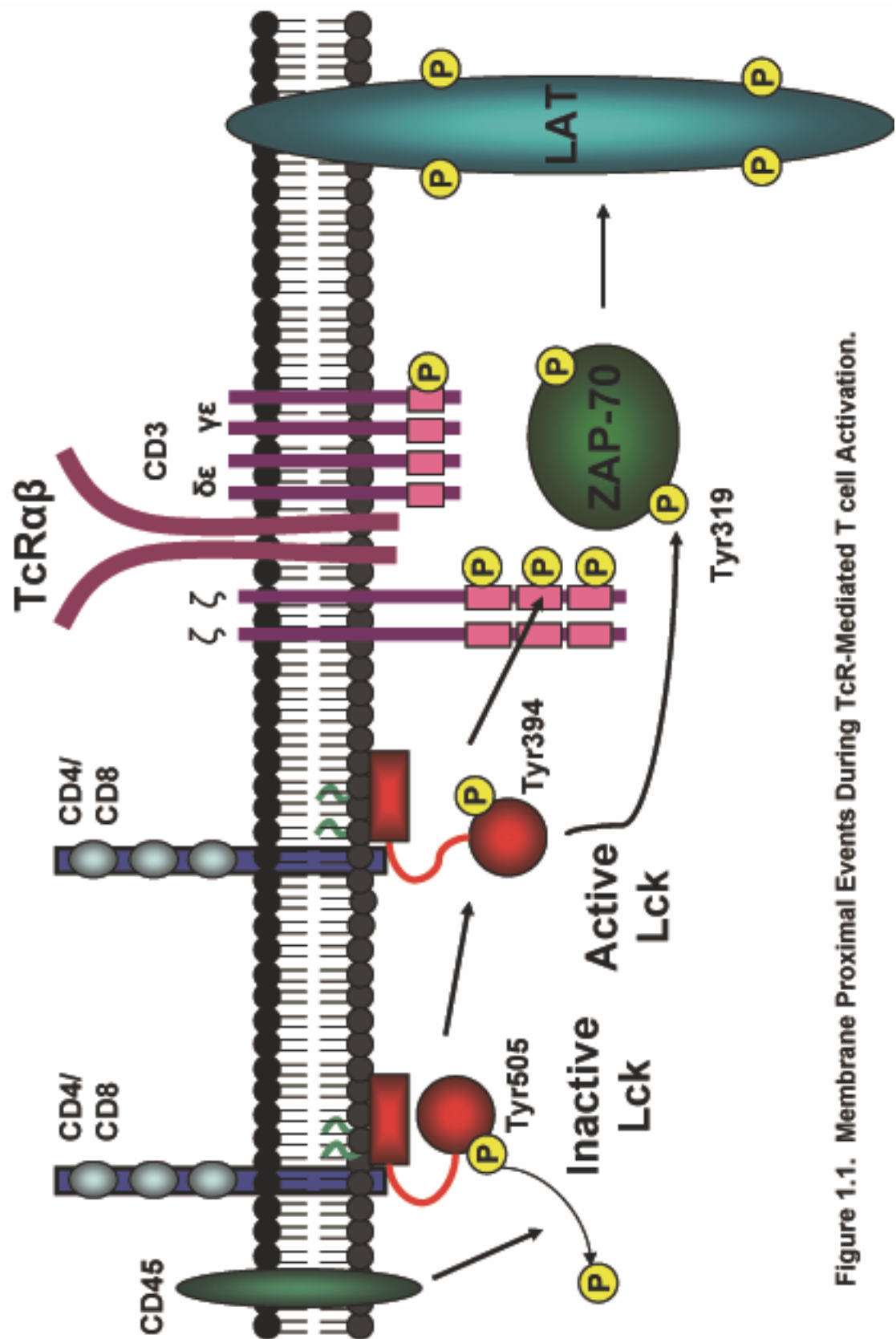


Figure 1.1. Membrane Proximal Events During TcR-Mediated T cell Activation.

initial event during T cell activation. This model was supported by the observations that crosslinking of the TcR complex with monoclonal antibodies (mAb) or soluble pMHC tetramers induces TcR signaling pathways (15). Recent studies, however, question the relevancy of receptor aggregation as the earliest event during T cell activation (16). Importantly, agonist pMHC is present at very low surface densities under physiological conditions, making it very unlikely that two agonist pMHC would be in close enough in proximity to promote TcR aggregation. In fact, as few as ten MHC molecules displaying agonist peptide on the surface of an APC are required to initiate T cell activation (17). Additionally, interaction of a single pMHC molecule with a TcR results in transient signaling, suggesting TcR aggregation is not necessary to initiate TcR signal transduction. Nevertheless, TcR clustering is important during T cell activation (18). TcRs form clusters rapidly after TcR triggering (19). In addition to TcRs, these clusters are enriched in numerous signaling molecules involved in TcR signal transduction, including activated Ick and Zap-70, both of which are required for sustained signaling.

It has also been postulated that the binding of the CD4 or CD8 co-receptors to invariant regions of the MHC during the TcR/pMHC interaction, which results in Ick being moved into close proximity of the ITAMs, is the initial signaling event in TcR activation (20). This co-receptor heterodimerization, although important to TcR activation, is unlikely to be the initiating event since TcR signaling can occur in the absence of CD4 and CD8. Interestingly, several studies have suggested that the pMHC-TcR interaction may trigger a conformational change in the TcR signaling complex. Recently, Xu *et al.* (21) provided evidence that ITAMs are hidden within the inner leaflet of the plasma membrane under resting conditions due to electrostatic interactions with acidic lipids. These findings suggest that a conformational change may be required for ITAMs to be revealed and phosphorylated during TcR activation. Moreover, Gil *et al.* (22) showed that the interaction of pMHC with TcR results in a conformation change within CD3 ϵ , which exposes a proline-rich region within the cytoplasmic tail. Consequently, the adaptor protein Nck was recruited to CD3 ϵ . The relevance of Nck signaling during

TcR activation, however, remains controversial (23). Although not definitive, these findings provide evidence that a conformational change could be the initiating event during T cell activation.

1.2.1.2 Downstream Signaling Pathways

Active ZAP-70 phosphorylates Tyr residues on two adaptor proteins that are essential to T cell activation: linker for activation of T cells (LAT) and Src homology 2 (SH2)-domain containing leukocyte protein of 76 kDa (SLP-76) (24, 25) (Figure 1.2). Phosphorylated LAT and SLP-76 nucleate a multimolecular signaling complex through the recruitment of numerous signaling molecules. This complex propagates downstream signaling pathways that promote T cell proliferation and differentiation into effector and memory cells.

LAT is localized to the plasma membrane where it recruits phospholipase C γ 1 (PLC γ 1), the p85 subunit of phosphatidylinositol-3 kinase (PI-3K), growth factor receptor-bound protein 2 (Grb-2), and Grb-2-related adaptor downstream of Shc (Gads) (26-29). Gads recruits the cytosolic adaptor protein SLP-76 to the signaling complex, which is phosphorylated by ZAP-70 at three Tyr residues (25, 30). Additional signaling molecules, including Vav1, Nck and interleukin (IL)-2 induced tyrosine kinase (Itk) are all recruited to phosphorylated SLP-76 via SH2 domains (31, 32).

PLC γ 1 binds to LAT through a SH2 domain and is phosphorylated by ZAP-70 and Itk (33, 34). SLP-76 and Vav1 also directly bind to PLC γ 1 (34). These interactions are thought to stabilize PLC γ 1 in a conformation that has optimal enzymatic activity. Active PLC γ 1 hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP₂) to yield inositol triphosphate (IP₃) and diacylglycerol (DAG), two second messengers that are essential for T cell activation (35). Protein kinase C θ (PKC θ) is activated by DAG and mediates the activation of the transcription factor nuclear factor κ B (NF κ B) (36). DAG also activates the Ras signaling pathway, which initiates a mitogen-activated protein kinase (MAPK) cascade culminating in the activation of extracellular signal-regulated kinase (ERK)1 and ERK2 (37). A major target of the ERK1/2 signaling pathway is Fos,

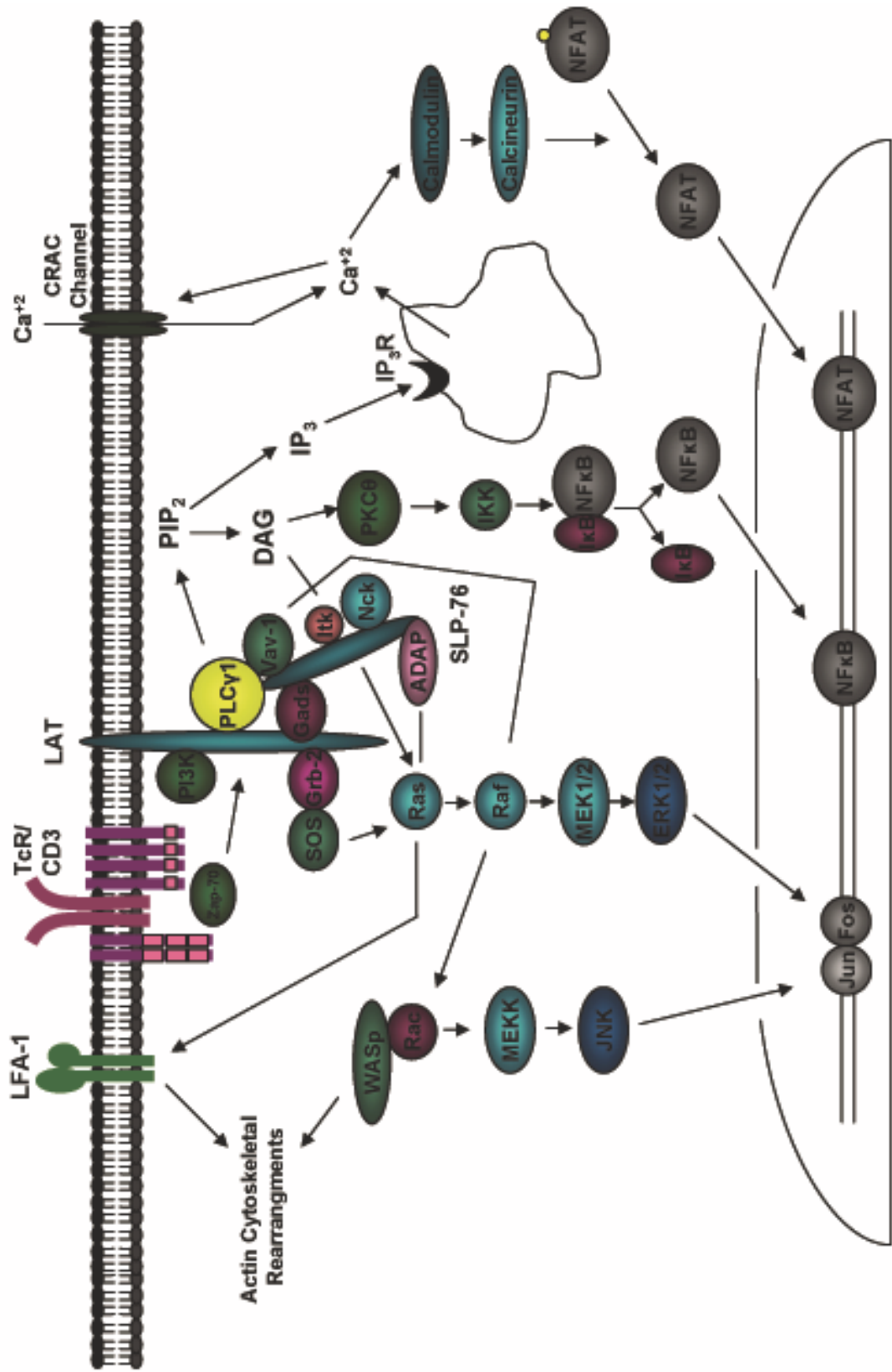


Figure 1.2. Downstream Signaling Events During TcR-Mediated T cell Activation.

which forms a transcription factor complex with c-Jun that is designated activator protein-1 (AP-1) (38, 39). ERK1/2 activation is also mediated by the adaptor protein Grb-2. Grb-2 is an adaptor molecule that also binds to LAT via a SH2 domain (29). Grb-2 recruits the guanine exchange factor (GEF) son of sevenless (SOS) to the LAT signaling complex, where it facilitates the activation of Ras. Meanwhile, the IP₃ generated by PLC γ 1 induces a rise in intracellular calcium (Ca²⁺) that activates the Ca²⁺ sensitive phosphatase, calcineurin (40). Calcineurin dephosphorylates the transcription factor nuclear factor of activated T cells (NFAT), enabling NFAT to translocate to the nucleus and modulate gene transcription.

In addition to new gene expression, cytoskeletal rearrangements and the activation of integrins are also important consequences of TcR signaling. During T cell activation, actin cytoskeletal rearrangements mediate the polarization of TcR signaling complexes to the interface between the T cell and APC (41). Disruption of actin polymerization severely inhibits T cell activation (42). Vav1 is a Rho GTPase-specific GEF that mediates actin remodeling through the activation of nucleation-promoting factors, Wiskott-Aldrich syndrome protein (WASp) and WAVE2 (43-45). TcR triggering also induces inside-out signaling that activates integrins, including leukocyte function-associated antigen-1 (LFA-1), required for cell to cell and cell to matrix adhesion. SLP-76 recruits adhesion and degranulation-promoting protein (ADAP) to the signaling complex, which mediates integrin activation (46, 47). PKC θ also mediates integrin activation through the activation of the serine-threonine protein kinase D1 (48).

1.2.1.3 Transcription Factors

The TcR signaling pathway ultimately leads to the activation of three key transcription factors, NF κ B, NFAT and AP-1, which initiate the expression of genes that are required for T cell proliferation, IL-2 production and differentiation into effector and memory cells.

1.2.2 Costimulation (Signal 2):

Engagement of the TcR alone is not usually sufficient for full T cell activation, which also requires a second signal known as a costimulatory signal.

In fact, TcR triggering in the absence of costimulatory signals typically results in a nonresponsive state known as anergy (49). T cells that are anergic have impaired proliferation and cytokine production upon subsequent exposure to cognate Ag. pAPCs that receive inflammatory signals upregulate a number of different costimulatory ligands that bind costimulatory receptors on the surface of T cells during T activation. In the absence of inflammation, however, APCs that present cognate Ag do not receive the necessary signals to upregulate the expression of costimulatory ligands and are therefore unable to activate T cells. Consequently, the requirement for costimulation is an additional layer of control, which ensures that T cells are only activated in response to dangerous non-self Ag.

The primary mechanism of costimulation is thought to be the amplification of signal 1. This amplification decreases the TcR signaling threshold necessary for T cell activation. As a result, T cells receiving costimulatory signals can respond to minimal doses of Ag. The requirement for the amplification of the TcR signal is evident from the observation that under certain circumstances TcR signaling alone can result in T cell activation (50, 51). In the presence of a sufficiently high Ag dose, enough TcRs are engaged to meet the signaling threshold required for T cell activation (50). Under normal physiological conditions, however, T cells receive signals from a limited number of agonist pMHC, which are unable to effectively activate a T cell, often leading to anergy instead. Costimulatory signals act by reducing the number of triggered TcRs that are required for T cell activation (52).

1.2.2.1 CD28 Signaling

Numerous cell surface receptors have been implicated in providing the costimulatory signals necessary for full T cell activation. CD28 is the best characterized of the costimulatory receptors and is required for optimal activation of naïve T cells (53). CD28 is a homodimeric, transmembrane protein that belongs to the immunoglobulin (Ig) gene superfamily (IgSF). The key role that CD28 plays in providing costimulatory signals during T cell activation is highlighted by studies with mice deficient in CD28, which show impaired proliferation and cytokine production in response to Ag stimulation (53-55).

Consequently, CD28-deficient mice have reduced responses against numerous types of pathogens (54, 56-59) and are resistant to the induction of T cell-mediated autoimmune diseases, including arthritis and experimental allergic encephalomyelitis (EAE), as well as allergic diseases, such as asthma (60-62). Moreover, a loss in CD28 expression by a significant percentage of human T cells correlates with reduced immune function in the elderly (63).

CD28 signaling is initiated upon ligation with the B7 family members, B7-1 (CD80) and B7-2 (CD86). B7-1 and B7-2 are type I transmembrane glycoproteins that belong to the IgSF and can be expressed on pAPCs (64-66). B7-1 is slowly upregulated on dendritic cells in response to a number of activating stimuli, including lipopolysaccharide (LPS) and proinflammatory cytokines (67, 68). In contrast, B7-2 is constitutively expressed and is rapidly upregulated upon activation. CD28-mediated signaling promotes T cell activation by several mechanisms. CD28-signaling promotes high levels of IL-2 production, both by inducing new gene transcription as well as by enhancing mRNA stability (69, 70). Through the upregulation of D cyclin expression and inhibition of cyclin-dependent kinase (CDK) inhibitors KIP1 and INK4C, CD28 signaling also facilitates entry into and progression through the cell cycle (71, 72).

Although the CD28 signaling pathways are far from being completely understood, numerous studies over the last two decades have led to several key findings. In keeping with CD28 providing an amplifying signal during TcR triggering, many of the signaling molecules implicated in CD28 signal transduction are also involved in TcR signaling. In fact, to date no signaling molecules that are unique to the CD28 signaling pathway have been discovered. Consequently, the transcription factors NF κ B, NFAT and AP-1, which are important in mediating T cell proliferation, survival and differentiation during TcR signaling, are all synergistically activated in the presence of CD28 costimulation (73-75).

The CD28 cytoplasmic tail contains at least three different motifs involved in propagating the CD28 costimulatory signal. One motif is a Tyr-methionine (Met)-asparagine (Asn)-met (YMNM) sequence that, like the ITAMs in the TcR

signaling complex, is Tyr phosphorylated by Lck upon cross-linking of the CD28 receptor (76). PI3K and Grb2 bind to the phosphorylated YMNM motif via SH2 domains. PI3K phosphorylates the inositol ring of membrane phosphoinositides, producing PI(4,5)P₂, PI(3,4,5)P₃ and PI(3,4)P₂ that bind the pleckstrin homology (PH) domains of numerous proteins (77). One such protein, phosphoinositide-dependent protein kinase 1 (PDK1), activates the serine/threonine kinase Akt. Activated Akt contributes to the activation of both NFκB and NFAT and is required for optimal IL-2 gene expression as well as enhanced cell survival through signaling for the expression of the anti-apoptotic proteins Bcl-2 and Bcl-XL (36, 78-81). CD28 signaling also prevents activation-induced cell death (AICD) by Akt pathway-mediated inhibition of the FS-7 cell-associated cell surface (Fas)/Fas Ligand (FasL) death receptor (DR) pathway (82, 83).

In addition to PI3K, Vav1 is a key signaling molecule in CD28-mediated costimulation. Binding of Grb-2 to the phospho (p)YMNM domain is required for recruitment and activation of Vav1 during CD28 signaling (76, 84). Vav1 signaling contributes to CD28-induced NFAT, NFκB and AP-1 activation (74, 85, 86), suggesting that CD28-mediated Vav1 activation is important in the amplification of almost all of the TcR signaling pathway. Due to GEF activity, Vav1 initiates a MAPK pathway leading to the activation of c-jun kinase (JNK) (39, 84). A major target of JNK is c-jun, which when phosphorylated becomes a key component of the AP-1 transcription factor complex (39). Importantly, CD28-mediated Vav1 activation may play a major role in augmenting the TcR signaling pathway through the enhancement and stabilization of TcR signaling complexes (74). In addition to being a GEF, Vav1 is also an adaptor protein. During T cell activation, Vav1 activated by CD28 signaling binds and stabilizes SLP-76/LAT complexes activated by TcR triggering (87, 88).

In addition to the YMNM motif, CD28 contains two proline rich repeats that contribute to CD28 signal transduction. One proline rich repeat is designated P¹⁷⁵RRPGP and recruits Tec family kinases, such as Itk (89). Although the significance of Itk recruitment during CD28 signaling is not well understood, it may enhance the activation of PLCγ1 (90). The second proline rich repeat is

designated P¹⁷⁵YAPA and recruits PKC θ and Lck (91). PKC θ -deficient mice have reduced IL-2 production and T cell proliferation in response to activation via TcR ligation (92). Accumulation of PKC θ within the immunological synapse (IS) is a key event during T cell activation (93). Interestingly, Yokosuka *et al.* have recently shown that CD28 plays a major role in recruiting PKC θ to the IS (91), suggesting yet another mechanism by which CD28 signaling amplifies TcR signaling.

As discussed above, TcR triggering in the absence of costimulation causes the T cell to enter an anergic state. Recent studies indicate that CD28-mediated costimulatory signals avert anergy by inducing PI3K/Akt signaling and promoting AP-1 activation (94). Buckler *et al.* showed that the expression of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is required for anergy induction (95). PTEN negatively regulates PI3K function by removing phosphate groups from PIP₃ molecules. In the absence of costimulation, TcR-mediated activation of PI3K may not be robust enough to overcome negative regulation by PTEN. In the presence of costimulation, however, PI3K activation may reach a threshold that precludes PTEN effects. Optimal PI3K signaling facilitates the downregulation of the KIP1 and allows cells to progress through the cell cycle and escape anergy (96).

TcR triggering in the absence of costimulation induces the expression of a number of proteins that mediate the anergic program (94). These anergy-associated proteins negatively regulate TcR signal transduction and gene transcription. Several proteins, including the E3-ubiquitin ligases Itch, Cbl-b, gene related to anergy in lymphocytes (GRAIL) and tumor necrosis factor receptor-associated factor (TRAF), as well as caspase 3, are implicated in promoting the proteolytic degradation of signaling molecules involved in TcR signal transduction (97, 98). As a result, anergic T cells are associated with a defect in PKC θ and PLC γ 1-mediated signaling pathways. TcR signal transduction also induces the expression of several proteins, including Erg2, Erg3, and Ikaros, that repress IL-2 gene transcription (99, 100), which explains why anergic cells are unable to produce IL-2 in response to activating signals.

CD28-mediated costimulation is thought to prevent the expression of these anergy-associated proteins through the activation of the AP-1 transcription factor (94). TcR-signaling alone promotes the activation of NFAT, but not the full Fos/c-jun dimer that constitutes active AP-1. Although TcR triggering sufficiently promotes the activation of Fos, a costimulatory signal is required to amplify TcR-mediated JNK activation required for c-jun activation. In the absence of active AP-1, NFAT preferentially promotes the transcription of anergy-associated proteins (101). In the presence of a costimulatory signal, however, NFAT cooperates with AP-1 to preferentially promote the transcription of genes such as IL-2 that are involved in T cell activation.

1.2.2.2 Other Costimulatory Molecules

Numerous other molecules have been implicated in providing costimulatory signals during T cell activation (102). Like CD28, some of these molecules belong to the IgSF, including CD2, inducible costimulatory molecule (ICOS) and the recently identified T cell Ig and mucin (TIM) domain family (102). Others belong to the tumor necrosis factor receptor (TNFR) family, including CD40, CD27, OX40, 4-1BB. LFA-1, a member of the integrin family, has also been shown to play a key role in providing costimulatory signals during T cell activation (102). Each costimulatory receptor/ligand pair has distinct expression profiles and kinetics and varies in function from modulating initial T cell activation to promoting effector and memory cell activation and function (102).

1.3 Dendritic Cells

There are two main categories of DCs; conventional DCs (cDCs) and plasmacytoid DCs (pDCs) (103). The former are well recognized pAPCs that play a major role in the activation of naïve T cells. In contrast, pDCs are associated with protection against viral infections through the production of large amounts of type I IFNs. A role for pDCs in Ag presentation, however, remains controversial (104).

cDCs are heterogeneous and can be divided into at least five different subsets based on cell surface marker expression, location, and function (105, 106). Epidermal Langerhans cells and interstitial DCs are found in the skin and

most other organs, respectively. These DCs sample the environment for Ag and upon receiving activation signals via PRRs migrate into draining lymph nodes (LN), where they can activate naïve T cells. CD8 α ⁻CD4⁻, CD8 α ⁻CD4⁺ and CD8 α ⁺CD4⁻ DCs are located within secondary lymphoid tissues. CD8 α ⁺CD4⁻ DCs are very efficient at cross-presenting Ag to CD8⁺ T cells (107, 108).

1.3.1 Dendritic Cells and T Cell Activation

DCs are sentinel cells of the innate immune system. Immature DCs are located in ideal positions within the body, including peripheral tissues such as the skin and mucosal linings as well as secondary lymphoid tissues, to continuously monitor for invading pathogens. DCs express numerous types of PRRs, including Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like helicases (RLHs) and C-type lectin receptors (CLRs) that enable the DCs to recognize and respond to different types of microorganisms (109). PRR-signaling induces a maturation program involving morphological and functional changes from generally spherical cells that specialize in phagocytosis to cells that have numerous long projections, or dendrites, and are excellent at presenting Ag to T cells.

DCs are unique in their ability to activate primary T cell responses. They induce naïve T cell activation better than any other type of pAPCs due to the expression of high levels of costimulatory ligands (CD80, CD86) and MHC molecules (4, 110). In response to maturation signals, DCs also upregulate the expression of chemokine receptors, such as CCR7, that enable their migration to secondary lymphoid tissues where naïve T cells typically reside (4). Additionally, depending upon the type of PRR signal that DCs receive and their environment at the time, DCs produce distinct cytokines that influence the nature of the subsequent T cell-mediated immune response. DCs also play a role in preventing autoimmune disease. DCs that present Ag to T cells in the absence of a PRR signal promote tolerance rather than activation, thus ensuring that self-reactive T cells that escape thymic selection are not activated in the periphery (111).

An important consequence of DC and T cell interactions is the formation of an IS at the contact site. The IS involves the rearrangement of signaling

molecules into three distinct domains that appear as a bulls-eye-like structure at the cell interface (112). This dynamic structure is necessary for stable T cell-DC interactions and the sustained TcR signaling that is required for the activation of naïve T cells. The center domain, designated central supramolecular complex (cSMAC), contains the majority of the signaling molecules involved in T cell activation, including the TcR-pMHC clusters and costimulatory molecules. The cSMAC is surrounded by a ring populated by the adhesion molecule LFA-1 and its ligand intercellular adhesion molecules 1 (ICAM-1), which is important in maintaining tight adherence between the T cell and DC. This ring is called the peripheral SMAC (pSMAC). The region outside of the pSMAC is known as the distal SMAC (dSMAC) and is enriched in the phosphatase (113). A recent study by Varma *et al.*, suggests that initial TcR-pMHC interactions occur within the dSMAC and move towards the cSMAC, where the TcR signal may be terminated (114). The continual formation and movement of TcR-pMHC complexes within the dSMAC to the cSMAC is required for sustained TcR signaling leading the T cell activation and differentiation into effector and memory cells.

1.4 Cytotoxic T Cells

CTLs are effector T cells that play a major role in cell-mediated immune responses through the identification and destruction of host cells that are either infected with pathogens or are cancerous. Naïve CD8⁺ T cells differentiate into CTLs upon recognition of antigenic peptide presented in MHC I by pAPCs in the presence of costimulatory signals. CTLs are armed with cytotoxic effector molecules, including perforin, granzymes, and FasL, that induce apoptosis in target cells (115, 116). All nucleated host cells continuously sample, process and display endogenous peptides on their cell surface in the cleft of MHC I molecules. Consequently, cells infected with viruses or intracellular bacteria display foreign antigenic peptides from that microorganism on their cell surface in MHC I and become targets for CTL-mediated destruction. Similarly, neoplastic cells display peptides derived from mutated proteins on their cell surface in MHC I molecules. CTLs that recognize cognate Ag receive antigenic stimulation from a target cell, thus engaging cytotoxic effector functions, which execute a cell death program

within the target cell mediated by two independent pathways, granule-mediated cytotoxicity and DR-mediated cytotoxicity (115, 116).

1.4.1 Granule-Mediated Cytotoxicity

Granule-mediated cytotoxicity involves the directional secretion of two key types of effector molecules, perforin and granzymes, from preformed cytotoxic granules towards the target cells. Perforin and granzymes released by the CTLs then act together to induce apoptosis within the infected or neoplastic cell.

Perforin is a 66 kDa pore-forming molecule that was first isolated and characterized from CTLs in 1985 (117, 118). A key role for perforin in cell-mediated immune responses is supported by studies with perforin^{-/-} mice that are unable to clear certain viral and intracellular bacterial infections and have decreased tumor immune surveillance (119, 120). T cells from perforin^{-/-} mice also have severely impaired cytotoxic activity (121), supporting a prominent role for perforin in T cell cytotoxic effector function.

Granzymes are a family of serine proteases expressed by CTLs and NK cells. Currently, ten different granzymes have been identified in the mouse. Of these, granzyme B is the most well characterized and has the strongest apoptosis-inducing function (122). Granzyme B induces apoptosis within target cells by activating the mitochondrial pathway (123). A major target of granzyme B is the pro-apoptotic Bcl-2 protein family member, Bcl-2 interacting domain death agonist (Bid) (124, 125). Granzyme B cleaves Bid into a truncated form (tBid), which destabilizes the mitochondrial membrane and results in the release of proapoptotic factors that activate caspase-dependent and -independent apoptosis. Granzyme B can also directly activate caspases involved in apoptosis (126).

Granzymes and perforin are only synthesized in CD8⁺ T cells following Ag recognition. Both of these molecules are stored together in secretory lysosomes, termed cytotoxic granules, allowing for a rapid response once the CTL recognizes a target cell (127). The TcR signaling threshold for CTLs to release their granules is much lower than that required for the activation of naïve T cells, since only a few TcRs need to be engaged (128). Interestingly, an IS forms at the

contact site between the CTL and target cell (129). In addition to a cSMAC and pSMAC, this IS also has a secretory domain adjacent to the cSMAC. TcR triggering promotes the polarization of the microtubule organizing complex (MTOC) towards the IS, which the cytotoxic granules move towards along microtubules. At the plasma membrane, the contents of the cytotoxic granules are then released at the secretory domain into a synaptic cleft formed between the CTL and the target cell (129). Granzymes then gain entry into the cytoplasm of the target cell with the help of the pore-forming activity of perforin, and then induce apoptosis.

1.4.2 Death Receptor-Mediated Cytotoxicity

The DR family is currently made up of eight transmembrane proteins defined by the presence of a death domain (DD) within the cytoplasmic tail (130). Upon ligation, DRs initiate the extrinsic pathway of apoptosis through the recruitment of signaling molecules to the DD.

The DR ligand FasL is a key effector molecule of CTLs. CTLs are armed with preformed FasL stored in vesicles that are distinct from the perforin- and granzyme-containing cytotoxic granules (131). FasL is expressed on the surface of CTLs that recognize cognate Ag through two different mechanisms. First, the preformed FasL packaged in vesicles is translocated to the cell surface within seconds. Additionally, FasL protein is also newly synthesized and transported to the cell surface, resulting in a more sustained expression for several hours (131).

FasL trimerizes the DR Fas on target cells, resulting in the generation of an apoptotic signal. Fas is constitutively expressed on a wide variety of tissues, including the liver, heart, kidneys, lungs and ovaries (132, 133). Fas is also upregulated on lymphocytes during their activation and is involved in the negative regulation of adaptive immune responses by AICD (134). In fact, the *lpr* (lymphoproliferation) strain of mice has an autosomal recessive mutation of Fas and exhibit lymphadenopathy and lupus-like autoimmunity partially due to uncontrolled T cell activation and proliferation (135).

The Fas/FasL signaling pathway is well characterized (115). FasL induces the trimerization of Fas molecules resulting in the recruitment of Fas-associated

death domain (FADD) adaptor molecules to the cytoplasmic DD of Fas. Procaspase-8 is then recruited to FADD resulting in the formation of the death-inducing signaling complex (DISC). The DISC mediates proteolytic cleavage and activation of procaspase-8 to caspase-8. Active caspase-8 then cleaves effector caspases, such as procaspase-3, into active forms and thus initiates a signaling cascade that leads to apoptosis. Bid is also a target of caspase-8 cleavage, and tBid initiates mitochondria-dependent apoptosis. Interestingly, certain cell types favor direct activation of effector caspases over Bid-mediated disruption of the mitochondria and *vice-versa* (136).

1.5 T Helper Cells

Th cells are CD4⁺ T cells whose main effector function is to produce cytokines that modulate the nature of ensuing immune responses. In a pivotal study, Mossman and Coffman (137) first showed that Th cells could be divided into two distinct subtypes, Th1 and Th2 cells, based on their cytokine signatures. Cytokines produced by Th cells have a myriad of functions including the recruitment and activation of innate immune cells, supporting B cell and CTL activation, and favoring class switching to distinct isotypes of antibodies during B cell activation (138-140). Each Th subtype develops in response to particular types of pathogens and promotes the most efficient type of immune response for eliminating that microorganism. The decision of which Th cell response will prevail is made during T cell activation. Numerous factors have been implicated in influencing this decision, including the nature and dose of Ag, route of administration, costimulatory signals provided and the cytokine milieu (141, 142). Of these, the cytokine milieu during Ag recognition plays a predominant role in the decision-making process. Additionally, the development of each Th subtype is reciprocally regulated, mainly through the action of cytokines. This ensures efficient activation of only the necessary effector mechanisms during a particular immune challenge. In the early 2000's, an additional functional subset of Th cells was described that was designated as Th17 cells (143, 144). As discussed below, the discovery of Th17 cells filled some gaps in the Th1/Th2 cell paradigm by providing an explanation for how certain types of pathogens, against which

Th1 and Th2 responses were not very effective, could be eliminated. In recent years, a number of other Th cell phenotypes with distinct cytokine profiles have also been identified, suggesting even more complexity to Th cell-mediated immune responses (145, 146).

1.5.1 Th1 Cells

Th1 cells are a subset of CD4⁺ Th cells characterized by the production of IFN γ , IL-2 and TNF and are critical for host defense against intracellular pathogens and cancer cells (137, 147). The primary role of Th1 cells is to orchestrate cell-mediated immune responses by promoting the recruitment and activation of phagocytic cells, especially macrophages, and to help promote the differentiation of CD8⁺ T cells into CTLs (147). Th1 cells also provide help to B cells, promoting class-switching to subtypes of antibodies involved in antibody-dependent cell-mediated cytotoxicity (ADCC) as well as opsonizing and complement-fixing antibodies, which further enhance phagocytosis-mediated clearance of pathogens (147). Although Th1 responses are effective at clearing intracellular pathogens, dysregulation of Th1 responses can also lead to destructive inflammatory conditions known as delayed-type hypersensitivity (DTH) reactions (147). Moreover, Th1 cells have been implicated in the pathogenicity of numerous types of organ-specific autoimmune diseases, including insulin-dependent diabetes mellitus (IDDM), multiple sclerosis (MS), rheumatoid arthritis (RA) and Crohn's disease (148-151).

1.5.1.1 Cytokine-Mediated Differentiation of Th1 Cells

Naïve CD4⁺ T cells differentiate into Th1 cells upon Ag recognition in the presence of IL-12 and IFN γ (152-154) (Figure 1.3). Initially, signal transducer and activator of transcription (STAT)1 activation through IFN γ signaling promotes low levels of expression of the transcription factor T-box expressed in T cells (T-bet), the master regulator of Th1 cell differentiation (152). Subsequently, T-bet induces IL-12 receptor (IL-12R) β 2 chain expression on the surface of the activated CD4⁺ T cells, making them responsive to IL-12 produced by activated macrophages and DCs (153). Macrophages and DCs express high levels of IL-12 in response to CD40 signaling initiated through interaction with CD40L on activated T cells

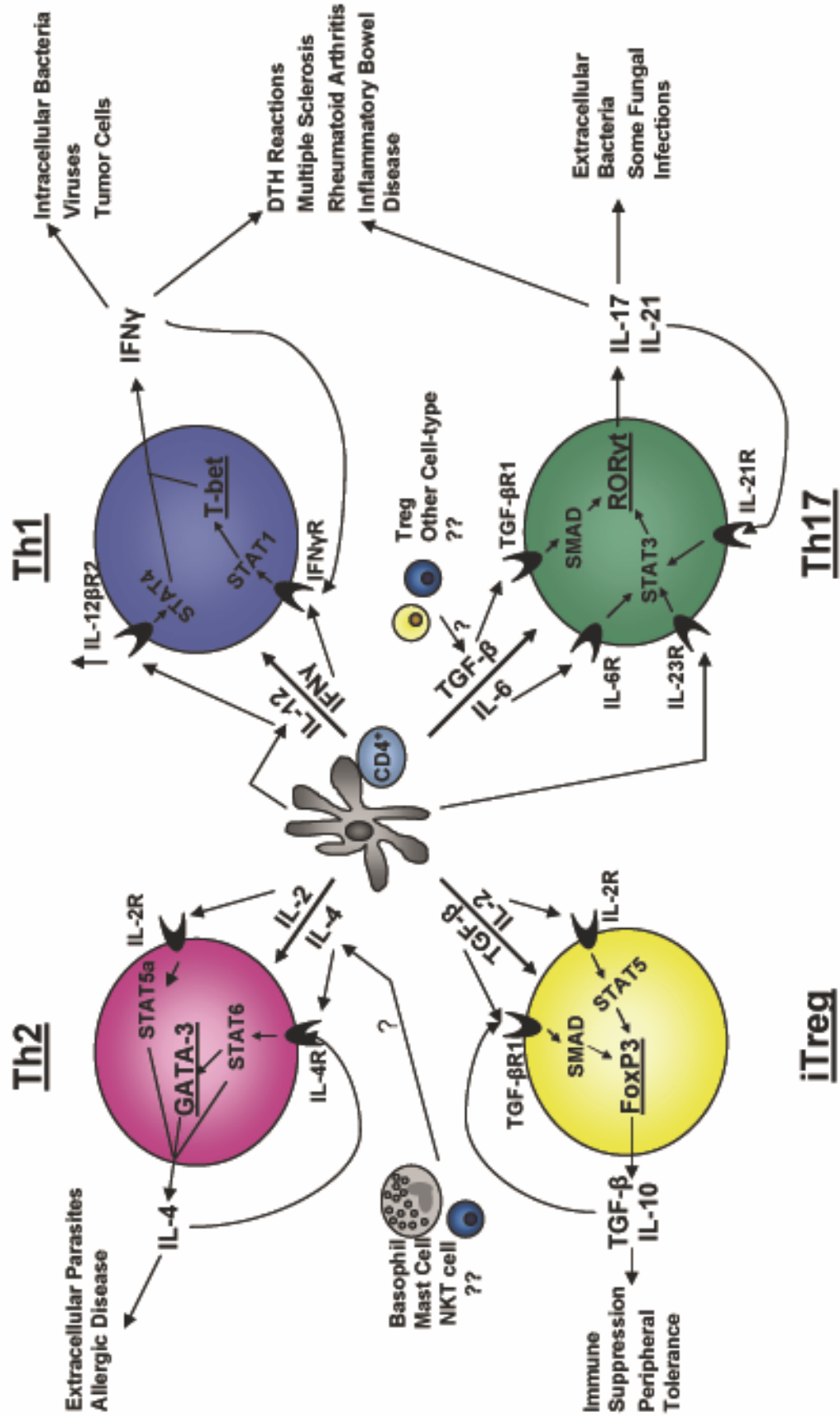


Figure 1.3. Differentiation of Naive CD4⁺ T Cells into Th1, Th2, Th17 and iTreg Cell Subsets

(155). IL-12 signaling activates STAT4, a transcription factor that promotes the production of IFN γ by the differentiating Th cell. IL-12-induced IFN γ further enhances T-bet expression in an autocrine fashion (154). STAT4 signaling also induces expression of the IL-18R (152). Together, IL-12 and IL-18, originating from activated APCs, synergistically increase early IFN γ production, and thus act together to further stabilize T-bet expression (156).

The important role of T-bet as the key lineage-specific transcription factor that regulates the distinct Th1 phenotype is well established. T-bet expression is found only in Th1 cells, and not Th2 or Th17 cells, and is upregulated within 24h of T cell activation in the presence of Th1 polarizing conditions (157). Ectopic expression of T-bet in CD4⁺ T cells induces the Th1 cell phenotype by preferentially inducing IFN γ expression over IL-4 and IL-5 (157). Moreover, CD4⁺ T cells deficient in T-bet are unable to differentiate into Th1 cells under polarizing conditions, and instead differentiate into Th2 cells (158). Pivotal studies have shown that T-bet, in addition to mediating Th1 differentiation by promoting the transcription of *IFN γ* and *IL-12R β 2* (152, 153, 157), promotes a stable Th1 phenotype by initiating remodeling of the *IFN γ* gene locus through the acetylation of lysine residues on histone proteins in nucleosomes, which makes it highly permissive to gene transcription (159). At the same time, T-bet promotes silencing of the Th2 cytokine gene loci and thus favors the expression of Th1 cytokines over Th2 cytokines (160). The final outcome is a Th cell that is programmed to preferentially make high levels of IFN γ and that will give rise to progeny of similar phenotype.

1.5.1.2 Role of IFN γ in Th1 Cell Function

IFN γ is a pleiotropic, homodimeric cytokine with proinflammatory activity (161). In addition to Th1 cells, IFN γ is produced by CD8⁺ T cells and NK cells. IFN γ exerts its activity by binding to a heterodimeric receptor made up of IFN γ receptor 1 (IFN γ R1) and IFN γ R2 subunits (161). Many of the responses initiated by IFN γ signaling are due to the activation of a Jak (Janus kinase)-STAT signaling pathway. Inactive Jak1 and Jak2 are constitutively associated with the cytoplasmic tails of the IFN γ R1 and IFN γ R2 subunits, respectively (162, 163).

Ligation of the IFN γ R results in oligomerization of the receptor subunits and activation of the Jaks by trans-phosphorylation (164). Upon activation, the Jaks then create a docking site for STAT1 through the phosphorylation of Tyr residues in the cytoplasmic tail of IFN γ R2 (165). Recruitment to the docking site brings STAT1 in close proximity to the Jaks, which activate STAT1 by Tyr phosphorylation. STAT1 then dissociates from the receptor and forms a homodimeric complex that translocates to the nucleus, where dimerized STAT1 binds to specific promoter elements in IFN γ -regulated genes and then either activates or represses gene transcription (166, 167). IFN γ signaling also involves the activation of STAT-independent pathways that are not as well characterized. PI3K and MAPK pathways, including p38 MAPK, ERK and JNK, are all activated by IFN- γ in a STAT1-independent manner (168-170). Interestingly, PI3K and p38 MAPK signaling enhances STAT1 activity by the phosphorylation of a serine residue on STAT1, suggesting cross-talk between the pathways (168, 169).

It has long been appreciated that IFN γ plays a key role in the induction of cell-mediated immune responses by regulating the expression of numerous genes in many different cell types (171). In fact, mice that are deficient in IFN γ signaling do not mount effective cell-mediated immune responses against intracellular bacteria or viral infections (172-175) as well as certain tumors (176, 177). One of the major targets of Th1-derived IFN γ is macrophages. Macrophages are mononuclear cells of the innate immune system that eliminate pathogens by phagocytosis and the release of toxic metabolites, and help activate T cells via Ag presentation (138). In response to IFN γ signaling, activated macrophages upregulate their microbicidal effector functions, including receptor-mediated phagocytosis (178) and the production of reactive oxygen species (ROS) (179) and reactive nitrogen intermediates (RNI) (180). IFN γ -activated macrophages also produce high levels of proinflammatory cytokines, including IL-12 (181), TNF α and IL-1 β , and chemokines, including IFN-inducible protein 10 (IP-10; CXCL10), monocyte chemoattractant protein-1 (MCP-1; CCL2), monokine induced by IFN γ (MIG; CXCL9), macrophage inflammatory protein-1 α (MIP-1 α ; CCL3), MIP-1 β (CCL4) and regulated on activation normal T

cell expressed (RANTES; CCL5) (182). Consequently, IFN γ -activated macrophages promote the trafficking of specific immune cells, in particular monocytes, CD4⁺ and CD8⁺ T cells, to sites of inflammation. Additionally, IFN γ also induces endothelial cells to produce similar chemokines (183, 184), as well as the adhesion molecules ICAM-1 and vascular cellular adhesion molecule 1 (VCAM-1) (185, 186), which are also involved in the recruitment of monocytes, T cells, and NK cells to sites of inflammation.

IFN γ also regulates adaptive immune responses by enhancing the expression of numerous molecules involved in Ag processing and presentation. The MHC class II Ag-presenting pathway is not only upregulated in pAPCs, but also in cells that do not normally present Ag to CD4⁺ T cells upon stimulation with IFN γ (187). As well, the MHC class I Ag-presenting pathway is upregulated by IFN γ , which enhances the recognition and destruction of infected cells by CTLs (188). As discussed above, IL-12 is a strong inducer of IFN γ . In turn, IFN γ strongly enhances IL-12 production by phagocytic cells and thus indirectly promotes its own production by a potent positive feedback mechanism (189). Moreover, IFN γ inhibits IL-4 production by Th2 cells (190). Consequently, IFN γ production further skews the immune response towards a Th1 phenotype by inhibiting Th2 responses. IFN γ signaling also modulates B cell responses. Cytokines secreted by Th cells during B cell activation influence the types of antibodies that will be produced. In mice, IFN γ induces class switching to antibody isotypes, such as IgG2a and IgG3, that are efficient at complement fixation, ADCC, and opsonization (191-193), and thus enhance the ability of phagocytes to destroy pathogens. At the same time, IFN γ also inhibits class switching to antibody isotypes that would be more suited to Th2 responses, such as IgE and IgG1 (194, 195).

1.5.2 Th2 Cells

Th2 cells are a subset of CD4⁺ T helper cells characterized by their production of IL-4, IL-5 and IL-13 (196). Th2 cells are critical to host defense against extracellular parasites such as helminthes. Additionally, Th2 cells also play a key role in the development of asthma and other allergic diseases. Th2

responses are classically known to induce humoral (or antibody-mediated) immune responses. B cells that receive help from Th2 cells produce large amounts of IgE and IgG1. Moreover, Th2 cells mediate the recruitment and activation of eosinophils, mast cells and basophils, all of which are involved in the immune response against helminthes, as well as in the pathogenesis of many allergic diseases.

1.5.2.1 Cytokine-Mediated Differentiation of Th2 Cells

Naïve CD4⁺ T cells differentiate into Th2 cells upon Ag recognition in the presence of IL-4 and IL-2 (197) (Figure 1.3). Currently, the cellular source of IL-4 *in vivo* is unclear and a subject of much debate (198). Several types of cells secrete IL-4, including mast cells, eosinophils, basophils and NKT cells. It has been hypothesized that any one of these cell types may be the source of IL-4 *in trans* during T cell activation by DCs (198). Naive T cells can also produce IL-4 upon activation and thus may initiate Th2 differentiation by an autocrine mechanism (199). In recent years, basophils have received an increasing amount of attention as a possible inducer of Th2 differentiation. Basophils migrate to draining lymph nodes under certain conditions and can present Ag via MHC II to naïve CD4⁺ T cells (200). Depletion of basophils inhibits Th2 responses against the cysteine protease antigens. However, basophils are not required for all Th2 responses. For example, depletion of basophils has no effect on Th2 immune responses against house dust mite Ag (201).

In response to IL-4 signaling, STAT6 is activated and translocates to the nucleus of the differentiating Th cell where it induces the expression of GATA binding protein-3 (GATA-3), the master regulator of Th2 cell differentiation (202, 203). STAT6 also transactivates the IL-4 gene and is involved in chromatin remodeling of the IL-4/IL-13 locus, and thus plays an important role in promoting IL-4 production by Th2 cells (204). In fact, CD4⁺ T cells that are deficient in STAT6 fail to express GATA-3 and do not develop into Th2 cells (202).

The role of GATA-3 as the key lineage transcription factor of Th2 cells is well established. GATA-3 is expressed in differentiated Th2 cells, but not by other types of Th cells (205, 206). Ectopic expression of GATA-3 in CD4⁺ T cells

induces the Th2 cell phenotype (207). Moreover, CD4⁺ T cells that are deficient in GATA-3 are unable to differentiate into Th2 cells (208-210).

GATA-3 promotes Th2 cell differentiation by several different mechanisms. GATA-3 directly activates the transcription of Th2-type cytokines, including IL-5 and IL-13 (211-213). A role for GATA-3 in directly promoting the transcription of IL-4, however, is less clear. Expression of antisense GATA-3 in Th2 cells inhibits IL-5 production but not IL-4 production (212). In addition, continuous expression of GATA-3 in fully differentiated Th2 cells is required only for IL-5 and IL-13 production, but not for IL-4 production (209). Together, these findings suggest that transcription factors other than GATA-3 must be involved in IL-4 promoter gene expression. However, as GATA-3 deficiency results in reduced IL-4 production by Th2 cells (209), GATA-3 clearly must play some role in the regulation of *IL-4* gene expression. In fact, several studies have shown the GATA-3 mediates chromatin remodeling during Th2 differentiation at the *IL-4/IL-13* gene locus, making these Th2 cytokine genes more permissive to transcription (209, 214). In addition to regulating Th2 cytokine expression, GATA-3 promotes Th2 differentiation by inhibiting the expression of genes involved in Th1 differentiation. Specifically, GATA-3 has been shown to initiate chromatin remodeling at the *IFN γ* gene locus, resulting in the repression of gene expression (215).

Several other transcription factors have also been implicated in Th2 development. The transcription factors c-Maf and JunB are preferentially upregulated in Th2 cells and play a critical role in promoting *IL-4* gene transcription (216, 217). Additionally, two members of the NFAT transcription factor family, NFATc and NFATp, may also have opposing roles in Th2 cell differentiation. NFATc-deficient mice have reduced Th2 responses, while NFATp-deficient mice have enhanced Th2 responses (218, 219).

IL-2-mediated STAT5 activation is also essential for Th2 differentiation. In a seminal study, Cote-Sierra *et al.* (220) showed that neutralization of IL-2 during T cell activation in the presence of Th2 polarizing conditions allows for normal proliferation but prevents Th2 cell differentiation. Th2 cell differentiation, however,

can be restored under these conditions when STAT5a is ectopically expressed (221). Moreover, STAT5a-deficient T cells also have severe defects in Th2 differentiation independent of IL-4-mediated STAT6 activation (220, 222). Interestingly, GATA-3 expression levels are not affected by either STAT5a overexpression or deficiency in STAT5a-signaling in the presence of IL-4 (220, 221, 223), suggesting that STAT5a contributes to Th2 differentiation independent of GATA-3. Consistent with this finding, Guo *et al.* (214) have recently shown that GATA-3 and STAT5a may regulate accessibility at the *IL-4* gene locus at different sites. This finding may explain the synergistic effect on IL-4 production observed when GATA-3 and STAT5a are overexpressed in the same cell (221). A number of cytokines, including IL-2, IL-7, IL-9 and thymic stromal lymphopoietin (TSLP), activate STAT5 in Th cells; however, their relative contribution to Th2 differentiation *in vivo* remains unclear (224). IL-2 neutralization *in vivo* results in impaired Th2 responses. As well, TSLPR-deficient mice are unable to mount effective Th2 responses against the intestinal pathogen *Trichuris muris* (225). These findings support a role for IL-2 and TSLP in Th2 differentiation *in vivo*.

1.5.2.2 The Role of IL-4 in Th2 Cell Function

IL-4 is a pleiotropic type I cytokine produced by Th2 cells, mast cells, basophils, eosinophils, NKT cells and $\gamma\delta$ T cells (139). IL-4 exerts its activity by binding to a heterodimeric receptor made up of the specific IL-4R α chain and the common γ chain. Similar to the IFN γ R, the IL-4R also signals by a Jak-STAT signaling pathway. The IL-4R, however, is associated with Jak1 and Jak3, which upon activation recruit and activate STAT6 (226).

In addition to promoting Th2 immune responses, IL-4 has numerous other functions in shaping the immune responses against parasitic infections and during allergic reactions. Importantly, IL-4 signaling controls the specificity of Ig class switching; thereby inducing B cells to produce high levels of IgE and IgG1 antibodies (194, 227). IgE production is important in the activation of mast cells and basophils, and hyperproduction of IL-4 and IgE is associated with atopic allergy (228). In addition, mice that are deficient in IL-4 signaling produce significantly less IgE and have diminished immunity against certain helminthic

parasites (229). IL-4 also enhances B cell activity by enhancing cell proliferation, survival and MHC II expression (230, 231). A major consequence of Th2-mediated immune responses is the recruitment and activation of eosinophils. IL-4 has been implicated in mediating the selective recruitment of eosinophils by inducing epithelial cells and fibroblasts to produce chemokines, such as eotaxins (232).

1.5.3 Th17 Cells

Seven years ago, a third type of CD4⁺ T helper cell characterized by its production of IL-17, IL-17F and IL-22 was discovered and designated as Th17 cells (143, 233). The identification of Th17 cells originated from the observation that induction of pathogenic Th1 responses by IL-12 could not solely explain the cause of organ-specific autoimmune diseases such as RA and MS and their respective animal models, collagen-induced arthritis (CIA) and EAE. A role for IL-12 and Th1 cells in CIA and EAE had previously been suggested by the observations that IL-12 and IFN γ levels are elevated in animals and patients with these diseases (234-236), and that the addition of recombinant mouse (rm)-IL-12 exacerbates disease onset while Ab-mediated neutralization of IL-12 prevents disease (237, 238). Studies with mice deficient in IL-12 signaling (lacking either IL-12 or the IL-12R), however, were conflicting. IL-12 is a heterodimeric cytokine consisting of two subunits, p40 and p35. While mice deficient in the p40 subunit are resistant to the development of both EAE and CIA (239), mice deficient in the p35 subunit actually develop similar disease as wild-type mice or even exacerbated disease (233, 240-242). Moreover, mice deficient in the IL-12R β 2 chain also develop exacerbated EAE (243). A major causative role for IFN γ in EAE and CIA was also not fully supported by studies showing that mice treated with neutralizing anti-IFN γ mAb, as well as IFN γ -deficient or IFN γ R-deficient mice also have exacerbated disease onset or increased susceptibility to disease induction (244-247).

The identification of a new heterodimeric cytokine designated IL-23, which shares the p40 subunit with IL-12 (248), shed considerable light on these seemingly conflicting studies. The observation that IL-23 and IL-12 share the p40

subunit combined with the divergent roles of the p40 and p35 subunits in disease severity provided strong evidence that IL-23 plays a major role in the pathogenesis of MS and RA (233, 241). In two pivotal studies, mice deficient in the IL-23 specific subunit p19 were shown to be resistant to the induction of both EAE and CIA (233, 242). Subsequent studies have shown that IL-23, produced by activated dendritic cells (248), monocytes and macrophages, promotes the stabilization of the Th17 cell phenotype (143). Moreover, pathogenic Th17 cells play a major role in promoting the detrimental inflammation seen in EAE (144) and CIA (249). In addition to MS and RA, Th17 cells have been implicated in the pathogenicity associated with other autoimmune diseases such as experimental autoimmune uveitis (EAU) (250), experimental autoimmune myocarditis (EAM) (251) and inflammatory bowel disease (IBD) (252). Th17 cells also play a role in the pathology of certain allergic responses such as allergy (253) and contact hypersensitivity (254).

Since the discovery of Th17 cells, the physiological role of these IL-17-producing cells in host defense against infectious disease has also been intensely studied. Prior to the identification of this new CD4⁺ T-cell lineage, the Th1/Th2 paradigm could not explain host immunity against all types of pathogens. While it was clear that Th1 cells defend against intracellular pathogens and Th2 cells defend against parasitic helminthes, the role that Th cells play in providing protective immunity against extracellular bacteria was less clear. Emerging evidence suggests that Th17 cells fill this gap. Ye *et al.* (255) showed that IL-17R-deficient mice had greater dissemination of the extracellular bacteria *Klebsiella pneumoniae* in the lung due to significant delays in neutrophil recruitment, resulting in greater mortality compared to wild-type mice. More recently, Th17 cells have also been implicated in providing protection against numerous other extracellular bacteria, including bacteria that infect the oral cavity (256, 257).

Th17 cells also play a protective role against fungal infections. In healthy individuals, *Candida albicans* is normally a commensal organism of the oral cavity and GI tract. In patients with immune deficiencies, such as HIV infection

and autosomal-dominant hyper-IgE syndrome, however, *C. albicans* often becomes pathogenic (258-260). IL-17 signaling has been shown to be necessary for the protection against fungal infections caused by *C. albicans*, while IFN γ signaling is dispensable (261). Interestingly, patients with hyper-IgE syndrome resulting from a mutation in the STAT-3 gene, which is essential to the differentiation of Th17 cells, are highly susceptible to *C. albicans* infections and have decreased numbers of IL-17-producing cells (259, 260). In addition to *C. albicans*, Th17 cells and IL-17 can defend against other fungal infections that colonize mucosal tissues including *Aspergillus fumigatus* (262), *Pneumocystis carinii* (263), *Cryptococcus neoformans* (264) and *Paracoccidioides braziliensis* (265). Overall, these data indicate that Th17 responses are important in the host defense against extracellular bacteria and fungi that colonize mucosal tissues.

Th17 responses to pathogens are not always beneficial to the host, and can actually cause severe inflammation and other deleterious host effects. *Helicobacter pylori* infection of the gastric mucosa leads to elevated levels of IL-17 and recruitment of neutrophils, resulting in persistent inflammation and gastritis (266). Pathogenic IL-17-mediated inflammatory responses have also been reported in response to intragastric *C. albicans* and intranasal *Aspergillus fumigatus* (267, 268). Additionally, Th17 cell infiltrates have been shown to contribute to pathological abscess formation during *Bacteroid fragilis* infection (269). Currently, it is not clear what factors determine the fine balance of whether a Th17 response to a pathogen will be protective or detrimental to the host. Further studies into control mechanisms for Th17 responses may allow for the design of therapies and vaccines that promote protective Th17 responses while limiting pathogenic responses.

1.5.3.1 Cytokine-Mediated Differentiation of Th17 Cells

Naïve CD4⁺ T cells were first shown to differentiate into Th17 cells in response to the combination of the immunoregulatory cytokine transforming growth factor (TGF)- β and the proinflammatory cytokines IL-6 and IL-1 β (270-274) (Figure 1.3). Subsequently, IL-21 has also been shown to promote the differentiation of Th17 cells in the presence of TGF- β signaling, although IL-21 is

not as effective as IL-6 in this regard (275). The combination of TGF- β and IL-6 or IL-21 induces the expression of retinoid-related orphan receptor (ROR) γ t and ROR α , which are key lineage-specific transcription factors that regulate the distinct Th17 phenotype (276, 277). While it is known that TGF- β is produced by numerous types of leukocytes and stromal cells, the source of TGF- β that promotes Th17 cell differentiation is not well defined. It appears that TGF- β comes from either autocrine or paracrine sources, as neutralizing antibody for TGF- β locally, but not systemically, inhibits Th17 cell differentiation (278). As well, mice deficient in T cell-derived TGF- β have decreased numbers of Th17 cells and are resistant to EAE induction, suggesting that T cells themselves are a source of TGF- β for Th17 cell differentiation (279). Interestingly, Treg cells also produce TGF- β and thus may promote Th17 cell differentiation (270, 280). While TGF- β in the absence of inflammatory signals promotes Treg cell differentiation (see below), IL-6 is produced during inflammatory conditions by pAPCs upon interaction with microbial products and thus acts as a molecular switch between tolerance and inflammation.

The mechanism by which TGF- β and IL-6 act together to promote the differentiation of naïve CD4⁺ T cells into Th17 cells is still not completely clear. TGF- β signaling alone promotes expression of both ROR γ t and FoxP3 (281). FoxP3 physically associates with ROR γ t and inhibits its function, favoring Treg cell differentiation. On the other hand, IL-6 signaling activates STAT3 which not only promotes ROR γ t expression further, but inhibits the FoxP3 expression promoted by TGF- β (282), which relieves the repression of ROR γ t and, thus allows for the Th17 transcriptional program. Furthermore, TGF- β has been shown to promote Th17 cell differentiation by repressing the activity of inhibitors of ROR γ t expression. STAT3 activation is normally regulated by the phosphatase SOCS3. TGF- β induces further ROR γ t expression by inhibiting the expression of SOCS3, and as a result enhancing the activity of STAT3 (283). Similarly, TGF- β signaling also represses the expression of T-bet and Gata-3, both of which have been shown to inhibit the transcription of ROR γ t (284). The end result of the combined action of TGF- β and IL-6 is a synergistic increase in ROR γ t expression

concurrent with the repression of FoxP3, T-bet and Gata-3, resulting in preferential Th17 differentiation over Treg, Th1 and Th2 cell differentiation, respectively.

Although IL-21 can promote differentiation of naïve T cells into Th17 cells when in the presence of TGF- β by promoting STAT3 activation *in vitro*, IL-21 probably plays more of a role in maintaining and amplifying the pool of Th17 cells, rather than promoting the initial differentiation program. Consistent with this, Th17 cells themselves produce copious amounts of IL-21 and are thus able to enhance their own differentiation in a positive feedback loop (285, 286). IL-21 is clearly not as important as IL-6 *in vivo*, as a role for IL-21 in Th17 cell differentiation only becomes apparent in the absence of both IL-6 and Treg cells (275).

The combination of TGF- β and IL-6 signaling results in the expression of the IL-23R, rendering the newly differentiating T cells responsive to IL-23 (281). IL-23R expression is dependent on the ROR γ t induced by TGF- β and IL-6, as well as STAT3 (271, 285, 287). Produced by pAPCs in response to certain microbial products (288-291) and other activating signals (289, 292), IL-23 signaling also promotes STAT3 activation in T cells (293), further enhancing expression of ROR γ t and the differentiation of Th17 cells. Although IL-23 is not required for the initial differentiation of Th17 cells, IL-23 signaling appears to be crucial to the expansion and maintenance of Th17 cells (144, 233, 242).

The mechanism by which ROR γ t and ROR α program naïve T cells to become Th17 cells is unclear. ROR γ t may promote IL-17 production by binding directly to the IL-17 promoter. A binding site for ROR γ t in the IL-17 promoter has been characterized, and recently it has been shown that ROR γ t can indeed bind this site and promote transcription of IL-17 *in vitro* (294). Using reporter constructs, the authors also showed that ROR γ t binding alone was not enough to induce optimal levels of *IL-17* gene transcription. In fact, a second transcription factor Runx1, was shown to also bind to the promoter and act in concert with ROR γ t to promote *IL-17* transcription. Runx1 further influenced Th-17 cell differentiation by inducing ROR γ t expression (294).

In addition to Runx1, several other transcription factors have been shown to collaborate with ROR γ t to promote Th17 cell differentiation. In addition to enhancing ROR γ t expression, STAT3 binds directly to the IL-17 and IL-21 promoters and thus cooperates with ROR γ t to induce IL-17 and IL-21 production (286, 295). Similar to Runx1, a nuclear inhibitor of NF κ B (I κ B) family member, I κ B ζ , alone is unable to induce Th17 cells, but instead cooperates with ROR γ t to promote the transcription of *IL-17* (296). Interferon regulatory factor 4 (IRF4), a member of the IRF transcription factor family, has also been implicated in Th17 cell differentiation. Brustle *et al.* (297) showed that IRF4-deficient mice are resistant to EAE because they fail to mount a Th17 response. These findings could be explained in part by failure of IRF-deficient T cells to upregulate ROR γ t, suggesting that IRF4 is required for ROR γ t expression. However, restoration of ROR γ t expression in IRF4-deficient T cells failed to fully induce Th17 cell differentiation, suggesting that IRF4 collaborates with ROR γ t to promote full commitment of T cells to the Th17 cell lineage. Indeed, IRF4 has recently been shown to be crucial for the IL-21-mediated steps of Th17 differentiation by a STAT3-independent mechanism (298).

1.5.3.1.1 The Role of IL-17 in Th17 Cell Function

The IL-17 cytokine family has six members, termed IL-17A (also known as IL-17) to IL-17F (140). IL-17 and IL-17F share the highest homology within the IL-17 cytokine family and, unlike the other family members, are located on the same chromosome (chromosome 1 in mouse and 6 in human). IL-17 and IL-17F are both homodimeric proinflammatory cytokines whose main function is to promote neutrophil-mediated inflammatory responses by inducing granulopoiesis, neutrophil recruitment and activation (140).

IL-17 and IL-17F exert their activity by binding to a heterodimeric receptor composed of the IL-17RA and IL-17RC subunits (299). The signaling pathways by which IL-17 and IL-17F mediate their effects are still being discovered. The cytoplasmic tails of the IL-17RA and IL-17RC contain a SEF/IL-17R (SEFIR) domain (300), which recruits the adaptor protein NF- κ B activator 1 (ACT1) upon ligation (301). ACT1 in turn recruits TRAF6 and TGF- β -activated kinase I (TAK1)

to a TRAF6-binding motif resulting in that activation of the NF κ B pathway (302). p38 MAPK is also activated by IL-17 via an ACT1-dependent, TRAF6-independent mechanism and has been shown to promote increased stability of mRNAs coding for several chemokines, including CXCL1 and MIP-2 (303, 304). IL-17 signaling also appears to involve ACT1-independent pathways, including the activation of JAK1 and PI3K (305).

The end result of IL-17 signaling is the production of proinflammatory cytokines, such as IL-6, TNF, and IL-1 β and hematopoietic cytokines, such as GM-CSF and G-CSF, by a broad range of cell types (306-310). These cytokines promote granulopoiesis and neutrophil recruitment and activation at the site of inflammation. Additionally, IL-17 signaling also stimulates the production of numerous chemokines involved in neutrophil chemotaxis, such as CXCL1, CXCL2, CXCL5, CXCL8 and CXCL10, which further promotes neutrophil recruitment (311-314). Monocytes can also be recruited by IL-17, as expression of CCL2 and CCL20, chemokines involved in monocyte chemotaxis, are also induced by IL-17 signaling (315, 316). Furthermore, signaling by TNF and IL-1 β , as well as certain TLR ligands, enhances the ability of IL-17 to promote cytokine and chemokine production (317-319).

In addition to the recruitment and activation of innate immune cells, IL-17 also enhances host defense against microorganisms by inducing the expression of various different antimicrobial peptides, including β -defensins, S100 proteins and acute phase proteins such as lipocalin 2/24p3 (320-322). One major symptom of RA is bone destruction and joint erosion. IL-17 signaling can promote this damage by stimulating the production of tissue remodeling-associated molecules such as receptor activator of NF κ B ligand (RANKL) (323). RANKL expression on osteoblasts induces osteoclastogenesis that results in bone destruction. IL-17 also promotes production of matrix metalloproteinases (MMP1, MMP9 and MMP13), which also play a role in tissue damage (324-326).

1.5.4 Other Th cells

In addition to Th1, Th2, and Th17 cells, several other Th cell phenotypes with unique functions have been characterized. Although IL-9 has previously

been viewed as a Th2 cytokine (327, 328), several recent studies suggest that a distinct Th cell subtype produces large amounts of IL-9. These Th9 cells can be induced *in vitro* from the activation of naïve CD4⁺ T cells in the presence of IL-4 and TGF- β (145). The *in vivo* relevance of Th9 cells is unclear; however, IL-9 has been implicated in allergic disease (329).

T follicular helper cells (Tfh) are a unique type of Th cell found within the follicles of secondary lymphoid tissues (146). Tfh cells express high levels of CXCR5 and, although their hallmark cytokine is IL-21, they can also produce IFN γ , IL-4 and IL-17 under certain conditions (146). Consistent with their location, the main function of Tfh appear to be providing help to B cells during the germinal center reaction (146).

1.6 T Regulatory cells

An additional lineage of CD4⁺ T cells, called Treg cells, play a major role in regulating the strength of adaptive immune responses against invading pathogens (7). Treg cells are also important in establishing and maintaining peripheral tolerance by ensuring that self-reactive T cells that have escaped elimination in the thymus are suppressed in the periphery, thus limiting autoimmunity. The two main types of Treg cells that have been characterized are natural Treg (nTreg) cells and inducible Treg (iTreg) cells. nTreg cells develop in the thymus, while iTreg cells are induced in the periphery from naïve CD4⁺ T cells following Ag recognition (330). Both types of Treg cells are characterized by their expression of the high affinity IL-2R α chain (CD25) and are thus CD4⁺CD25⁺ T cells. Two types of iTreg include Th3 cells and Tr1 cells which produce high levels of TGF- β or IL-10, respectively (331, 332). The essential role that Treg cells play in controlling immune responses is evident in individuals who carry a mutation in FoxP3, the key lineage determining transcription factor for the Treg cell phenotype (333). These patients have a multi-organ lymphoproliferative disorder known as immune dysregulation, polyendocrinopathy, enteropathy X-linked syndrome (IPEX) due to an aberrant adaptive immune system (334). Importantly, some autoimmune diseases are associated with abnormally low levels of Treg cells, as well as dysregulation of Treg cell function (335).

Developing therapies that enhance Treg cell differentiation and function is currently a promising area of research for the treatment of autoimmune diseases.

1.6.1 Cytokine-Mediated Differentiation of Treg Cells

The mechanism by which nTreg cells develop in the thymus is still poorly understood, although nTreg cell development appears to be largely dependent on strong TcR signaling in the presence of CD28-mediated costimulation and IL-2R signaling in thymocytes expressing competent $\alpha\beta$ TcRs (336, 337). CD28 costimulation provided by thymic DCs is required for the induction of IL-2 responsiveness through the upregulation of CD25 on Treg cell precursors. IL-2R signaling then induces the expression of FoxP3 (338, 339).

FoxP3⁺ iTregs develop in the periphery from naïve CD4⁺ T cells in response to Ag stimulation in the presence of TGF β and IL-2 (340)(Figure 1.3). As discussed previously, the differentiation of both iTreg and Th17 cells is dependent on TGF β signaling, and proinflammatory cytokines, such as IL-6, that act as a switch to determine whether an iTreg or Th17 response will prevail. TGF β signaling induces the expression of FoxP3 and ROR γ t; however, in the absence of proinflammatory cytokine signals ROR γ t expression is eventually eliminated and FoxP3-induced Treg cell differentiation prevails (281). IL-2-dependent activation of STAT5b may enhance FoxP3 expression (340) and prevent Th17 cell differentiation (131).

The crucial role of FoxP3 in regulating the Treg cell phenotype is well established (341, 342). The molecular mechanism by which FoxP3 mediates the Treg cell phenotype, however, is less clear. FoxP3 is a transcriptional repressor of several cytokine genes including *IL-2*, *IFN γ* and *IL-4* (343). Similarly, FoxP3 inhibits Th17 differentiation by binding to and repressing the transcriptional activity of ROR γ t and ROR α (281). FoxP3 may also directly activate the transcription of immunoregulatory proteins, including glucocorticoid-induced TNF receptor (GITR) (344, 345).

1.6.2 Mechanisms of Treg Cell-Mediated Suppression

Treg cells inhibit immune responses by two main mechanisms; contact-dependent and contact-independent suppression. The contact-independent

mechanism of Treg cell-mediated suppression involves the secretion of the immunosuppressive cytokines TGF β and/or IL-10 (346, 347). Recent studies suggest that Treg cells may also inhibit T cell-mediated immune responses through the production of immunosuppressive adenosine. Treg cells express two ectoenzymes on their surface, CD39 and CD73, which convert adenosine nucleosides into adenosine (348, 349), which then binds to receptors on the surface of effector T cells, inhibiting their function (350, 351).

Treg cells from mice and humans have been shown to express cytotoxic granules containing perforin and granzymes, and can target and destroy effector T cells in a contact-dependent manner (352-354). As well, Tregs express cytotoxic T lymphocyte antigen-4 (CTLA-4) on their surface and may inhibit DC function through interaction with CD80 and CD86 expressed on the DC surface (355). Moreover, Tregs can also suppress effector T cell function by transfer of the inhibitory second messenger cyclic adenosine monophosphate (cAMP) to effector T cells cytoplasm in a contact-dependent manner (356).

1.7 GPI-Anchored Proteins

The plasma membrane (PM) of most eukaryotic cells contains numerous proteins that are anchored to the outer leaflet by a glycolipid structure known as glycosylphosphatidylinositol (GPI). All GPI anchors have a conserved core structure with side-chains and lipid moieties that can vary between proteins and species (357). GPI biosynthesis is a complex process that occurs on the endoplasmic reticulum (ER) membrane and involves at least nine different enzymatic reactions, which convert PI into the GPI anchor (358). Newly synthesized proteins that are destined to become GPI-anchored have a GPI attachment signal peptide at their C-terminus that is recognized by a transamidase within the ER (359, 360). The GPI transamidase is a multi-subunit enzyme that mediates the attachment of the protein to the GPI anchor (361). Subsequently, GPI-anchored proteins (GPI-AP) undergo glycan remodeling and are then transported to the Golgi complex where fatty acid remodeling occurs. GPI-APs are then transported to the cell surface.

A unique feature of most mammalian GPI-APs is the presence of two saturated fatty acids within the GPI structure. Consequently, most GPI-APs are localized within detergent-resistant membrane microdomains, known as lipid rafts. Lipid rafts are nanoscale-sized assemblies enriched with cholesterol, sphingolipids and GPI-APs that are important for signal transduction. Although there has been a considerable amount of controversy over whether lipid rafts actually exist or are merely an *in vitro* artifact, most recent studies strongly support a role for lipid rafts in the structure and function of membranes in living cells (362).

1.7.1 Function of GPI-Anchored Proteins

At least 150 GPI-APs have been identified so far, which have a multitude of biological functions (357, 363). GPI-APs include cell surface Ags, receptors, adhesion molecules, various enzymes, and complement regulators. The importance of GPI-AP function in mammalian development is highlighted by the fact that GPI-deficiency results in embryonic lethality in mice (364). Due to the requirement of GPI-APs in development, only two disorders involving GPI anchor deficiency in humans have been identified; paroxysmal nocturnal hemoglobinuria (PNH) and inheritable GPI deficiency (IGD).

PNH results from the clonal expansion of HSCs that harbor an acquired somatic mutation in the X-linked gene encoding for phosphatidylinositol glycan-class A (PIG-A) (365). PIG-A is a protein required for the first step of GPI anchor biosynthesis and cells carrying the PIG-A mutations do not express GPI-APs (358). Patients with PNH exhibit complement-mediated hemolysis and arterial or venous thrombosis due to lack of the GPI-APs CD59 and CD55, which normally protect host cells from complement-mediated lysis (366). The mechanism of clonal expansion of PNH clones is currently unclear (367). A considerable amount of evidence suggests that the lack of GPI-APs does not give an intrinsic proliferative advantage to PNH clones over healthy cells. In fact, healthy individuals normally have a small percentage of hematopoietic stem cells carrying a PIG-A mutation that do not expand and cause disease (368). Currently, it is believed that clonal expansion of PNH cells may occur through

selection due to an immune-mediated attack on HSCs (367). PNH cells are able to escape this attack due to a survival advantage. A recent study by Savage *et al.* (369) strongly supports an intrinsic survival advantage by PNH clones during immune-mediated attacks. PNH clones are more resistant to both allogeneic and autologous cell-mediated killing than healthy HSCs from the same donor. Similarly, a GPI-AP^{-/-} myeloid cell line, due to a mutation in the PIG-A gene, was more resistant to apoptosis induced by allogenic and autologous effectors, NK cells, TNF α and γ -irradiation than wild-type cells. These findings suggest a possible role for GPI-APs in negatively regulating apoptosis-inducing signals.

1.7.2 GPI-Anchored Proteins and T Cell Activation

Several lines of evidence support a role for GPI-APs in T cell activation. GPI-AP^{-/-} T cells from PNH patients have a marked reduction in proliferation and IL-2 production in response to mitogens, allogeneic stimulation and anti-CD3 ϵ monoclonal antibody (mAb)-mediated activation compared to GPI-AP^{+/+} T cells from the same patients (370, 371). The decreased responsiveness of PNH clones to T cell activation was attributed to reduced Lck activation. GPI-APs can be removed from the cell surface by treatment with PI-specific phospholipase C (PI-PLC). T cells treated with PI-PLC have dramatically reduced proliferation in response to stimulation with the T-cell mitogen, Concanavlin A (ConA), although anti-CD3 mAb-mediated activation was unaffected (372). Similarly, GPI^{-/-} T cell lines show severely impaired Lck and Zap-70 activation in response to TcR stimulation (373). Together, these observations support a common role for GPI-APs in membrane proximal signaling events in T cell activation.

The crosslinking of several GPI-APs, including Ly-6, Qa-2, CD48, Thy-1, CD59, CD55, CD73, and cellular prion proteins (PrPc) on the T cell surface results in the transduction of signals within the cell that can promote both proliferation and IL-2 production (374). GPI-APs have been implicated in providing both signal 1 and costimulatory signals during T cell activation (375, 376), indicating that GPI-APs may play an important role in the induction of T cell immune responses. The role of GPI-APs might play in the regulation of T cell effector function, however, is far from being understood.

1.8 Thy-1

Thy-1 (CD90, θ) is a 25 kDa glycoprotein belonging to the IgsF, which is attached to the surface of many different cell types by a GPI anchor (377). Thy-1 was identified over 45 years ago (378); however, the true functions of this GPI-AP are just starting to be elucidated. In recent years, Thy-1 has been shown to be an important signaling molecule capable of activating signaling cascades under a variety of situations. Consequently, Thy-1 signaling has been implicated in numerous biological processes, including T cell activation, apoptosis, neurite outgrowth, wound healing, fibrosis, and tumor suppression (379). Interestingly, although the ability to provide activating signals to T cells was one of the first functions attributed to Thy-1, there still remain many questions about the exact role of Thy-1 in T cell function.

1.8.1 Thy-1: Tissue Distribution and Expression

Thy-1 was first identified by Reif and Allan in 1964 and was found to be expressed in the murine thymus and brain (378). Since then, Thy-1 has been shown to be expressed at high levels on murine thymocytes and peripheral T cells, as well as on neurons, fibroblasts, epithelial cells, epidermal cells, keratinocytes, osteoblasts and myoblasts (380-383). In humans, Thy-1 is expressed on neurons, glial cells, fibroblasts, activated endothelial cells, osteoblasts, ovarian cells, a small subset of CD34⁺ hematopoietic progenitor cells and hepatic progenitor cells (384-388). Several types of cancers can also express Thy-1, including melanoma, embryonal carcinoma cells, leukemias, and gastrointestinal stromal tumors (389-393). Interestingly, Thy-1 has been implicated as a tumor suppressor for human ovarian cancer (393-395), indicating that Thy-1 signaling may prevent cell proliferation under certain circumstances.

Thy-1 is highly conserved and can be found on the fibroblasts and neurons of numerous different species, including rodents, humans, squid, frogs, chickens and dogs (382, 396), although there is some variation in the expression of Thy-1 between species. Importantly, Thy-1 is expressed on thymocytes and peripheral T cells in the mouse. In humans, however, Thy-1 is absent on mature T cells and found only on a small subset of cortical thymocytes (397).

The *Thy-1* gene is located on chromosome 9 in mice, and chromosome 11q22.3 in humans (382, 398). There are two different allelic variants of murine Thy-1, designated Thy-1.1 and Thy-1.2. Thy-1.1 is limited to the AKR/J and PL mouse strains, whereas Thy1.2 is expressed by all other mouse strains. Structurally, Thy1.1 and Thy1.2 differ by only one amino acid in position 89. This position is occupied by arginine Thy1.1, and glutamine in the Thy1.2 (396). The murine Thy-1 gene encodes for a 25 kDa core protein consisting of 111 or 112 amino acids. Thy-1 proteins are post-translationally modified by N-glycosylation, which contributes to nearly 30 percent of its molecular mass (382).

1.8.2 Thy-1 and T Cell Activation

After Thy-1 was initially discovered in 1964, it was used for many years as a marker for murine T cells. It was not until 1979 that Thy-1 was implicated in T cell activation. In the seminal study by Norcross and Smith (399), it was shown that Thy-1-specific antiserum generated by immunizing rabbits with mouse brain (rabbit anti-mouse brain or RAMB antiserum) induced the proliferation of T cells isolated from LN and spleens. Subsequently, Gunter *et al.* (400) characterized an anti-Thy-1 mAb (clone G7) that induces potent proliferation, IL-2 production and CD25 expression in a T cell hybridoma and primary T cells. A number of other anti-Thy-1 mAb have been characterized that can induce T cell activation; however, many of these require the presence of accessory cells, crosslinking by a secondary Ab, or costimulation by the phorbol ester, PMA (401).

Thy-1-deficient mice have diminished DTH reactions to haptens in comparison to wild-type mice (402). Since DTH reactions are T cell-mediated, this observation further supports a role for Thy-1 in T cell activation. Indeed, peripheral T cells isolated from Thy-1-deficient animals exhibit diminished Ca^{2+} flux, proliferation and IL-2 production in response to immobilized anti-CD3 mAb in comparison to wild-type T cells (402). Intriguingly, Thy-1-deficient thymocytes are hyper-responsive to TcR stimulation (403), indicating that Thy-1 may also negatively regulate TcR signaling under certain circumstances.

The ability of Thy-1 to initiate an antigen-independent signal 1 has clearly been demonstrated. In the initial study by Norcross and Allan (399), RAMB

antisera were only able to activate T cells in the presence of B cells, which may enable T cell activation by anti-Thy-1 mAb through Fc receptor crosslinking. Consistently, many anti-Thy-1 mAbs require crosslinking by Fc receptor-expressing accessory cells or a secondary Ab (401). An alternative explanation, although not mutually exclusive, is that the accessory cells provide costimulatory signals that enable a TcR-like signal 1 emanating from Thy-1 to promote T cell activation. A recent study by our lab provides strong evidence for the latter possibility (375). Highly purified T cells were strongly activated by the G7 anti-Thy-1 mAb in the presence of LPS-matured syngeneic bone marrow-derived DCs (BMDCs). Blocking CD28-signaling with mAbs against CD80 and CD86 severely impaired T cell proliferation, indicating that Thy-1-mediated T cell activation requires costimulatory signals. Moreover, microbeads coated with anti-Thy-1 (clone G7) and anti-CD28 mAbs induced T cell proliferation similar to beads coated with anti-CD3 and anti-CD28 mAbs.

Thy-1 may also provide costimulatory signals during TcR-mediated T cell activation. Leyton *et al.* showed that Thy-1 signaling augments anti-CD3-induced activation in hybridoma T cell and EL-4 thymoma cell lines (404). Consistently, we have shown that a mAb that blocks Thy-1 signaling (clone 30-H12) also prevents TcR-mediated activation of T cells in the presence of B cells (376). This observation not only suggests that Thy-1 signaling can provide a costimulatory signal, but that B cells may express a ligand for Thy-1. Finally, we have shown that microbeads coated with anti-CD3 and anti-Thy-1 mAbs are sufficient to activate T cells similar to beads coated with anti-CD3 and anti-CD28 (375). Together, these findings indicate that Thy-1 has the unique ability of providing either signal 1 or signal 2 during T cell activation.

1.8.3 Thy-1-Mediated Signal Transduction

A major difficulty in studying the role of Thy-1 in T cell function is that the ligand responsible for stimulating Thy-1 on T cells has not yet been identified. Consequently, much of our knowledge of Thy-1 signal transduction emanates from studies using stimulatory mAbs. Crosslinking of many receptors using Abs results in similar responses to those induced by their physiological ligands,

suggesting that this is a valuable technique for examining Thy-1 function. For example, TcR-triggering with mAbs results in comparable T cell responses to TcR-triggering by pMHC. Nevertheless, the physiological function of Thy-1 expressed on T cells will not be fully understood until its immunological ligand is discovered.

The mechanism by which GPI-APs like Thy-1, which do not extend across the plasma membrane, can trigger intracellular signal transduction pathways is also unclear. Many of the signaling molecules activated by TcR triggering are also required for Thy-1 signal transduction in T cells. Thy-1 signaling involves Ca^+ flux, the activation of the PTKs Lck and Fyn, and the activation of the MAPKs, ERK1/2, JNK and p38 MAPK (405). Moreover, we have shown that selective pharmacological inhibition of many signaling molecules involved in TcR signaling, including PTKs, calcineurin, PLC γ , PKC, PI3K, prevents Thy-1-mediated activation of T cells when costimulatory signals are provided by splenic accessory cells (406). Together, these observations combined with the fact that Thy-1 can substitute for signal 1, has led to the hypothesis that Thy-1 may somehow signal through the TcR complex (Figure 1.4A). Consistent with this, Gunter *et al.* (407), showed that expression of the complete TcR complex is required for full Thy-1-mediated T cell activation. However, Thy-1 can trigger some signaling pathways in the absence of TcR. For example, Kroczek *et al.* (408) showed that crosslinking Thy-1 transfected into B lymphoma cells results in a rapid increase in intracellular Ca^{+2} levels. Thy-1 may also transmit its signal through other signaling molecules (Figure 1.4A). Coimmunoprecipitation studies show that Thy-1 associates with Fyn, G-proteins, CD45, a p100 protein and Csk binding protein (CBP, PAG-85) (409-413), suggesting a possible role for some or all of these molecules in Thy-1 signaling. CD45 associates with both Thy-1 and TcR and CD45^{-/-} T cells do not respond to either Thy-1 or TcR triggering (411). These findings suggest that CD45 may provide the link between the TcR and Thy-1. However, Thy-1 and TcR have not yet been found to coimmunoprecipitate together (411). CBP is a transmembrane adaptor protein that contains nine Tyr residues within the cytoplasmic portion that are phosphorylated by Fyn (414).

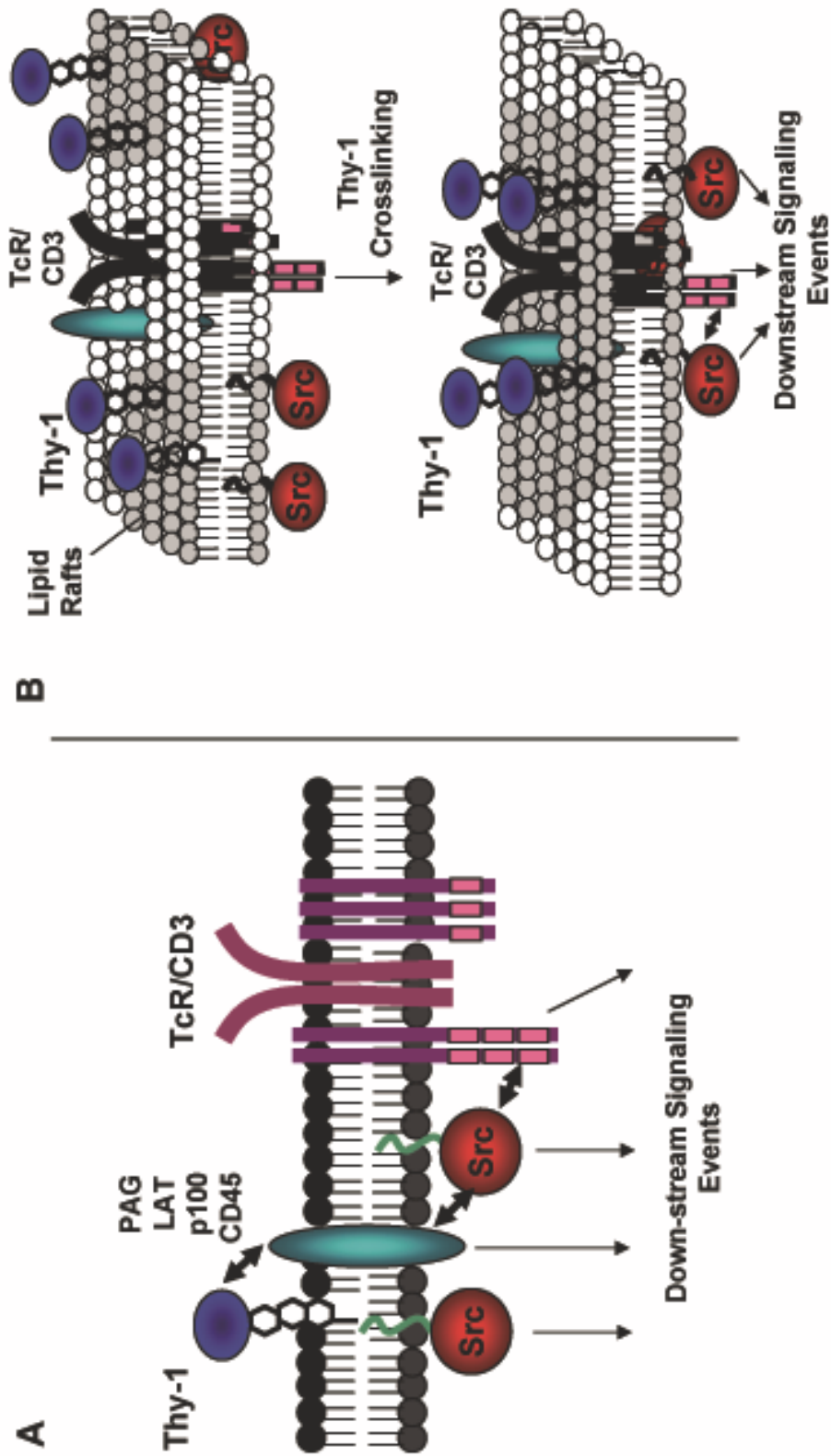


Figure 1.4. Possible Mechanisms of Thy-1-Mediated Signal Transduction. (A) Conventional signaling model; Thy-1 signals through one or more transmembrane signaling molecule(s). (B) Lipid raft aggregation model; Thy-1 crosslinking results in the reorganization of lipid rafts leading to the aggregation and activation of signaling molecules.

CBP has been implicated in negatively regulating SFK activity through recruitment of the phosphatase Csk (415). In a recent study by Chen *et al.* (416), compelling evidence is presented that suggests Thy-1 can transmit signals to the cytoplasm through CBP. Short-hairpin (sh) RNA knockdown of CBP prevented the transient anchoring of Thy-1 clusters to the actin cytoskeleton that is induced by crosslinking with mAbs. This finding indicates that CBP may be one link through which Thy-1 can communicate with cytoplasmic proteins. The authors, however, did not examine the role of CBP in other aspects of T cell activation, such as T cell proliferation and IL-2 production. Future studies are needed to determine if CBP is required for the activation of many of the other signaling molecules involved in Thy-1-mediated T cell activation. Finally, the observation that GPI-APs localize within lipid rafts has led to the proposal that crosslinking of GPI-APs induces T cell activation signals by promoting lipid raft aggregation (417) (Figure 1.4B). Coalescence of lipid rafts may aggregate other signaling molecules, such as PTKs, that are associated with these membrane domains, leading to their activation and transmission of signals (Figure 1.4B).

1.8.4 Thy-1: Induction of Effector T cell Functions

Considering the unique ability of Thy-1 to provide signal 1 or signal 2 for T cell activation, it is not surprising that Thy-1 has been implicated in the regulation of T cell effector functions. Thy-1 triggering in the presence of costimulatory signals provided by DCs promotes the expression of perforin and granzyme B in resting T cells similar to TcR signaling (375), indicating that Thy-1 signaling is sufficient to induce the expression of effector molecules involved in CTL function. In line with this, blocking Thy-1 with the nonstimulatory anti-Thy-1 mAb (30-H12) severely diminishes TcR-mediated upregulation of perforin and granzyme B (376). These findings suggest that Thy-1 signaling may play an important role in the induction of CTL responses.

Several studies have also explored the role of Thy-1 signaling during the effector phase of CTL responses. The ability of CTLs to recognize and destroy target cells can be studied *in vitro* using redirected lysis assays, where mAb against stimulatory molecules on the surface of CTLs are used to direct killing

toward FcγR-bearing target cells. Importantly, anti-Thy-1 mAbs are unable to induce granule-mediated exocytosis in redirected lysis assays using Thy-1-induced CTLs (375). The addition of anti-CD3 mAbs to the redirected lysis assays, however, enables Thy-1-induced CTLs to destroy the target cells. These findings suggest that the TcR provides a signal for granule-mediated exocytosis that is absent from the Thy-1 signaling cascade. Consistent with this idea, Thy-1 triggering using the same anti-Thy-1 mAb (clone G7) was sufficient to allow CTL clones to destroy target cells in a redirected lysis assay (418). Since the CTL clones would have received TcR signaling during initial recognition of cognate Ag, they may have already received the necessary TcR signal. It is unclear, however, what might be missing from the Thy-1 signaling pathway that can be compensated for by TcR signaling to enable CTL-mediated lysis. It is also possible that Thy-1 signaling might simply not be strong enough to induce granule-mediated exocytosis (419).

The role of Thy-1 signaling in the regulation of cytokine production and Th effector cell differentiation has not been well studied, although it is clearly established that Thy-1 signaling in the presence of costimulation upregulates IL-2 production and IL-2 responsiveness (400, 407, 420). Thy-1 signaling also induces IFN γ production by CTL clones (421). The ability of Thy-1 stimulation to induce IL-4 and IL-17 expression, however, is less clear. Intriguingly, stimulation of CD4⁺CD25⁻ T cells with immobilized anti-Thy-1 mAb in the absence of costimulatory signal results in the induction of CD25⁺ T cells with regulatory cell function (422). These Thy-1-induced T regulatory cells are different from nTreg and iTreg in that they do not express FoxP3 or make copious amounts of IL-2. The mechanism of suppression of Thy-1-induced suppressor cells, however, is at present unclear.

1.9 Rationales, Objectives and Specific Aims

Despite the many years of research that has been aimed at understanding the role of Thy-1 signaling in T cell activation, the true physiological function of T cell-associated Thy-1 remains elusive. In particular, the fact that the true physiological ligand(s) has not been identified makes it difficult to determine

under what circumstances T cells receive Thy-1 signals as well as the true nature of the Thy-1-mediated signal transduction. Consequently, most studies employ Abs that cross-link Thy-1 in order to gain some understanding of the role Thy-1 signaling plays during T cell activation. Although it is difficult to evaluate the relevance of studying Ab-mediated Thy-1 signaling in T cells until the ligand(s) is identified, the fact that crosslinking other signaling molecules with mAbs results in similar responses to those induced by their physiological ligands lends credence to studying Thy-1 in this model.

A major outstanding question in our current understanding of the role that Thy-1 plays in T cell responses is the effect of Thy-1 signaling on T cell effector functions. It has been shown that Thy-1 can provide a TcR-like signal during T cell activation in the context of strong costimulatory signals provided by BMDCs. This promotes the expression of cytotoxic effector molecules but is not sufficient to trigger the delivery of these molecules to target cells. It is unclear, however, what is missing from the Thy-1 signal that is provided by TcR signaling that makes Thy-1 provide an insufficient signal in this context. A thorough comparison of TcR and Thy-1 signal transduction may therefore elucidate differences in these signal transduction pathways that could account for this disparity. Moreover, it remains to be determined if the Thy-1 signaling also plays a role in Th cell differentiation and function. In particular, no studies to date have examined the role that Thy-1 plays in the modulation of Th cell cytokine production.

The objective of this work is to compare Thy-1 and TcR signaling in the presence of costimulatory signals provided by LPS-matured syngeneic BMDCs and examine differences and similarities in T cell activation and differentiation into T effector cells. Specific aims are: 1) determine the kinetics of Thy-1 vs. TcR activation, 2) determine why Thy-1 signals are not sufficient to induce granule-mediated exocytosis in the absence of TcR signals, and 3) determine the effect of Thy-1 signaling on cytokine production and the development of different Th cell phenotypes.

Chapter 2. Materials and Methods

2.1 Animals

Adult female C57BL/6 mice (6-8 weeks-old) were purchased from Charles River Canada (Lasalle, PQ, Canada). Mice were housed in the Carleton Animal Care Facility at Dalhousie University and were fed standard rodent chow and water supplied ad libitum. Animal protocols were consistent with the Canadian Council on Animal Care guidelines and were approved by the Dalhousie University Committee on Laboratory Animals.

2.2 Medium

Complete Roswell Park Memorial Institute 1640 medium (cRPMI) was made by supplementing RPMI (Sigma-Aldrich; Oakville, ON, Canada) with 5% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mmol/L L-glutamine and 5 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4; Invitrogen; Burlington, ON, Canada). Medium used to culture BMDCs (BMDC medium) was RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mmol/L L-glutamine, 200 U/ml penicillin, 200 µg/ml streptomycin, 5 mmol/L HEPES buffer and 50 µM β-mercaptoethanol (Sigma-Aldrich).

2.3 Cytokines and Antibodies

rmGM-CSF was purchased from R&D Systems Inc. (Minneapolis, MN, USA). rmlL-2, rmlFN-γ, rmlL-12, rmlL-4, rmlL-6 and recombinant human (rh)TGF-β1 were purchased from Peprotech (Rocky Hill, NJ, USA). Functional grade purified anti-Thy-1 mAb (clone G7, rat IgG2c), fluorescein isothiocyanate (FITC) anti-Thy-1.2 mAbs (clones 30-H12 and 53-2.1, rat IgG2b and rat IgG2a) and functional grade purified rat IgG2c isotype control were purchased from BD Pharmingen (Mississauga, ON, Canada). Functional grade purified anti-TcRβ mAb (clone H57-597, ham IgG), anti-Thy-1.2 mAb (30-H12, rat IgG2b), anti-Ly6A/E mAb (clone D7, rat IgG2a), anti-CD48 mAb (clone HM48-1, ham IgG), anti-IL-4 (clone 11B11, rat IgG1), anti-IFNγ (clone R4-6A2, rat IgG1), anti-IL-12/IL-23 p40 subunit (clone C17.8, rat IgG2a) and ham IgG isotype control were purchased from ebioscience, Inc. (San Diego, CA, USA). FITC anti-MHC class II

(clone M5, rat IgG2b), FITC anti-CD86 (clone GL1, rat IgG2a), FITC anti-CD80 (clone 16-10A1, ham IgG), FITC anti-CD69 (clone H1.2F3, ham IgG), FITC anti-CD4 (clone GK1.5, rat IgG2b), Phycoerthrin (PE) anti-CD11c (clone N418, ham IgG), PE anti-CTLA-4 (clone UC10-4B9), PE anti-CD8a (clone 53-6.7, rat IgG2a), PE anti-Gata-3 (clone TWAJ, rat IgG2b), PE anti-T-bet (clone 4B10, mouse IgG1) as well as FITC ham IgG, FITC rat IgG2b, FITC rat IgG2a, FITC rat IgG1, PE ham IgG, PE mouse IgG1, PE rat IgG2b and PE rat IgG2a isotype controls were also purchased from ebioscience. FITC anti-CD3 ϵ (clone 145-2C11, ham IgG), FITC anti-CD25 (clone PC61.5.3, rat IgG1), PE anti-TcR $\alpha\beta$ (clone H57-597, ham IgG) were purchased from Cedarlane Laboratories Inc. (Hornby, ON, Canada). Anti-survivin (clone 71G4B7), anti-CDK6 (clone DCS83), anti-CyclinD3 (clone DCS22), anti-ZAP-70 (clone 99F2), anti-phospho-ZAP-70 (Tyr³¹⁹), anti-Lck (#2752) and anti-phospho-Lck (Tyr⁵⁰⁵) were purchased from New England BioLabs (Pickering, ON, Canada). Anti-actin (clone I-19), anti-phospho-ERK1/2 (clone E-4), anti-ERK1/2 (clone K-23), anti-CD25 (clone M-19), HRP-conjugated bovine anti-goat IgG, HRP-conjugated goat anti-Rat IgG, HRP-conjugated donkey anti-rabbit IgG, and HRP-conjugated goat anti-mouse IgG were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phosphoTyr (clone 4G10) was from Millipore (Billerica, MA, USA).

2.4 Cell Lines

The murine mastocytoma cell line P815 (H-2^d) was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) and was maintained in cRPMI at 37°C and 5% CO₂.

2.5 T Cell Isolation

Mice were sacrificed by cervical dislocation and spleens or lymph nodes (inguinal, axillary, and brachial) were isolated using aseptic technique. Cell suspensions were prepared in ice-cold phosphate buffered saline (PBS, pH 7.2) solution using a tissue homogenizer. Tissue debris were removed and cell suspensions were centrifuged at 500 xg for 5 min. Erythrocytes were then depleted from the resulting cell pellets by hypo-osmotic shock. A hypotonic salt solution (4 ml of 0.2% sodium chloride (NaCl)) was first added to the cells for 20

seconds to induce erythrocyte lysis. An equal volume of a hypertonic salt solution (1.6% NaCl) was then added to restore NaCl to physiological levels (isotonic) and stop cellular lysis. Cellular debris were removed and the cell suspensions were centrifuged at 500 xg for 5 min. Cells were resuspended in MACS® buffer (2% bovine serum albumin (BSA), 2 mM ethylenediaminetetraacetic acid (EDTA) in PBS, pH 7.2) and then run through a MACS® Pre-Separation Filter (30 µm nylon mesh) (Miltenyi Biotec; Auburn, CA) in order to remove any remaining debris and to ensure a single-cell suspension. Cells suspensions were centrifuged at 500 xg for 5 min, resuspended in MACS® buffer and cell number was determined by trypan blue (Invitrogen) counts using a hemocytometer.

2.5.1 CD3⁺ T Cell Isolation

Unlabeled highly purified CD3⁺ T cells were isolated using the negative selection Pan T Cell Isolation MACS® kits from Miltenyi Biotec according to the manufacturer's instructions with slight modifications. Briefly, 1.35×10^8 splenocytes were resuspended in 450 µl of MACS® buffer and treated with 50 µl of a cocktail of biotin-conjugated mAbs against unwanted cells [anti-CD45R (B220), anti-CD49b (DX5), anti-CD11b (Mac-1) and anti-Ter-119 to remove B cells, NK cells, dendritic cells, macrophages, granulocytes, and erythroid cells] for 10 min at 4°C. An additional 400 µl of MACS® buffer was added plus 100 µl of anti-biotin microbeads. Cell suspensions were then incubated for 15 min at 4°C to allow binding between the biotin-conjugated mAbs and the anti-biotin-coated magnetic beads. The volume was then topped up to 10 ml with additional MACS® buffer and the cell suspensions were pelleted at 500 xg for 5 min in order to remove excess Ab and beads. Cell pellets were resuspended in 1 ml of MACS buffer and run through an LS column placed in a magnetic field. Cells associated with the microbeads were retained in the column, while the desired CD3⁺ T cells were eluted. The column was washed three times with 3 ml of MACS® buffer and the negatively selected CD3⁺ T cells were resuspended in cRPMI and counted using trypan blue.

2.5.2 CD4⁺ and CD8⁺ T Cell Isolation

Unlabeled highly purified CD4⁺ or CD8⁺ T cells were isolated using the negative selection isolation MACS® kits from Miltenyi Biotec according to the manufacturer's instructions. Briefly, 1×10^8 splenocytes or lymph node cells were resuspended in 400 μ l MACS® buffer and treated with 100 μ l of a cocktail of biotin-conjugated mAbs against unwanted cells [either anti-CD8a or anti-CD4 and anti-CD45R (B220), anti-CD49b (DX5), anti-CD11b (Mac-1) and anti-Ter-119] for 10 min at 4°C. An additional 300 μ l of MACS® buffer was added plus 200 μ l of anti-biotin microbeads. Cell suspensions were incubated for 15 min at 4°C to allow binding between the biotin-conjugated to the Abs and the anti-biotin attached to the magnetic beads. Cell suspensions were washed once in MACS® buffer and run through LS columns as above.

2.6 Culturing of BMDCs

BMDCs were cultured from C57BL/6 mice as previously described Lutz *et al.* (423) with slight modifications. Mice were sacrificed by cervical dislocation and tibias and femurs were isolated using aseptic technique. Bone marrow was flushed from the tibia and femurs using 10 ml of ice cold PBS (pH 7.2) and a 27G1/2 PrecisionGlide® needle (Becton Dickinson & Co. Mississauga, ON, CA;). Single cell suspensions were made by forcing the bone marrow through an 18G1 PrecisionGlide® needle (Becton Dickinson & Co.) and then cells were centrifuged at 300 xg for 5 min. Erythrocytes were depleted from the resulting cell pellets by hypo-osmotic shock (see above) and the cell suspensions were centrifuged at 300 xg for 5 min. Cell pellets were resuspended in BMDC medium and cell number was determined by trypan blue counts. Bone marrow cells (1×10^6 cells/well) were cultured in 6-well tissue culture plates (Sarstedt, Inc.; Newton, NC, USA) containing 5 ml BMDC medium supplemented with 20 ng/ml rmGM-CSF at 37°C in 5% CO₂. On day 3, an additional 5 ml of fresh BMDC medium supplemented with 20 ng/ml rmGM-CSF was added to each well. On day 6, half of the BMDC medium in each well was removed, centrifuged at 300 xg for 5 min, resuspended in fresh medium supplemented with 20 ng/ml rmGM-CSF and placed back into the original well. On day 8, non-adherent cells were transferred

into fresh 6 well-plates and half the medium was replaced with new medium supplemented with 10 ng/ml rmGM-CSF. On day 8 or 9, 1 µg/ml lipopolysaccharide (LPS, Sigma-Aldrich) was added to the cells to promote maturation. LPS-matured BMDC (nonadherent cells) were harvested on day 9 or 10 for use in subsequent experiments. BMDC were typically ≥80% pure as determined by CD11c, MHC-II, CD80 and CD86 expression using flow cytometry.

2.7 T Cell Activation

T cells were seeded in 96-well U-bottom plates (250,000 cells/well) (Sarstedt) and activated with or without LPS-matured syngeneic BMDC (8,000 cells/well) and either anti-TcRβ mAb, anti-Thy-1 mAb, anti-Ly6A/E, anti-CD48 or the appropriate isotype control in a final volume of 200 µl/well. This ratio was chosen since it was shown to promote high levels of anti-Thy-1 mAb-mediated T cell proliferation in a previous study from our laboratory (375). For larger experiments, T cells were seeded in 24-well flat-bottom plates (1.25-2.5 x 10⁶ cells/well) (Sarstedt) and activated with or without LPS-matured syngeneic BMDC (40-80 x 10³ cells/well) and either anti-TcRβ mAb, anti-Thy-1 mAb, or the appropriate isotype control in a final volume of 1 ml. All cultures were incubated at 37°C and 5% CO₂ for indicated times.

2.8 Tritiated-Thymidine Incorporation Assay

Cells in 96-well U-bottomed plates were pulsed with 0.25 or 0.50 µCi of methyl ³H-thymidine ([³H]TdR; MP Biomedicals, Irvine, CA) during the final 6 h of culture. DNA from the cell cultures was then harvested onto glass fiber filter mats (Skatron; Sterling, VA) using a Titer-Tek cell harvester (Skatron). Liquid scintillation counting was used to determine [³H]TdR incorporation which was indicative of DNA synthesis.

2.9 Analysis of Cellular Division

T cells were labeled with either 2.5 µM carboxyfluorescein diacetate, succinimidyl ester (CFSE) or 2.5 µM Oregon green in PBS (pH 7.4, 37°C) for 10 min to allow uptake of dye into the cells and conjugation of dye to proteins within the cells (424). Excess unconjugated dye was quenched by addition of 4 ml of prewarmed heat-inactivated FCS (37°C). Cells were then pelleted by

centrifugation at 500 xg for 5 min, resuspended in 10 ml of fresh prewarmed cRPMI (37°C) and incubated for 30 min at 37°C and 5% CO₂. Cells were then washed once in cRPMI, counted using trypan blue and seeded into 96-well U-bottom plates. Cells were harvested at the indicated time points following activation and the fluorescence intensity of individual cells was analyzed by flow cytometry.

2.10 Cell Surface Staining

Cells ($2.5-5 \times 10^5$) were placed into 12 x 75 mm round-bottomed polystyrene tubes and washed twice in cold fluorescence-activated cell sorting (FACS) buffer (1% BSA and 0.2% sodium azide in PBS). Cells were then incubated with FITC-, PE-, or APC-conjugated mAbs against the indicated cell surface marker or the appropriate isotype control at either concentrations recommended by the manufacturer or 1 µg/100 µl. Tubes were incubated on ice and in the dark for 45 min. Cells were then washed three times with cold FACS buffer and then fixed with 1% paraformaldehyde in PBS. Cell surface expression was then analyzed by flow cytometry.

2.11 Annexin V and Propidium Iodide Staining

Resting or activated T cells ($2.5-5 \times 10^5$) were placed in 12 x 75 mm round-bottomed polystyrene tubes and washed once with cold PBS. Cells were resuspended in Annexin-V-Fluos labeling solution containing propidium iodide (Roche Diagnostics; Laval, Quebec) according to the manufacturer's instructions and incubated for 15 min at room temperature. Annexin V and propidium iodide staining intensity was then analyzed by flow cytometry. Positive staining for Annexin V only was indicative of early apoptosis, while positive staining for both Annexin V and propidium iodide was indicative of late apoptosis or necrosis.

2.12 Intracellular Staining

Resting or activated T cells ($2.5-5 \times 10^5$) were placed in 12 x 75 mm round-bottomed polystyrene tubes and washed once with cold PBS. Cells were then permeabilized and fixed using the FoxP3 Staining kit (ebioscience) according to the manufacturer's instructions. Briefly, cells were resuspended in 500 µl of the fixation/permeabilization working solution and incubated at 4°C for 18 h in the

dark. Cells were then washed twice with 1 ml permeabilization buffer and the stained with either PE anti-T-bet (0.5 µg) mAb or PE anti-Gata-3 (0.06 µg) mAb or the appropriate isotype control in a final volume of 100 µl of permeabilization buffer for 45 min on ice in the dark. Cells were washed twice with 1 ml permeabilization buffer and then resuspended in 400 µl of FACS buffer. Fluorescence intensity was then analyzed by flow cytometry and was indicative of transcription factor expression.

2.13 Flow Cytometry

Fluorescence intensity of individual cells was determined using a FACSCaliber flow cytometer with CellQuest software (version 3.3) (Beckson Dickson; Mississauga, ON). Flow cytometry data were analyzed using FCS Express software (version 3.0; De Novo Software, Thornhill, ON). Progressive halving of the cellular fluorescence compared to resting T cells was indicative of cellular division in activated cell cultures.

2.14 Redirected Lysis Assay

Cytotoxicity mediated by redirected lysis was measured by the JAM (DNA fragmentation) assay (425). Briefly, P815 target cells were labeled with [³H]TdR (5 µCi/ml) for 4 h at 37°C in a 5% CO₂ humidified atmosphere. Following 3 washes, radiolabeled cells were resuspended in cRPMI and added in quadruplicate (5x10⁴ cells/well) into 96-well round bottom tissue culture plates (Starstedt). Radiolabeled P815 target cells were cocultured in redirected lysis assays at different target:effector cells with resting, anti-Thy-1 mAb- or anti-TcR mAb-activated T cells with LPS-matured B cells for varying lengths of time at 37°C in a 5% CO₂ humidified atmosphere. DNA was harvested onto glass fiber filtermats using a multiple sample harvester (Skatron). Radioactivity was measured in counts per minute (cpm) by liquid scintillation counting. Percent specific DNA fragmentation of P815 target cells was calculated by the formula $1 - [(C_{\text{cpm}} - E_{\text{cpm}})/C_{\text{cpm}}]$, where E is intact DNA from P815 target cells cocultured with effector cells and C is intact DNA from P815 target cells alone.

2.15 Cell Lysate Preparation

Resting or activated T cells were washed once with ice-cold PBS, centrifuged at 500 xg for 5 min and then lysed with 35 µl of ice-cold radioimmunoprecipitation (RIPA) buffer (50 mM Tris-HCL, pH 7.5, 150 mM NaCl, 50 mM Na₂HPO₄, 0.25% sodium deoxycholate [w/v], 0.1% Nonidet P-40 [v/v], 5 mM EDTA, and 5 mM ethylene glycol tetraacetic acid (EGTA)) supplemented with fresh protease and phosphatase inhibitors (1 mM Na₃VO₄, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin) for 15-30 min on ice. Cellular debris was removed from the lysates by centrifugation at 10,000 xg for 10 min at 4°C. Total protein concentration was then determined for each sample using the Bradford Assay (Bio-Rad, Hercules, CA) and then equalized to the sample with the lowest concentration. Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) sample buffer (200 mM Tris-HCL [pH 6.8], 30% glycerol [v/v], 6% SDS [w/v], 15% β-mercaptoethanol [v/v], and 0.001% bromophenol [w/v]) was added and then the lysates were heated at 90-100°C for 5 min to promote denaturation. Lysates were stored at -80°C until future use.

2.16 SDS PAGE Electrophoresis

Tris-HCL acrylamide resolving gels (7.5%-15% acrylamide, 375 mM Tris-HCL [pH 8.8], 0.1% SDS [w/v], 0.1% ammonium persulfate [APS, w/v] and 0.15% N,N,N',N'-tetramethylethylenediamine [TEMED, v/v]) with 4% acrylamide stacking gels (125 mM Tris-HCL [pH 6.8], 0.1% SDS [w/v], 0.3% TEMED [v/v] and 0.1% APS [w/v]) were cast. Cell lysates (10-20 µg protein/well) and prestained protein standards (Bio-Rad and Invitrogen) were then loaded into the gels and electrophoresed at 200 V for 1 h in SDS-PAGE running buffer (20 mM Tris-HCL [pH 8.3], 200 mM glycine, and 0.1% SDS [w/v]).

2.17 Western Blotting

Proteins separated on Tris-HCL acrylamide resolving gels were transferred onto nitrocellulose membranes using the iBlot® Dry Blotting System (Invitrogen). Membranes were washed once in Tris-buffered saline (TBS)-Tween-20 (TBST; 20 mM Tris-HCL [pH 7.6], 200 mM NaCl, 0.05% Tween-20 [v/v]) and then

blocked in TBST containing 5% fat-free milk powder [w/v] for 1 h at room temp or overnight at 4°C with gentle rocking. Membranes were washed three times with fresh TBST and then incubated in the appropriate primary Ab (typically a 1:200-1:1000 dilution in TBST containing blocking solution [5% fat-free milk powder or BSA] according to the manufacturer's instructions) for 1 h at room temperature or overnight at 4°C with gentle rocking. Membranes were washed six times with fresh TBST and then incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary Ab (typically a 1:1000 dilution in TBST containing 5% fat-free milk powder) for 1 h at room temperature with gentle rocking. Membranes were washed six more times with fresh TBST and then reacted with enhanced chemiluminescence (ECL) or ECL PLUS® reagents (GE Healthcare, Baie d'Urfe, Quebec, CA) for 1 or 5 min, respectively. Excess reagent was removed and membranes were exposed to X-ray film in the dark for varying times. Film was then developed in a Kodak X-OMAT 1000A automated X-ray developer.

2.18 Antibody Immobilization onto Carboxylate Microspheres

Polybead® carboxylate microspheres (10 µm; Polysciences, Warrington, PA) at 4.5×10^6 microspheres/ml were combined with 5 µg/ml anti-Thy-1 mAb or anti-TcRβ mAb in 1.5 ml microtubes (Sarstedt). Tubes were rotated end-over-end overnight at room temperature. Microspheres were washed three times in PBS and then resuspended in cRPMI. The number of microspheres was then determined by trypan blue counts.

2.19 T Cell Polarization

CD4⁺ T cells were seeded in 24-well flat-bottom plates (1.25×10^6 cells/well) and activated with LPS-matured syngeneic BMDC (40×10^3 cells/well) and either anti-TcRβ mAb or anti-Thy-1 mAb in the presence of different polarizing conditions: Th0 – medium alone; Th1 – 5 ng/ml rIL-12 and 10 µg/ml anti-IL-4 (clone 11B11); Th2 – 10 ng/ml rIL-4, 10 µg/ml anti-IL-12 and 10 µg/ml anti-IFNγ (clone XMG); Th17 – 100 ng/ml rIL-6 1 ng/ml rhTGFβ1, 10 µg/ml anti-IFNγ, 10 µg/ml anti-IL-4 (426). On day 3, half the media was removed and replaced with fresh media containing half the original concentration of the

respective recombinant cytokines and blocking mAbs. On day 6, T cells were harvested, washed, resuspended in cRPMI and allowed to rest for 4 h at 37°C and 5% CO₂. T cell viability was determined by trypan blue dye exclusion and T cells were seeded into 96-well U-bottom plates (1.25 x 10⁵ viable cells/well). T cells were then restimulated with either 5 ng/ml PMA and 500 ng/ml ionomycin, or anti-TcRβ mAb, anti-Thy-1 mAbs or appropriate isotype controls (alone, immobilized on microbeads or in the presence of costimulation provided by syngeneic LPS-matured BMDC) and were incubated at 37°C and 5% CO₂ for the indicated times.

2.20 Cytokine Array

Supernatants from activated T cell cultures were harvested and assayed for the presence of 22 different cytokines (G-CSF, GM-CSF, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 p40p70, IL-12p70, IL-13, IL-17, IFN-γ, MCP-1, MCP-5, RANTES, stem-cell factor (SCF), sTNF-RI, TNFα, Thrombopoietin) simultaneously using RayBio® Mouse Cytokine Array I (Raybiotech, Inc.; Norcross, GA) following the manufacturer's instructions. Briefly, each membrane containing a panel of immobilized capture Ab specific to the above cytokines was incubated for 30 min in blocking buffer at room temperature with gentle rocking. The blocking buffer was removed by gentle pipetting and the membranes were then incubated with 1 ml of the supernatants for 2 h at room temperature with gentle rocking. Membranes were washed three times (5 min/wash with gentle rocking) with Wash Buffer I, two times with Wash Buffer II and then incubated in a cocktail of biotin-conjugated anti-cytokine primary Abs diluted in the blocking buffer for 2 h at room temperature with gentle rocking. Membranes were washed three times with Wash Buffer I and two times with Wash Buffer II and then incubated with HRP-conjugated streptavidin diluted (1:1000) in the blocking buffer for 2 h at room temperature with gentle rocking. Membranes were washed again as above and then reacted with Detection Buffer at room temperature for 2 min. Excess Detection Buffer was removed and membranes were exposed to X-ray film in the dark for varying times. Film was then developed in a Kodak X-OMAT 1000A automated X-ray developer.

2.21 Enzyme-Linked Immunosorbant Assay

Supernatants from activated T cell cultures were harvested and assayed for IFN γ and IL-2 using a sandwich enzyme-linked immunosorbant assay (ELISA) kit from BD Biosciences according to the manufacturer's instructions. IL-4 and IL17A levels in the supernatants were measured using sandwich ELISA kits from ebioscience according to the manufacturer's instructions. Absorbance readings for all ELISAs were recorded at 450 nm with a wavelength correction for 570 nm using an ELx800 UV universal microplate reader (BioTek Instruments, Inc., Winooski, VT) and KCjunior software (version 1.17; BioTek Instruments, Inc.). SOFTmax[®] PRO software (version 4.3; Molecular Devices Corp., Sunnyvale, CA) was used to determine cytokine concentrations from the absorbance readings.

2.22 RNA Isolation

Cells were lysed in 1 ml of cold Trizol[®] reagent (Invitrogen) for 5-10 min at room temperature. Chloroform (0.2 ml) was added; the mixture was inverted 15 times and then incubated at room temperature for 2-3 min. The samples were then centrifuged for 15 min at 12,000 xg and 4°C to separate the mixture into aqueous and organic phases. The aqueous phase was gently transferred to a new ribonuclease (RNASE) free microcentrifuge tube and mixed with 0.5 ml of ice-cold isopropyl alcohol to precipitate out the ribonucleic acid (RNA). Following 10 min incubation at room temperature, the RNA was pelleted by centrifugation at 10,000 xg for 10 min and 4°C. The RNA was washed twice with ice-cold 75% ethanol (v/v) in water treated with 0.1% diethylpyrocarbonate (DEPC; [v/v]), air-dried and resuspended in 35 μ l of pyrogen-free water. RNA was quantified using a spectrophotometer. Purity of the RNA was determined based on the resulting A260/A280 ratio (~1.8-2.0 = good purity). The total RNA was also resolved on a 1.5% agarose gel using (Tris-acetate-EDTA) TAE running buffer (40 mM Tris-acetate, 1 mM EDTA [pH 8.0]) in order to assess the purity and quality of the RNA. RNA samples were stored at -80°C until future use.

2.23 First-Strand cDNA Synthesis

Approximately 1 µg of total RNA was reverse transcribed using Moloney murine leukemia virus (M-MLV) reverse transcriptase (RT) (Invitrogen). Total RNA was first mixed with 1 µg of random primer oligonucleotides (mostly hexamers) and 1 µl of 10 mM deoxynucleotide triphosphates (dNTPs) in a final volume of 12 µl in a nuclease-free microcentrifuge tube. The mixture was heated to 65°C for 5 min and was followed by a quick chill on ice. The contents of the tube were collected by quick centrifugation and then 4 µl of 5X First-Strand Buffer, 2 µl of 0.1 M dithiothreitol (DTT) and 1 µl of nuclease-free water were added to each sample. 200 U (1 µl) of M-MLV RT was added and samples were mixed by gentle pipetting. The reaction was incubated at 37°C for 50 min in an automatic thermal cycler (MJ Research, Inc., Watertown, MA) and then terminated by heating to 70°C for 15 min. The cDNA was used immediately or stored at -80°C for future use.

2.24 Real-time Polymerase Chain Reaction

Real-time polymerase chain reactions (RT-PCR) were carried out using the Quantifast SYBR-green RT-PCR kit (Qiagen; Mississauga, ON). A 1 µl volume of each cDNA sample was combined with 10 µl 2X Master Mix and 125 nM forward and reverse primers for IL-4, IFN γ , IL-17A or RNA POL II in a final volume of 25 µl. Negative controls did not contain any cDNA. All PCR reactions were performed in duplicate using an MX3000P quantitative PCR machine (Stratagene; La Jolla, CA). Cycling conditions included a 10 min activation step at 95°C followed by forty amplification cycles at 95°C for 10 s and 60°C for 30 sec. Data were analyzed using Stratagene MxPro version 3.0. The primers were selected because they span introns. The integrity and size of PCR products was checked using melt curve analysis and by running products on 3% agarose gels. Relative concentrations of mRNA were determined using the standard curve method whereby standard curves were generated using serial dilutions of the cDNA from activated T cells. Cytokine mRNA levels were normalized to RNA Pol II mRNA levels.

Primer sequences and amplicon sizes were as follows:

IFN γ (427) (F): 5'-ATG AAC GCT ACA CAC TGC ATC-3'
(182bp)

(R): 5'-CCA TCC TTT TGC CAG TTC CTC-3'

IL-4 (F): 5'-ACT TGA TGA GAG AGA TCA TCG GCA-3'
(208 bp)

(R): 5'-AGC TCC ATG AGA ACA CTA GAG TT-3'

IL-17(428) (F): 5'-CTC CAG AAG GCC CTC AGA CTA C-3'
(100 bp)

(R): 5'-AGC TTT CCC TCC GCA TTG ACA CAG-3'

RNA Pol II(429) (F): 5'-GCG GAT GAG GAT ATG CAA TAT GA-3'
(133bp)

(R): 5'-ACC AAG CCT TTC TCA AAA TA-3'

2.25 Statistics

Data were analyzed using the InStat statistics program (GraphPad Software Inc., San Diego, CA). Statistical comparisons were performed using the Student's t-test or one-way analysis of variance (ANOVA) and the Bonferroni multiple comparisons test.

Chapter 3. Results: Thy-1-Mediated Signal Transduction is Weaker and Has Delayed Signaling Kinetics Compared to TcR-Mediated Signal Transduction.

Over the past 30 years since Thy-1 was first implicated in T cell activation, a significant amount of evidence has emerged that has suggested that the Thy-1 signal is very similar to that provided by the TcR. Like the engagement of the TcR, crosslinking of Thy-1 with specific antibodies in the presence of costimulatory signals results in T cell proliferation, IL-2 production, CD25 expression, and the expression of the CTL effector molecules perforin and granzyme (376, 400). It is thus not surprising that numerous signaling molecules involved in TcR signal transduction, including calcineurin, PTKs, MAPKs, PI3K, and PKC, are also involved in Thy-1 signaling (406). It is important to note, however, that several differences between TcR and Thy-1 signaling do exist. For example, our lab has shown that although Thy-1 signaling can induce resting T cells to differentiate into fully-armed CTLs, the Thy-1 signal is not sufficient to allow these CTLs to mobilize their granules and destroy target cells (375). Why Thy-1 is unable to provide the necessary signal(s) to initiate granule-mediated exocytosis is unclear, however, it suggests that one or more signaling molecules are sufficiently activated by the TcR but not by Thy-1.

The kinetics of signal transduction can play an important role in determining a particular cellular response (430-432). Interestingly, a direct comparison of TcR and Thy-1 signaling kinetics has never been performed. In this section, I explored the kinetics of TcR- versus Thy-1-mediated activation of resting T cells in the presence of strong costimulation provided by LPS-matured BMDC, in order to gain a more thorough understanding of Thy-1 signaling. I show evidence that Thy-1 provides a weaker signal and has delayed signaling kinetics compared to TcR signaling. Consequently, I also show that Thy-1 may actually trigger granule-mediated exocytosis, but with delayed kinetics compared to the TcR.

3.1 Purity of Highly Purified CD3⁺ T cells Selected Using Miltenyi MACS® Pan T cell Isolation Kits.

Previous studies examining the function of Thy-1 on T cells have used two main methods for T cell isolation from spleens and lymph nodes; nylon wool

columns or T cell-enrichment immunocolumns. Both methods enrich T cell populations by removing B cells and macrophages. Nylon wool binds B cells and macrophages through binding $\beta 2$ integrins, allowing T cells and NK cells to be collected from the flow-through product (433). This method is not very effective, is time-consuming, and a second passage of the cell suspension through a fresh nylon wool column is often required for optimal removal of B cells and macrophages. On the other hand, T cell-enrichment immunocolumns contain glass beads coated with anti-Ig. The Fab portion of anti-Ig binds surface Ig on B cells, whereas the Fc region of anti-Ig binds Fc receptors on macrophages. These columns are more effective and less time-consuming at removing unwanted cells than nylon wool. Both methods, however, do not remove NK cells and an additional step of complement-mediated lysis is usually utilized to deplete NK cells. In our lab, nylon wool enrichment of splenic T cells typically results in a purity of ~81% CD3⁺ cells, whereas the immunocolumn enrichment results in ~95% CD3⁺ cells (405, 406). For my project, I adopted a T cell isolation protocol utilizing the Miltenyi MACS® magnetic separation kits. For the isolation of untouched T cells from spleens, I used the Pan T cell Isolation kit. In this system, magnetic microbeads coated with a cocktail of mAbs binds to unwanted cells, which are subsequently removed using a column in a magnetic field. There are several benefits to this isolation approach in comparison to the other two methods. It is faster, has better recovery, and removes virtually all non-T cells that could confound data interpretation. Figure 3.1A shows the typical purity of T cells isolated using this method was ~98% CD3⁺ cells, with ~96% of the cells also staining positive for Thy1.2 (Figure 3.1B).

3.2 Purity of LPS-Matured BMDCs.

DCs are the most potent pAPC at activating naïve T cell responses, in part because they express high levels of costimulatory molecules (4). If Thy-1 plays a physiological role in the activation of naïve T cells, as suggested by the unique ability of Thy-1 to provide an antigen-independent signal 1, it is conceivable that DCs may be a source of costimulatory signals during Thy-1-dependent T cell

activation. For this reason, I chose to study Thy-1 signaling in the presence of mature DCs.

Because DCs are present in very small numbers within tissues, they are very difficult and expensive to isolate in sufficient numbers for functional assays. Consequently, researchers have developed techniques for the generation of DCs in culture from precursors that are more readily available. The most commonly used method to generate murine DCs is the differentiation of bone marrow (BM) cells into DCs using GM-CSF (423). These BMDCs express high levels of MHC II, CD80, and CD86 upon maturation with LPS. For my studies, I generated syngeneic BMDCs using BM cells isolated from C57BL/6 mice. After LPS-maturation, BMDCs used for all experiments were typically >80% pure based on CD11c and MHC II expression (Figure 3.2A). The majority of the cells (>80%) also expressed high levels of CD80 and CD86 (Figure 3.2B).

3.3 Thy-1 Signaling Induces Less DNA Synthesis in Highly Purified CD3⁺ T cells than TcR Signaling.

It is well established that Thy-1 requires costimulatory signals in order to elicit substantial T cell proliferation and IL-2 production in highly purified murine T cell cultures. This is illustrated by the observations that Thy-1 is able to activate T cells only in the presence of accessory cells, phorbol esters or immobilized anti-CD28 mAb (375, 400, 401). In fact, Thy-1 signaling in the absence of costimulatory signals does not promote appreciable T cell proliferation and instead induces suppressor cell function (429). Consistent with a requirement for costimulation during Thy-1-mediated T cell activation, our lab has previously shown that murine T cells strongly proliferate and produce IL-2 in response to anti-Thy-1 mAb in the presence of LPS-matured BMDC (375). How Thy-1 signaling compares to TcR signaling in the presence of LPS-matured BMDCs in terms of strength and kinetics, however, has not been examined. To explore this question, I first performed dose-response and time-course studies using the tritiated-thymidine [³H]-TdR incorporation assay to measure new DNA synthesis, which is indicative of cells being in the S phase of the cell cycle. In kinetic studies, Gunter *et al.* (400) previously showed that the anti-Thy-1 mAb (clone G7) induces maximal DNA synthesis at 72 h in T cell-enriched spleen cell

preparations. To determine the concentration of anti-Thy-1 (clone G7) or anti-TcR β mAbs that induce a maximal mitogenic response when in the presence of mature BMDCs, I performed dose-response experiments at this time point (Figure 3.3A). Anti-Thy-1 mAb induced DNA synthesis in the T cell cultures in a dose-dependent manner, reaching the maximal response at 0.5 $\mu\text{g/ml}$. In contrast, anti-TcR β mAb induced the same amount of DNA synthesis at all of the concentrations tested, indicating that the maximal response had been reached. In separate dose-response experiments that included concentrations of anti-TcR β mAb that induced suboptimal levels of DNA synthesis, 1 $\mu\text{g/ml}$ of anti-TcR β mAb was shown to elicit maximal proliferation (Appendix 1.1). Anti-TcR β mAb consistently induced higher levels of DNA synthesis than anti-Thy-1 mAb (Figure 3.3A), suggesting that anti-Thy-1 mAb-mediated signaling is weaker than anti-TcR mAb-mediated signaling in its ability to induce T cell proliferation. In other dose-response experiments using different lots of anti-Thy-1 mAb (clone G7) purchased from the same company (BD Biosciences), there was some variability observed in Thy-1-induced DNA synthesis at 0.5 to 3.0 $\mu\text{g/ml}$ anti-Thy-1 mAb (data not shown); however, in all experiments 6 $\mu\text{g/ml}$ anti-Thy-1 mAb consistently resulted in a maximal response (Figure 3.3A and data not shown). Consequently, 6 $\mu\text{g/ml}$ mAb was chosen to compare optimal Thy-1 signaling to optimal TcR signaling in all future experiments.

The kinetics of Thy-1-induced proliferation is variable depending upon the source of costimulatory signals. Anti-Thy-1 mAb induces maximal DNA synthesis at 72 h in T cell cultures containing splenic accessory cells (400). In contrast, microbeads coated with anti-Thy-1 and anti-CD28 mAbs induce maximal DNA synthesis in highly purified T cells at 48 h (405). To determine the kinetics of Thy-1-induced proliferation in the presence of LPS-matured BMDC, I performed time course studies using the optimal concentration of Thy-1 mAb. Similar to T cell cultures containing splenic accessory cells, anti-Thy-1 mAb induced DNA synthesis that was detectable at 24 h, maximal at 72 h and had declined at 96 h in T cell cultures activated in the presence of LPS-matured BMDC (Figure 3.3B). TcR signaling had similar kinetics to Thy-1 signaling, also causing maximal DNA

synthesis at 72 h. However, Thy-1 signaling induced significantly less DNA synthesis at 48 and 72 h than TcR signaling. Taken together, my results indicate that Thy-1 signaling is weaker than TcR signaling in its ability to induce T cell proliferation.

3.4 Thy-1 Signaling Induces Less CD3⁺ T Cellular Division than TcR Signaling.

To confirm that Thy-1 signaling induces less proliferation than TcR signaling, T cell division induced by anti-Thy-1 or anti-TcR mAbs was examined using CFSE dye staining (424). CFSE is a cell-permeable fluorescent dye that covalently couples to intracellular molecules so that with each cellular division, CFSE fluorescence intensity is halved. The number of cellular divisions can be visualized by flow cytometry as decreasing fluorescence intensity. In this study, T cells were labeled with CFSE and then washed prior to incubation with stimulatory mAbs and BMDCs, which allows for the measurement of cellular division of T cells only. In contrast, the [³H]-TdR incorporation assay labels all of the cells in the culture and thus could be measuring proliferation by the BMDCs. Cellular division was not observed in either TcR- or Thy-1-stimulated T cells until 48 h of culture (Figure 3.4A). Consistent with the lower levels of DNA synthesis observed (Figure 3.3), Thy-1 signaling induced at least one less cellular division at 48, 72 and 96 h than TcR signaling. Interestingly, by 72 h a similar fraction of the initial population of resting T cells had undergone at least one cellular division. Cell counts using trypan blue exclusion dye corroborated the flow cytometry data, showing that although stimulation with either anti-Thy-1 or anti-TcR β mAbs increased the cell numbers in cultures from 48h-96h, Thy-1 signaling induced a significantly smaller increase in cell number (Figure 3.4B).

3.5 Thy-1 Signaling Induces Similar Levels of Cell Death as TcR Signaling.

It was possible that the lower levels of proliferation observed in T cell cultures activated with anti-Thy-1 mAb versus T cells activated with anti-TcR β mAb could also result from increased cell death. In fact, Thy-1 signaling has been shown to induce apoptosis in murine thymocytes (434). To determine whether Thy-1 signaling in T cell cultures was causing more cell death than TcR signaling,

T cells were stained with annexin V and propidium iodide (PI) at different time points following mAb stimulation. Annexin V binds to phosphatidylserine (PS) residues that are present on the inner leaflet of the plasma membrane in healthy cells, but flip to the outer leaflet early in apoptosis and can therefore be detected with fluorochrome-conjugated annexin V (435). PI is a membrane impermeable dye that binds to nucleic acids, and thus positive staining with PI is indicative of the loss of membrane integrity that occurs both in necrosis and late apoptosis. Lower numbers of Annexin V⁺PI⁺ T cells were observed in cultures when BMDCs were present in comparison to T cells alone between 24-96 h after initial culture. This observation was likely due to survival signals provided by the BMDCs through costimulatory signals or cytokines. Importantly, at all time points tested, there were similar percentages of cells staining Annexin V⁺PI⁻ and AnnexinV⁺PI⁺ in Thy-1- and TcR-stimulated T cell cultures (Figure 3.5). These findings suggest that there were similar levels of early apoptosis and late apoptosis or necrosis, respectively, in cultures of Thy-1- and TcR-activated T cells.

3.6 Thy-1 Signaling Induces Similar Levels of Survivin Expression as TcR Signaling.

Survivin is a member of the Inhibitor's of Apoptosis (IAP) family, whose expression is upregulated during TcR-mediated T cell activation and has been implicated in both T cell survival and cellular division (436-439). To determine if Thy-1 signaling also induces survivin upregulation, western blotting was performed (Figure 3.6B). Thy-1 and TcR signaling induced comparable levels of survivin expression at all time points examined, suggesting that the differences seen in the abilities of Thy-1 and TcR to induce T cell division were not due to differences in survivin expression.

3.7 Thy-1 Signaling Induces Similar Levels of CDK6 Expression as TcR Signaling.

Cell cycle progression is controlled by the sequential activation of a family of serine/threonine specific kinases, designated cyclin-dependent kinases (CDKs) (440). The activation of CDKs requires interaction with a family of proteins that are synthesized and degraded at different stages of the cell cycle, termed cyclins. Upon receiving activation signals through the antigen receptor, resting T cells

transition from the quiescent G_0 state into the first phase of the cell cycle, i.e., G_1 . Progression through early G_1 is regulated by CDK4 and CDK6, which are activated by D-type cyclins. CDK6 mRNA is first detectable in resting T cells within 5 h of their activation by immobilized anti-CD3 mAb and reaches peak levels by 20 h (414). To determine if Thy-1 signaling results in less proliferation than TcR signaling due to the induction of lower levels of CDK6, western blotting was performed. Analysis of CDK6 protein levels showed low levels of CDK6 protein within resting T cells (Figure 3.7A). CDK6 protein in T cells reached maximal levels 48 h after stimulation with anti-TcR mAb in the presence of costimulation provided by LPS-matured BMDC and then decreased by 72 h (Figure 3.7B). Similar kinetics of CDK6 protein expression was seen in Thy-1-stimulated T cells. Interestingly, Thy-1 signaling induced comparable levels of CDK6 protein as TcR signaling at all time points tested. Taken together, these findings suggest that the lower level of proliferation induced by Thy-1 signaling compared to TcR signaling is not due to a difference in the induction of the cell cycle regulator CDK6.

3.8 Thy-1 Signaling Induces Lower Levels of CyclinD3 Expression than TcR Signaling.

CDK6 is activated through interaction with D-type cyclins (440). Three D cyclins have been identified, designated D1, D2 and D3. T cells have been shown to express cyclinD2 and cyclinD3 during the G_1 phase of the cell cycle (440). In response to stimulation with immobilized anti-CD3 mAb, T cells upregulate the expression of cyclinD2 and cyclinD3 mRNA, which reach peak levels at 10 h and 20 h after activation, respectively (441). To determine if Thy-1 signaling results in less proliferation than TcR signaling due to the induction of lower levels of cyclinD3, western blotting was performed (Figure 3.8A). Cyclin D3 protein was detected at low levels in resting T cells. In response to anti-TcR stimulation in the presence of costimulation provided by LPS-matured BMDC, cyclinD3 protein was upregulated within 24 h, reached maximal levels at 48 h and then diminished after 72 h. Cyclin D3 expression most likely decreased after 48 h due to a decrease in the percentage of total cells that were in the G_1 phase of the cell cycle. Although Thy-1 signaling induced a comparable increase in cyclinD3

protein levels after 24 h of activation, at 48 h Thy-1 signaling induced significantly less cyclinD3 than TcR signaling (Figure 3.8B). Thus, an inability of Thy-1 signaling to induce as much cyclin D3 as TcR signaling could contribute to the weaker mitogenic response of T cells to Thy-1 stimulation.

3.9 Thy-1 Signaling Induces Lower Levels of High Affinity IL-2 Receptor α Chain (CD25) Expression Than TcR Signaling.

A hallmark of T cell activation is expression of the high affinity IL-2 receptor α chain (CD25) and the concomitant production of IL-2 by T cells. It is well established that Thy-1 signaling in T cells induces IL-2 production and CD25 expression (400). How Thy-1 signaling compares to TcR signaling in its ability to promote CD25 expression and IL-2 production, however, has not been studied. Time course assays using western blotting showed that CD25 protein was detected in T cells activated with either anti-TcR β or anti-Thy-1 mAb in the presence of costimulatory signals provided by LPS-matured BMDC as early as 12 h after stimulation (Figure 3.9A). Murine CD25 was detected as multiple protein bands at approximately 55 kDa most likely due to heterogenous post-translational modifications at multiple N- and O-glycosylation sites (442). Similar to proliferation, the kinetics of CD25 expression was comparable for both Thy-1 and TcR signaling. In response to both stimuli, CD25 expression was detectable by 12 h, reached maximal levels after 48 h and began to diminish by 72 h. Consistent with being a weaker activating signal, however, Thy-1 signaling also induced significantly lower levels of CD25 protein at 24 and 48 h following activation than TcR signaling (Figure 3.9B). CD25 must be expressed on the cell surface with the IL-2R β and common gamma (γ_c) chains in order for T cells to become highly sensitive to IL-2 (443). Since Western blotting measures all CD25 protein within the cell irrespective of its location, I next measured cell surface protein levels of CD25 by flow cytometry following T cell activation (Figure 3.9C). Although both TcR and Thy-1 signaling induced CD25 expression on the surface of T cells 24 h following activation, at all time points tested (24, 48 and 72 h) the percentage of CD25 positive cells was less in Thy-1 activated T cell cultures. Moreover, 24 h and 48 h following activation, the mean channel fluorescence (MCF) of CD25 expression in Thy-1-activated T cells was significantly lower than

that in TcR-activated T cells (Figure 3.9D). Since MCF is the measurement of the mean fluorescence that the cells within a sample emit and is therefore an indicator of the average amount of protein expressed by each cell within a given population, these data indicate that on average Thy-1-activated T cells express lower levels of CD25 than TcR-activated T cells.

3.10 Thy-1 Signaling Induces Less IL-2 Production Than TcR Signaling.

To determine how Thy-1 signaling compares to TcR signaling in its ability to promote IL-2 production, sandwich ELISAs were performed. Anti-TcR β mAb stimulation induced high levels of IL-2 production in T cells activated in the presence of LPS-matured BMDCs for 24 h (Figure 3.10A). IL-2 levels then diminished 48 and 72 h after activation. This reduction in IL-2 protein was most likely due to increased consumption of the IL-2 by the higher numbers of activated T cells. Anti-Thy-1 mAb induced similar kinetics of IL-2 production, i.e. high levels of IL-2 were seen following 24 h of activation and diminished by 48 and 72 h. Consistent with Thy-1 providing a weaker signal, T cells stimulated with anti-Thy-1 mAb produced significantly less IL-2 than T cells stimulated with anti-TcR β mAb at all time points examined. A major function of IL-2 during T cell activation is the induction of T cell proliferation (443). IL-2 signaling in T cells also enhances IL-2 responsiveness by increasing cell surface expression of CD25 (444). It is plausible that Thy-1 signaling may induce lower levels of proliferation and CD25 expression than TcR signaling because of its weaker ability to induce IL-2 production. To explore this question, rIL-2 was added to T cells activated with anti-Thy-1 mAb to determine if additional IL-2 would allow Thy-1-stimulated T cells to proliferate at similar levels as TcR-stimulated T cells. Figure 3.10B shows that the addition of rIL-2 at 25-100 U/ml did not significantly increase Thy-1-induced T cell proliferation, indicating that reduced IL-2 levels do not account for reduced T cell proliferation following Thy-1 signaling in comparison to TcR signaling. The IL-2 activity was verified through the successful culture of the IL-2-dependent murine T cell line CTLL-2 (data not shown).

3.11 Thy-1 Signaling Induces Weaker and Delayed Protein Tyrosine Phosphorylation Compared to TcR Signaling.

One of the earliest biochemical events during TcR signal transduction is the phosphorylation of Tyr residues on proteins by PTKs. It has previously been shown that PTKs also play an important role in Thy-1-mediated T cell activation. Indeed, inhibition of src-family PTKs with the inhibitor Herbamycin A prevents both TcR- and Thy-1-induced proliferation in T cells (406). To determine if differences exist in the abilities of TcR and Thy-1 signaling to activate PTKs in terms of strength, I examined protein Tyr phosphorylation patterns by western blotting for various times following T cell activation in the presence of costimulatory signals provided by LPS-matured BMDCs. In short time-courses (Figure 3.11A), increases in the Tyr phosphorylation of multiple proteins (~115 kDa, 70 kDa, 55 kDa, 50 kDa, 35 kDa) were observed in TcR-activated cultures as early as 5 min following activation when compared to T cells and BMDCs cultured alone. It is worth noting that the BMDCs constituted only 3.2% of the cell culture. Since the BMDCs make up such a small portion of the cell culture, it is unlikely that the changes observed in protein phosphorylation were due to changes within the BMDCs. Rather; such changes can most likely be attributed to the T cells. In Thy-1-stimulated T cell cultures, no changes in protein Tyr phosphorylation were observed at early time points following activation when compared to T cells and BMDCs cultured alone. To determine if Thy-1 signaling would induce similar levels of protein Tyr phosphorylation to TcR at later time points, I next performed a longer time course (Figure 3.11B). Protein Tyr phosphorylation was not observed in response to anti-Thy-1 mAb-mediated T cell activation until 60 min following initial activation. At this time point, there was enhanced phosphorylation of a ~55 kDa protein that was also observed within the TcR-activated cultures. Tyr phosphorylation of this protein in Thy-1-activated T cells was weaker at this time point in comparison to TcR-activated T cells. Thy-1 signaling induced maximal levels of Tyr phosphorylation 120 and 240 min after T cell activation on ~115, 76, 70 and 55 kDa proteins that were also phosphorylated in TcR-activated T cells. Interestingly, the ~35 kDa protein was phosphorylated in TcR-activated T cell cultures at early time points was not phosphorylated at

any time point examined in Thy-1-activated T cell cultures and is consistent with differential signaling mediated by Thy-1 and the TcR. Both Thy-1- and TcR-induced protein Tyr phosphorylation diminished 480 min after activation. Taken together, these results are consistent with Thy-1 signaling being weaker and having delayed signaling kinetics in promoting protein Tyr phosphorylation in comparison to TcR signaling.

3.12 Thy-1 Signaling Induces Delayed ZAP-70 Phosphorylation Compared to TcR Signaling.

An important membrane proximal event following engagement of the TcR is the Tyr phosphorylation and activation of ZAP-70 (14). Considering the fact that Thy-1 was implicated in providing T cell activation signals over 30 years ago (399), it is surprising that a role for ZAP-70 in Thy-1-mediated T cell activation has never been established. Having observed the Tyr phosphorylation of a ~70 kDa protein in both Thy-1 and TcR-activated T cells (Figure 3.11B), I hypothesized that Thy-1 signaling induced the phosphorylation and activation of ZAP-70, which is a 70 kDa protein. Full activation of ZAP-70 requires phosphorylation of at least two different Tyr residues, Tyr319 and Tyr493, through both transphosphorylation by the src family kinase Ick and autophosphorylation (14). As shown in Figure 3.12A, ZAP-70 is phosphorylated at Tyr319 as early as 5 min after T cell activation with anti-TcR β mAb. No phosphorylation of ZAP-70, however, was observed in Thy-1-stimulated T cells within the first 60 min of activation. In longer time course studies, ZAP-70 phosphorylation was not observed until 60 min following Thy-1-stimulation of T cells (Figure 3.12B). Combined optical density ratios of phospho-ZAP-70 to total ZAP-70 from 3 separate short and 3 separate long time courses revealed that Thy-1 consistently induced maximal phosphorylation of ZAP-70 at a level comparable to maximal TcR-induced ZAP-70 phosphorylation only after 120 min of activation (Figure 3.12C). These results show that, although Thy-1 signaling is able to induce equal levels ZAP-70 phosphorylation to TcR signaling, the kinetics of anti-Thy-1-induced ZAP-70 activation are significantly delayed.

3.13 Thy-1 Signaling Induces Delayed Lck Serine Phosphorylation Compared to TcR Signaling.

During TcR-mediated T cell activation, Lck is responsible for the initial phosphorylation and activation of ZAP-70 (14). A possible functional role for Lck in Thy-1-mediated T cell activation has also been implicated by studies showing that Lck can be coimmunoprecipitated with Thy-1 in murine 2B4 hybridoma cells and thymocytes (409, 445). The exact role that Lck may play in Thy-1-mediated T cell activation, however, has not been elucidated. In order to try to further clarify the role of Lck in Thy-1 signaling, I employed western blotting to determine if Thy-1 and TcR signaling have a differential effect on Lck activation. It is widely believed that Lck is activated upon dephosphorylation of the inhibitory Tyr residue Tyr505 by the phosphatase CD45, which is accompanied by autophosphorylation at the activating Tyr residue Tyr394 (11, 12). Unexpectedly, there was no discernible difference in the phosphorylation status of Tyr505 of Lck in T cells after activation at various time points with either anti-TcR or anti-Thy-1 mAb compared to T cells that did not receive stimulation (Figures 3.13A and B). Unfortunately, antibodies against phosph-Tyr394 are not commercially available. Interestingly, several higher molecular weight (MW) bands appeared on blots probed with Ab against total Lck following activation with anti-TcR mAb for 60 min (Figure 3.13B). Similar patterns of higher molecular weight bands appeared in Thy-1-stimulated T cells after 60 min of T cell activation; however, the density of the high MW bands were not as strong as those seen in TcR-stimulated T cells until 240 min after activation. The same banding patterns were seen at the same time points in blots probed with the specific mAb against Lck Tyr505, suggesting that these Lck species were phosphorylated at the inhibitory Tyr residue. These banding patterns upon activation of T cells have previously been shown to be due to the phosphorylation of several serine residues on Lck (446, 447). One such serine phosphorylation event has been shown to be mediated by ERK1/2. Although these results do not shed any light on the ability of Thy-1 signaling to promote initial Lck activation, they do suggest that downstream signaling pathways such as the ERK pathway, which modify Lck through serine phosphorylation, are activated by Thy-1 signaling in a fashion similar to TcR signaling. Putative Thy-1-mediated

serine phosphorylation of Ick, however, is weaker and delayed compared to TcR-mediated and may suggest that Thy-1 elicits delayed activation of ERK.

3.14 Thy-1 Signaling Induces Delayed ERK-1/2 Phosphorylation Compared to TcR Signaling.

The ERK signaling pathway plays an important role in TcR-mediated T cell activation, survival and cytotoxic effector function (448-450). The ERK signaling pathway is also activated in response to Thy-1 and CD28 signaling, and pharmacological inhibition of ERK prevents Thy-1-induced T cell proliferation and IL-2 production (405). How ERK activation induced by Thy-1 signaling compares to TcR signaling in terms of strength and kinetics has not yet been examined. Figure 3.14A shows that at early time points following T cell activation, TcR signaling induced ERK1/2 phosphorylation as early as 5 min after stimulation. In contrast, there was no detectable increase ERK1/2 phosphorylation above basal levels in response to Thy-1 signaling as late as 60 min following activation. At even later time points, increased ERK1/2 phosphorylation was initially observed in Thy-1-stimulated T cells 120 min following activation. Combined optical density ratios of phospho-ERK1/2 to total ERK1/2 from 3 separate short and 3 separate long time courses revealed that Thy-1 signaling consistently induced maximal phosphorylation of ERK1/2 at a level comparable to that of maximal TcR-induced ERK1/2 phosphorylation only after 240 min of activation (Figure 3.14C), indicating that Thy-1 signaling induces delayed activation of ERK1/2 compared to TcR signaling.

3.15 Thy-1 Stimulation Induces CTLs That Destroy Target Cells, Although With Delayed Kinetics Compared to TcR-Induced CTLs.

Naïve CD8⁺ T cells differentiate into CTLs, which are capable of recognizing and destroying target cells, after receiving TcR and costimulatory signals. Fully differentiated CTLs then require an additional TcR signal in order to mobilize their cytotoxic granules and destroy target cells. Consistent with the hypothesis that Thy-1 provides a TcR-like signal, our lab has previously shown that Thy-1 signaling with strong costimulation provided by LPS-matured BMDC promotes the differentiation of resting T cells into fully-armed CTLs that express both perforin and granzyme B (375). In this study, ⁵¹Cr-release redirected lysis

assays were employed to determine if Thy-1-induced CTLs could destroy FcRγ-bearing P815 target cells when triggered by Thy-1 signaling. Surprisingly, although Thy-1-induced CTLs formed stable conjugates with the target cells, the lytic granules did not polarize towards the site of contact and the CTLs were unable to destroy the target cells within the 4 h experiment. In contrast, TcR-induced CTLs polarized their granules and destroyed target cells with 20%-80% specific lysis depending upon the effector cell to target cell ratio (2.5:1 – 50:1, respectively). Based on these findings, Haeryfar *et al.* (375) concluded that the Thy-1 signal is insufficient to trigger granule-mediated exocytosis and that Thy-1 must therefore provide an incomplete signal 1 during T cell activation. We have hypothesized that some key signaling pathway required for granule-mediated exocytosis is not sufficiently activated by Thy-1 signaling. Thy-1-induced CTLs, however, were not incubated with target cells for longer than 4 h and so a delayed response could not be ruled out.

The observation that stimulation of resting T cells, with a concentration of anti-Thy-1 mAb that induces a maximal mitogenic response in the presence of costimulatory signals provided by LPS-matured BMDCs, results in the delayed activation of several key signaling molecules involved in TcR signaling, led me to hypothesize that Thy-1-induced CTLs might be able to destroy target cells with delayed kinetics. To confirm this hypothesis, I measured DNA fragmentation in [³H]TdR-labeled P815 target cells exposed to Thy-1 or TcR-induced CTLs in redirected lysis assays. Since DNA fragmentation is a hallmark of apoptosis, this method allows for the specific detection of apoptosis triggered in target cells by CTLs (425). Since DNA fragmentation is an earlier event in apoptosis than membrane disintegration, these assays are more sensitive than ⁵¹Cr release assays at detecting CTL-mediated cytotoxicity. Moreover, ⁵¹Cr leaks out of cells at later time points whereas [³H]TdR does not. In Figure 3.15A, resting T cells were first differentiated into CTLs by stimulation with either anti-TcRβ or anti-Thy-1 mAbs for 72 h. To confirm our previous results, I incubated TcR- or Thy-1-induced CTL effector cells with [³H]TdR-labeled P815 target cells for 4 h at increasing effector cell to target cell ratios (2.5:1 to 50:1). As expected, TcR-

induced CTLs promoted DNA fragmentation in target cells that was more robust with increasing effector to target ratios, reaching approximately 60% specific DNA fragmentation at a ratio of 50:1. Consistent with our previous findings, Thy-1-induced CTLs did not induce DNA fragmentation in P815 target cells at any of the effector cell to target cell ratios tested at 4 h. Interestingly, if Thy-1-induced CTLs were incubated with the P815 target cells for longer periods of time, a significant amount of DNA fragmentation was observed. Figure 3.15B shows that significant Thy-1-induced DNA fragmentation was seen 12 and 24 h after exposure of P815 target cells to Thy-1-induced CTLs when compared to the negative control. In contrast, TcR-induced CTLs triggered maximal amounts of DNA fragmentation after only 4 h of culture. Although nonspecific killing by CTL necrosis and release of cytolytic granule contents has not been ruled out, these results are consistent with Thy-1 providing a signal sufficient to trigger cytotoxic effector function, albeit with delayed kinetics compared to TcR signaling.

3.16 Thy-1 Signaling Promotes Delayed Acquisition of Cytotoxic Effector Function When Compared to TcR Signaling.

Our group has previously shown that 72 h following either Thy-1- and TcR-signaling with strong costimulation by LPS-matured BMDC, T cells express comparable levels of perforin and granzyme mRNA by semi-quantitative polymerase chain reaction (PCR) (375). How Thy-1- and TcR-signaling compare in terms of the kinetics of the differentiation of naïve T cells into CTLs in terms of kinetics has not been explored. Based on the above findings, I hypothesized that Thy-1-activated T cells would acquire the ability to destroy target cells with delayed kinetics compared to TcR-activated T cells. To confirm this hypothesis, I activated T cells with either anti-Thy-1 or anti-TcR β mAb and costimulation provided by LPS-matured BMDC for 0, 4, 18 or 24 h and then analyzed their ability to destroy P815 target cells by the DNA fragmentation assay. In Figure 3.16A, activated T cells were incubated with P815 target cells for 4 h and then DNA fragmentation was measured. T cells gained the ability to trigger DNA fragmentation in target cells as early as 18 h following activation with anti-TcR β mAbs. Consistent with my previous observations, T cells cultured with anti-Thy-1 mAbs for as long as 24 h were not able to trigger significant DNA fragmentation

in target cells within 4 h. Interestingly, T cells initially activated with anti-Thy-1 mAbs for as little as 18 h had acquired the ability to induce DNA fragmentation in target cells in 24 h DNA fragmentation assays (Figure 3.16B). It is important to note that following the initial activation, T cells were washed and counted with trypan blue prior to plating with P815 target cells in order to ensure equal numbers of viable effector cells were added to each well. BMDCs made up 3.2% of the cells at the start of activation but they could not be removed with any confidence. As a result, the DNA fragmentation assay contained activated T cells, P815 target cells and any residual BMDCs. Combined with the observation that a significant amount of anti-Thy-1 mAb and anti-TcR β mAb stayed on the T cell surface for as long as 72 h following initial activation (375), the presence of BMDCs in the DNA fragmentation assays means that the T cells were likely still receiving Thy-1 or TcR signals combined with costimulatory signals. Consequently, in experiments where resting T cells were only briefly (0 h initial activation) incubated with anti-TcR β mAb and BMDC and then immediately washed and plated with P815 target cells for 24 h, ~80% DNA fragmentation was observed. This was comparable to the percentage of DNA fragmentation observed when T cells were initially activated for 24 h with anti-TcR β mAb and BMDC and then exposed to P815 cells for 4 h (Figure 3.16A and B) and was likely due to the presence of BMDCs in the DNA fragmentation assays. It is also difficult to determine the exact amount of time required for Thy-1 signaling to differentiate T cells into CTLs. What is clear, however, from these results is that T cells require a longer duration of Thy-1 signaling than TcR signaling to acquire cytotoxic effector function.

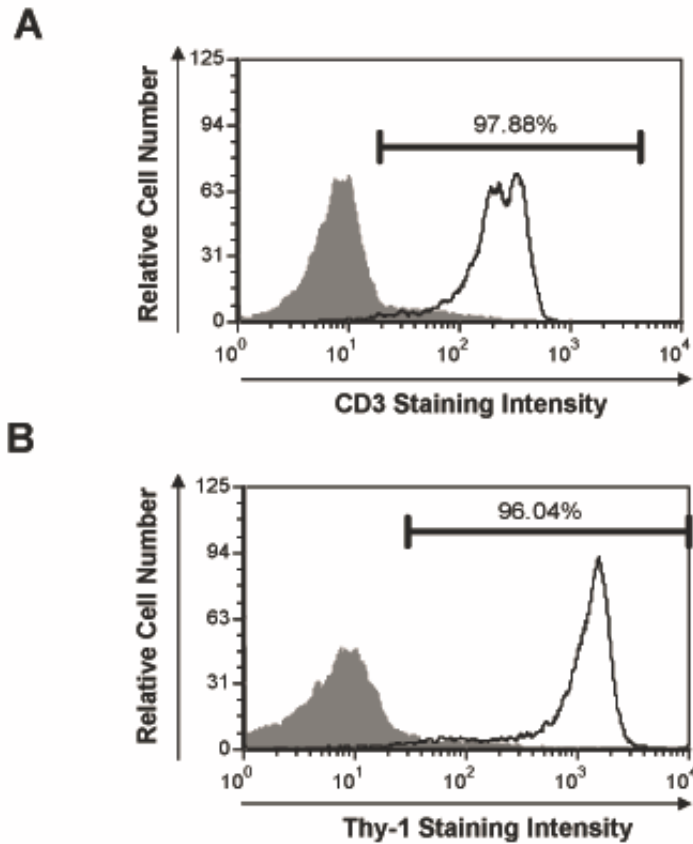


Figure 3.1. Purity of Highly Purified CD3⁺ T cells Obtained Using Miltenyi MACS® Pan T cell Isolation Kits. Highly purified CD3⁺ T cells were isolated from spleens taken from C57BL/6 mice using negative selection Pan T cell isolation kits from Miltenyi Biotech®. (A) Purity of T cells was assessed by labeling cells with anti-CD3-FITC or Hamster IgG-FITC and analyzing the cells by flow cytometry. (B) Cells were labeled with anti-Thy-1 (30-H12)-FITC or the appropriate FITC-labeled isotype control and then analyzed by flow cytometry.

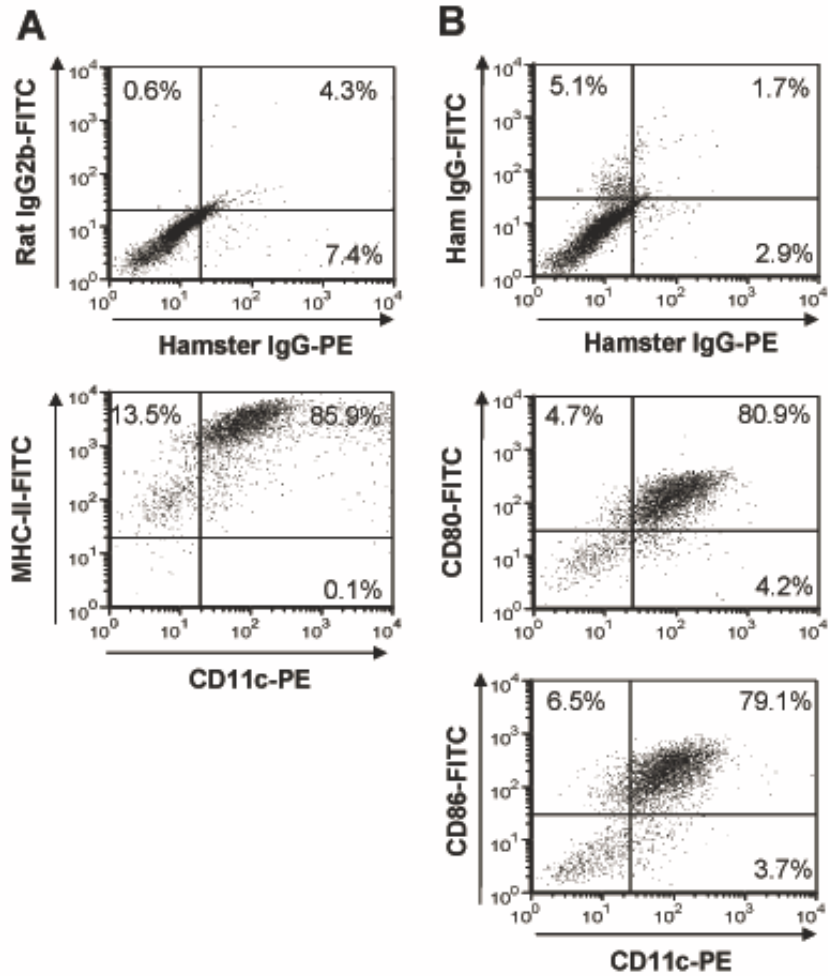
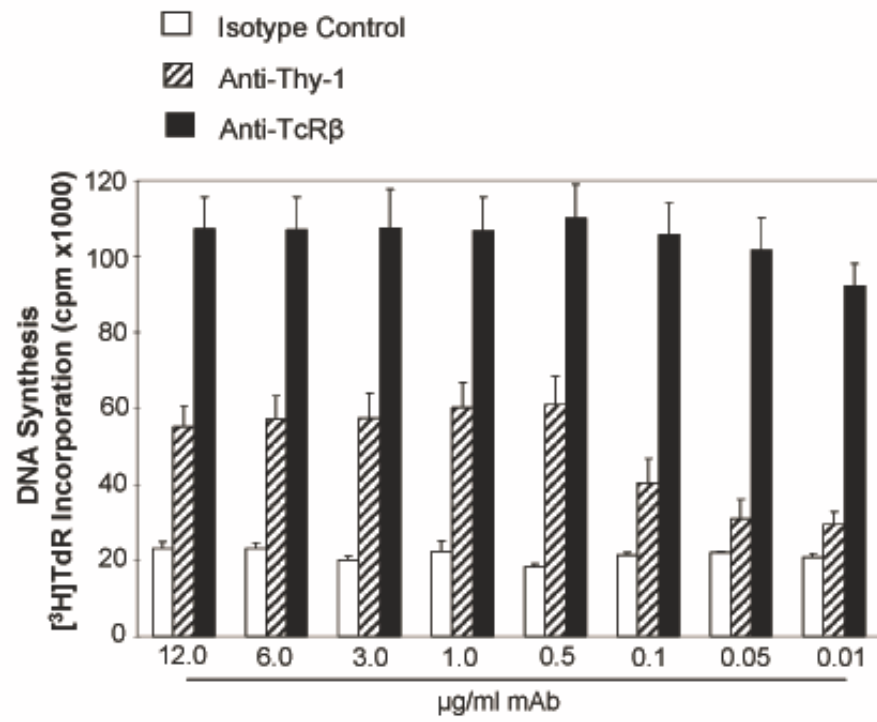


Figure 3.2 Purity of LPS-Matured BMDCs. Bone marrow cells from C57BL/6 mice were cultured for 10 days in the presence of GM-CSF. On day 9, the cells were treated with 1 $\mu\text{g/ml}$ LPS in order to promote maturation of the BMDC. Cells were harvested on day 10 and stained with (A) MHC-II-FITC and CD11c-PE or the appropriate FITC- or PE-labeled isotype controls and (B) either CD80- or CD86-FITC and CD11c-PE or the appropriate FITC- or PE-labeled isotype control. Cells were then analyzed by flow cytometry.

Figure 3.3. Thy-1 Signaling Induces Less DNA Synthesis in Highly Purified CD3⁺ T cells than TcR Signaling. Highly purified CD3⁺ T cells (2.5×10^5) with or without LPS-matured BMDCs (8×10^3 cells/well) were seeded in triplicate into 96-well round-bottom plates. **(A)** T cells were then cultured in the presence of the indicated concentrations of anti-Thy-1 mAb (clone G7), anti-TcR β mAb or isotype control (rat IgG2c) for 72 h. or **(B)** in the presence of 6 μ g/ml of anti-Thy-1 mAb, anti-TcR β mAb or the appropriate isotype control for the indicated times. Wells were pulsed with [³H]TdR 6 h before the endpoint at which time the cells were harvested and DNA synthesis was determined based on [³H]TdR incorporation. Data are the mean of 3 independent experiments \pm SEM. * denotes $p < 0.05$ as determined by the Bonferroni multiple comparisons test.

A



B

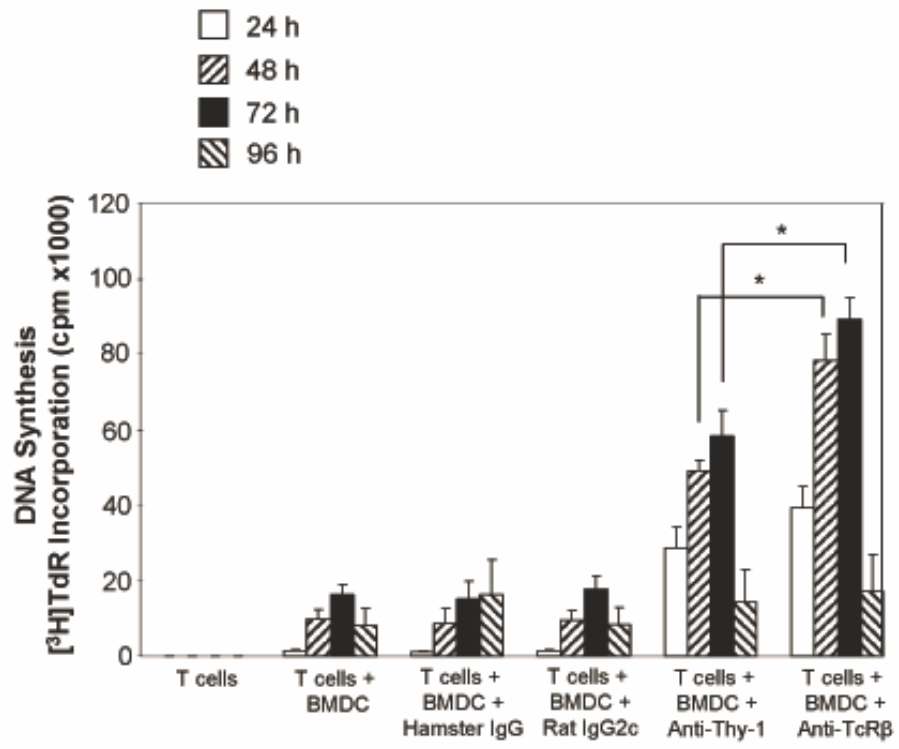
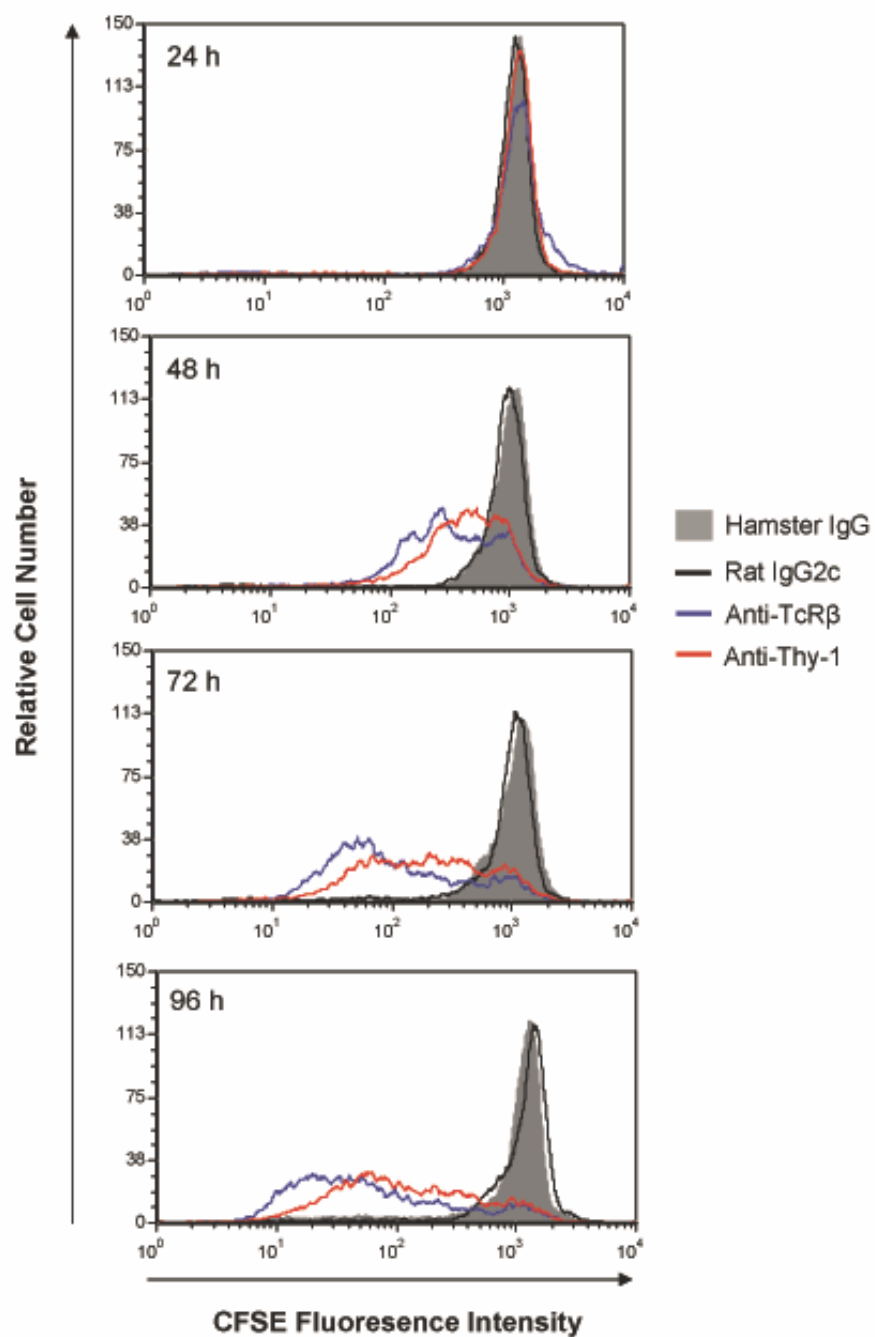


Figure 3.4. Thy-1 Signaling Induces Less Highly Purified CD3⁺ T Cell Division Than TcR Signaling. (A) Highly purified CD3⁺ T cells were labeled with 5 μ M CFSE and seeded in duplicate into 96-well round-bottom plates (2.5×10^5 cells/well) with or without LPS-matured BMDCs (8×10^3 cells/well). T cells were then cultured in the presence of 6 μ g/ml anti-Thy-1 mAb (clone G7), anti-TcR β and/or the appropriate isotype control for the indicated times. Wells were pooled and CFSE staining was measured by flow cytometry. The number of cellular divisions are indicated by decreasing fluorescence intensity. Data from one representative experiment are shown (n=3). (B) Highly purified CD3⁺ T cells (2.5×10^5 cells/well) were seeded in quadruplicate into 96-well round-bottom plates with or without LPS-mature BMDCs (8×10^3 cells/well). T cells were then cultured in the presence of 6 μ g/ml anti-Thy-1 mAb, anti-TcR β mAb and/or the appropriate isotype control for the indicated times. Wells were pooled and the number of cells in each treatment was determined under light microscopy using trypan blue. Average total number of cells per well was then calculated. Data from three independent experiments are expressed as mean total number of cells \pm SEM; * denotes $p < 0.05$, ** denotes $p < 0.01$ and *** denotes $p < 0.001$ as determined by the Bonferroni multiple comparisons test.

A



B

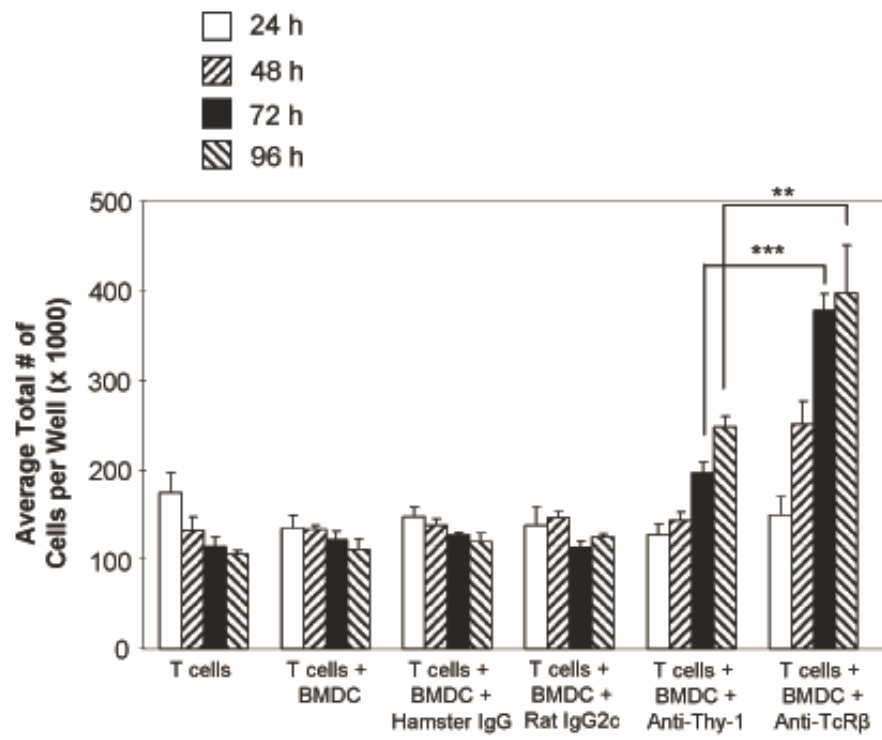


Figure 3.5. Thy-1 Signaling Induces Similar Levels of Cell Death as TcR Signaling. Highly purified CD3⁺ T cells (2.5×10^5 cells/well) with or without LPS-matured BMDCs (8×10^3 cells/well) were seeded in duplicate into 96-well round-bottom plates. T cells were then cultured in the presence of 6 $\mu\text{g/ml}$ anti-Thy-1 mAb (clone G7), anti-TcR β mAb and/or the appropriate isotype control for the indicated times. Cells were then collected, stained with annexin V-FITC and PI and then analyzed by flow cytometry. (A) Data shown are from the 72 h time point and are representative of three independent experiments. (B) The percent of cells that were early apoptotic (Annexin V⁺PI⁻) and late apoptotic/necrotic (Annexin V⁺PI⁺) were determined from three independent experiments and are expressed as the mean \pm SEM. Differences between anti-TcR β mAb-stimulated T cells and anti-Thy-1 mAb-stimulated T cells were not significant as determined by the Bonferroni multiple comparisons test.

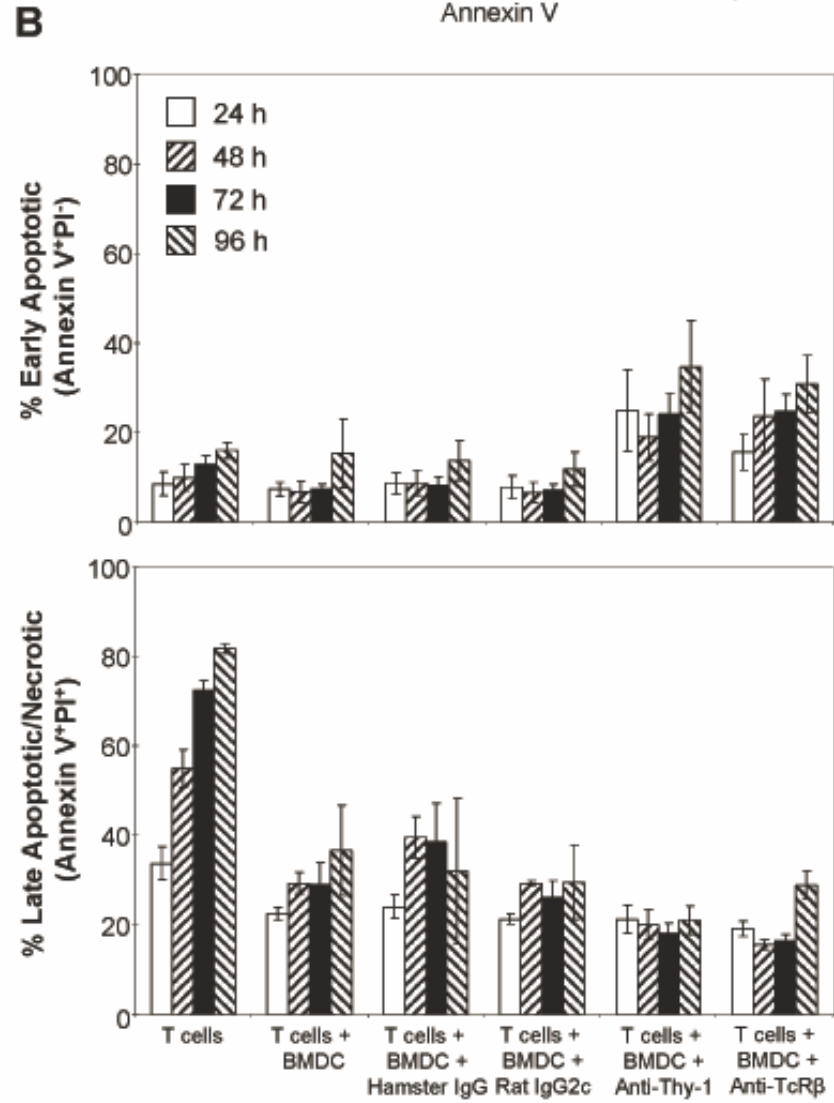
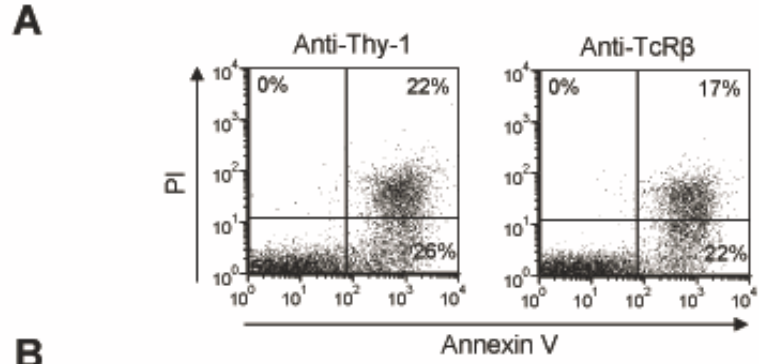


Figure 3.6. Thy-1 Signaling Induces Similar Levels of Survivin Expression as TcR Signaling. (A) Highly purified CD3⁺ T cells (2.5×10^6 cells/well) with or without LPS-matured BMDCs (8×10^4 cells/well) were seeded into 24-well plates. T cells were then cultured in the presence or absence of 6 $\mu\text{g/ml}$ anti-Thy-1 mAb (clone G7), anti-TcR β mAb and/or the appropriate isotype control for the indicated times. Cell lysates were prepared and survivin protein levels (16 kDa) were assessed by western blotting. Blots were then reprobbed with antibodies specific for actin (42 kDa) to confirm equal loading. Data are representative of three independent experiments. (B) Optical density ratios were calculated by comparing the density of individual survivin bands from three independent experiments with the corresponding actin band. The 0 h time point represents T cells treated with hamster IgG or Rat IgG isotype controls. Data are shown as mean \pm SEM; ns denotes not significant as determined by the Student's t-test.

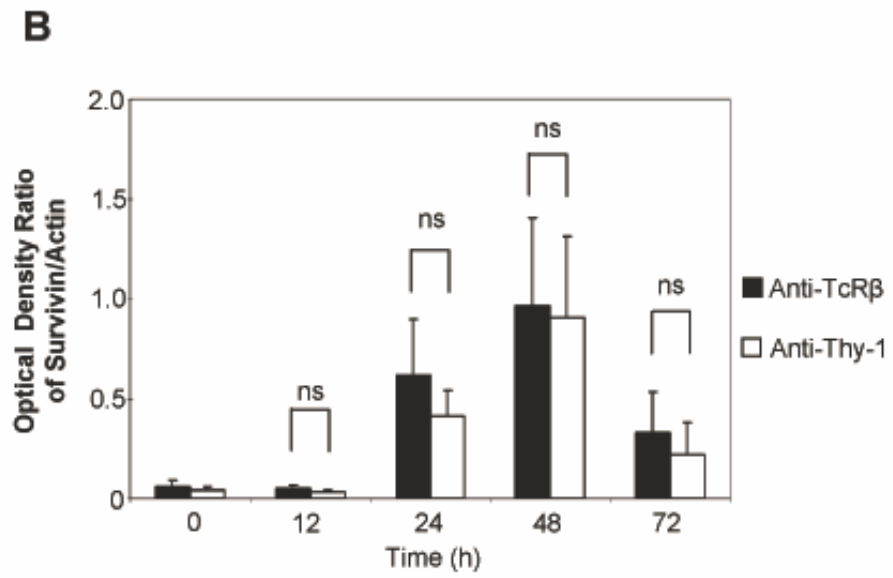
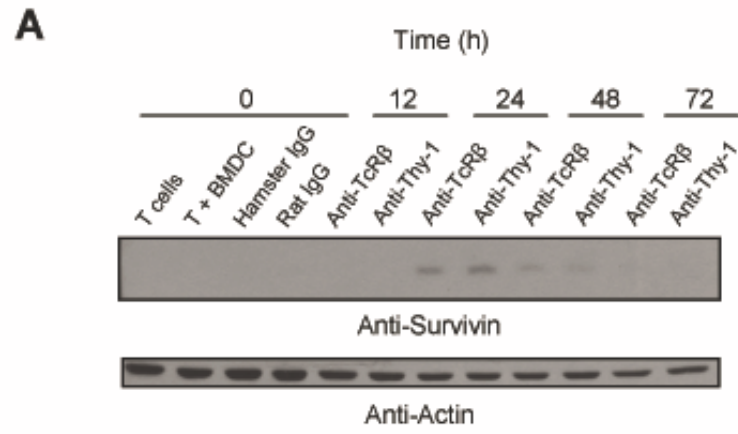


Figure 3.7. Thy-1 Signaling Induces Similar Levels of CDK6 Expression as TcR Signaling. (A) Highly purified CD3⁺ T cells (2.5×10^6 cells/well) with or without LPS-matured BMDCs (8×10^4 cells/well) were seeded into 24-well plates. T cells were then cultured in the presence or absence of 6 μ g/ml anti-Thy-1 mAb (clone G7), anti-TcR β mAb and/or the appropriate isotype control for the indicated times. Cells lysates were prepared and CDK6 protein levels (36 kDa) were assessed by western blotting. Blots were then reprobbed with antibodies specific for actin (42 kDa) to confirm equal loading. Data are representative of three independent experiments. (B) Optical density ratios were calculated by comparing the density of individual CDK6 bands from three independent experiments with the corresponding actin band. The 0 h time point represents T cells treated with hamster IgG or rat IgG isotype controls. Data are shown as mean \pm SEM; ns denotes “not significant” as determined by the Student’s t-test.

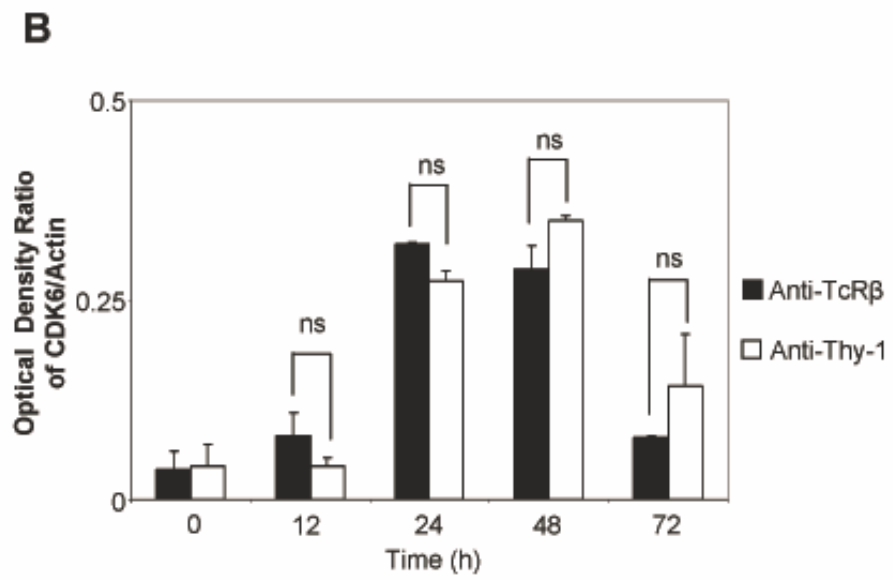
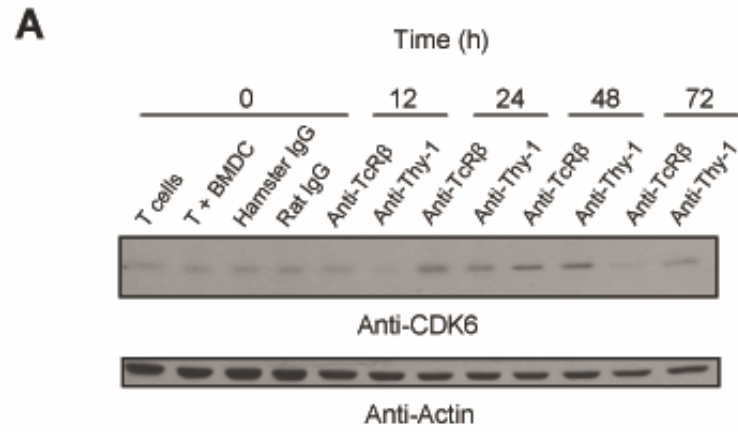


Figure 3.8. Thy-1 Signaling Induces Lower Levels of CyclinD3 Expression Than TcR Signaling. (A) Highly purified CD3⁺ T cells (2.5×10^6 cells/well) with or without LPS-matured BMDCs (8×10^4 cells/well) were seeded into 24-well plates. T cells were then cultured in the presence or absence of 6 $\mu\text{g/ml}$ anti-Thy-1 mAb (clone G7), anti-TcR β mAb and/or the appropriate isotype control for the indicated times. Cell lysates were prepared and CyclinD3 protein levels (31 kDa) were assessed by western blotting. Blots were then reprobbed with antibodies specific for actin (42 kDa) to confirm equal loading. Data are representative of three independent experiments. (B) Optical density ratios were calculated by comparing the density of individual CyclinD3 bands from three independent experiments with the corresponding actin band. The 0 h time point represents T cells treated with hamster IgG or rat IgG isotype controls. Data are shown as mean \pm SEM; ns denotes “not significant” as determined by the Student’s t-test.

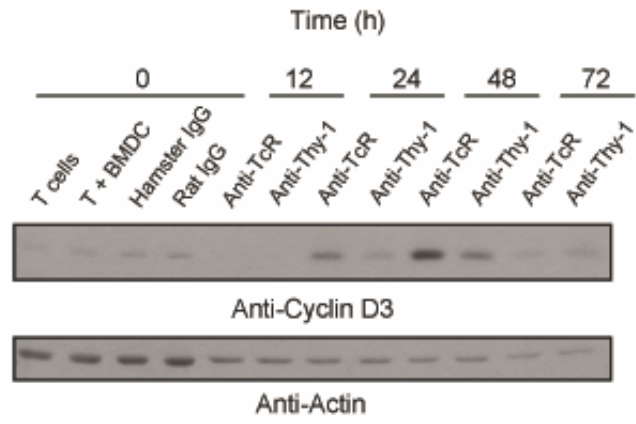
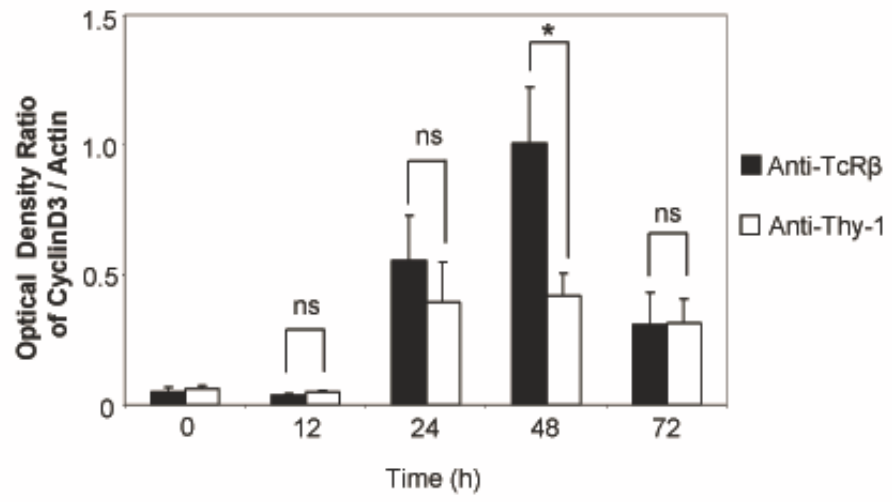
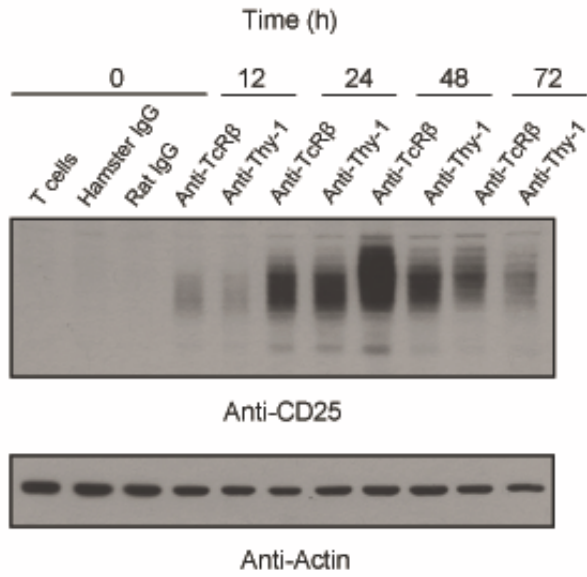
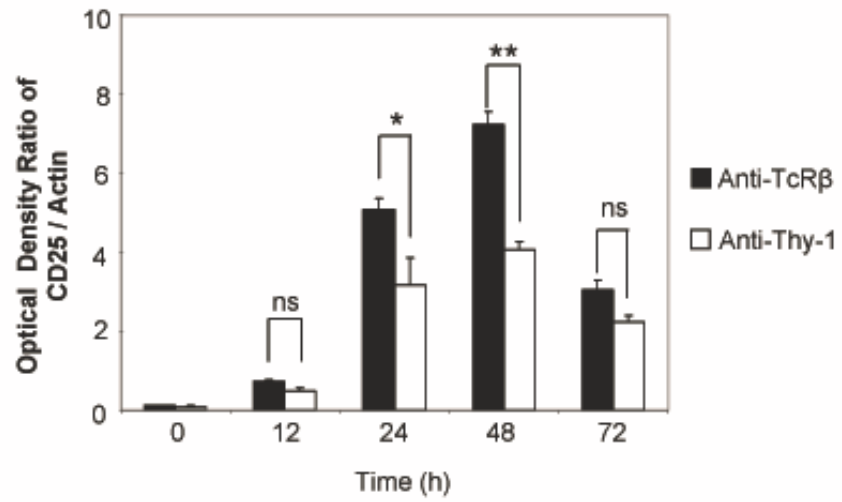
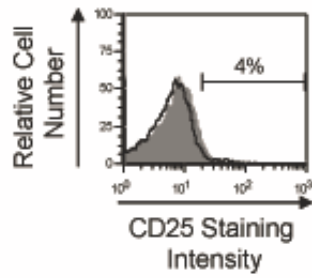
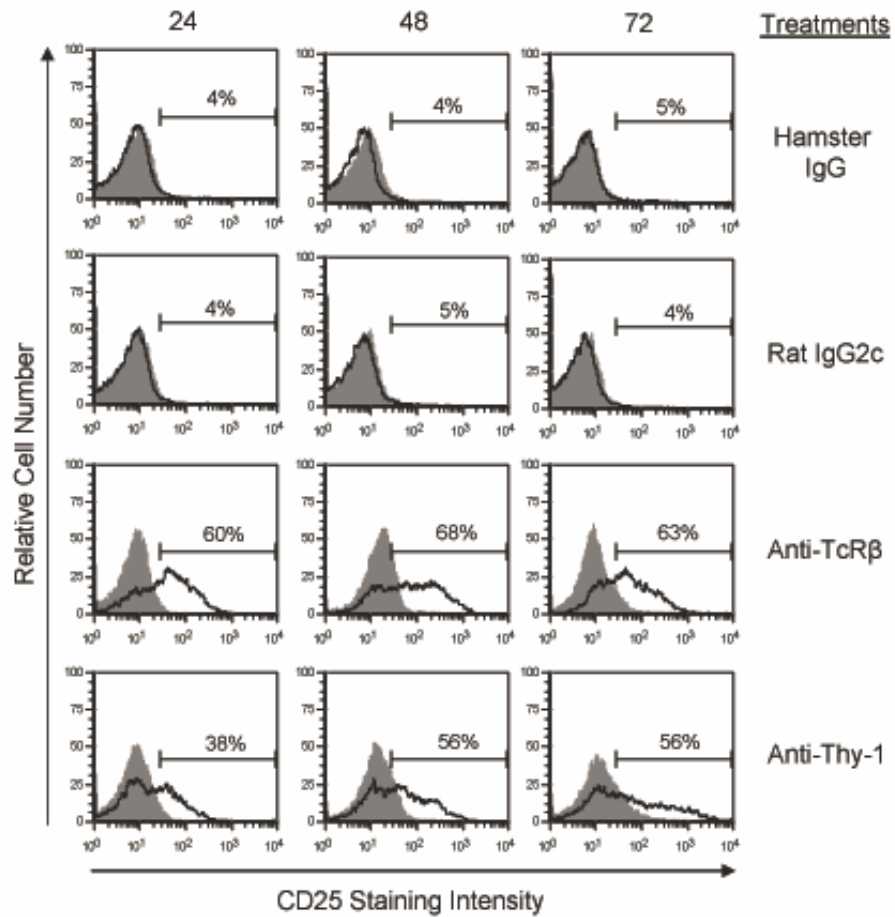
A**B**

Figure 3.9. Thy-1 Signaling Induces Lower Levels of CD25 Expression Than TcR Signaling. (A) Highly purified CD3⁺ T cells (2.5×10^6 cells/well) with or without LPS-matured BMDCs (8×10^4 cells/well) were seeded into 24-well plates. T cells were then cultured in the presence or absence of 6 μ g/ml anti-Thy-1 mAb (clone G7), anti-TcR β mAb and/or the appropriate isotype control for the indicated times. Cell lysates were prepared and CD25 protein levels (55 kDa) were assessed by western blotting. Blots were then stripped and reprobed with antibodies specific for actin (42 kDa) to confirm equal loading. Data are representative of three independent experiments. (B) Optical density ratios were calculated by comparing the density of individual CD25 bands from three independent experiments with the corresponding actin band. The 0 h time point represents T cells treated with hamster IgG or Rat IgG isotype controls. Data are shown as mean \pm SEM; ns denotes “not significant” as determined by the Student’s t-test. (C) Highly purified CD3⁺ T cells (2.5×10^5 cells/well) with or without LPS-matured BMDCs (8×10^3 cells/well) were seeded in duplicate into 96-well round-bottom plates. T cells were then cultured in the presence of 6 μ g/ml anti-Thy-1 mAb (clone G7), anti-TcR β mAb and/or the appropriate isotype control for the indicated times. Cells were stained for surface expression of CD25 with rat anti-mouse CD25-FITC (open peak) or rat IgG1-FITC (closed peak) and fixed with 1% paraformaldehyde. CD25 expression was then measured by flow cytometry. Data are representative of three independent experiments. (D) Mean channel fluorescence values and % of cells staining positive for CD25 were calculated from three separate experiments and are shown as mean \pm SEM. * denotes $p < 0.05$, ** denotes $p < 0.01$ and ns denotes “not significant” as determined by the Student’s t-test, when compared to T cells activated with anti-TcR β mAb.

A**B**

C**Resting T cells****Time After Stimulation (h)**

D

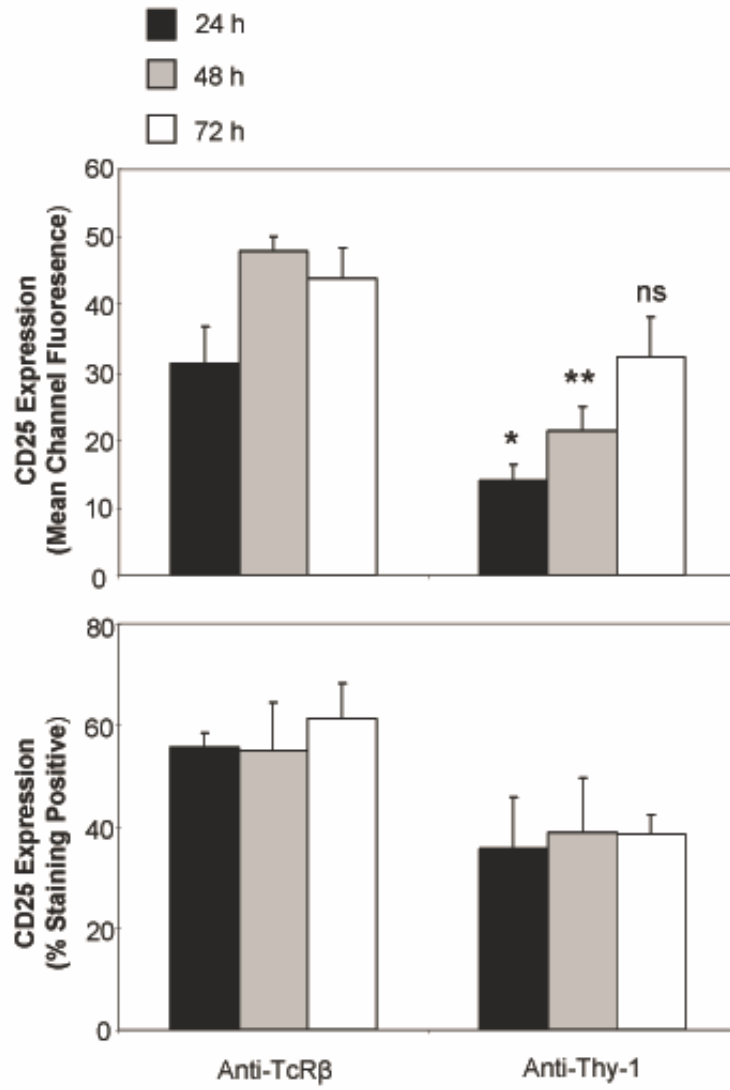


Figure 3.10. Thy-1 Signaling Induces Less IL-2 Production Than TcR Signaling. (A) Highly purified CD3⁺ T cells (2.5×10^5 cells/well) with or without LPS-matured BMDCs (8×10^3 cells/well) were seeded in quadruplicate into 96-well round-bottom plates. T cells were then cultured in the presence of 6 μ g/ml anti-Thy-1 mAb (clone G7), anti-TcR β mAb and/or the appropriate isotype control for the indicated times. Supernatants were then collected and assayed for IL-2 protein by sandwich ELISA. Data are representative of three independent experiments; *** denotes $p < 0.0001$ as determined by the Bonferroni multiple comparisons test. (B) Highly purified CD3⁺ T cells (2.5×10^5 cells/well) with or without LPS-matured BMDCs (8×10^3 cells/well) were seeded in quadruplicate into 96-well round-bottom plates. T cells were then cultured in the presence of 6 μ g/ml anti-TcR β or anti-Thy-1 mAb (clone G7) with the indicated concentrations of recombinant mouse IL-2 for 72 h. Wells were pulsed with [³H]TdR 6 h before the endpoint at which time the cells were harvested and DNA synthesis was determined based on [³H]TdR incorporation. Data are representative of three independent experiments; ns denotes “not significant” as determined by the Student’s t-test.

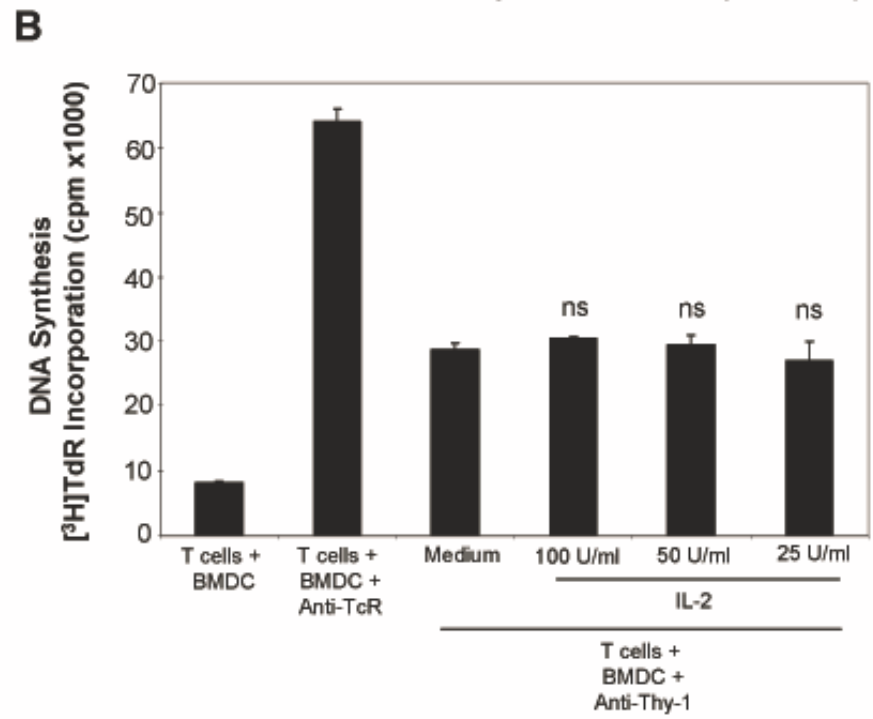
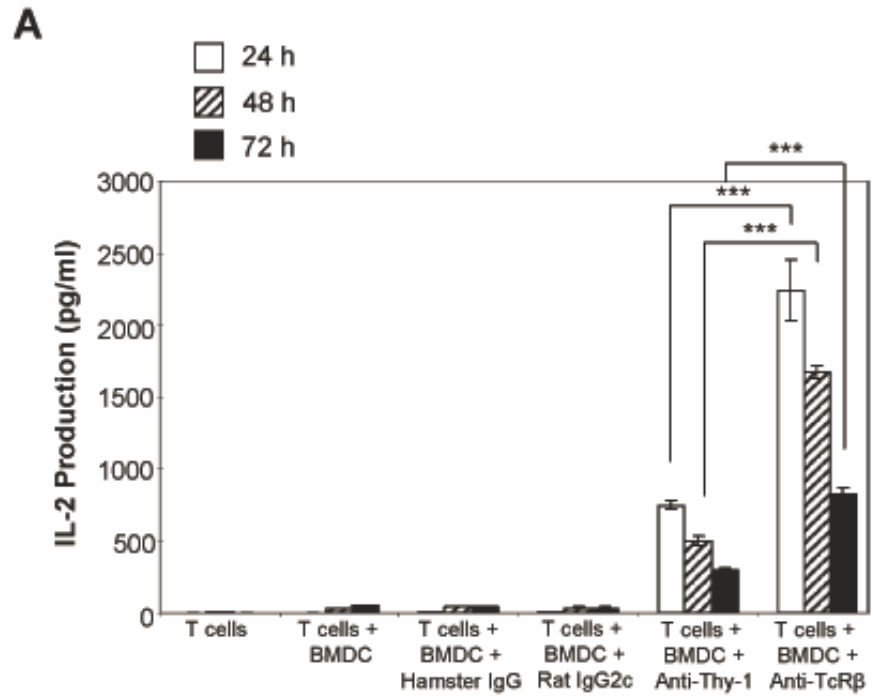
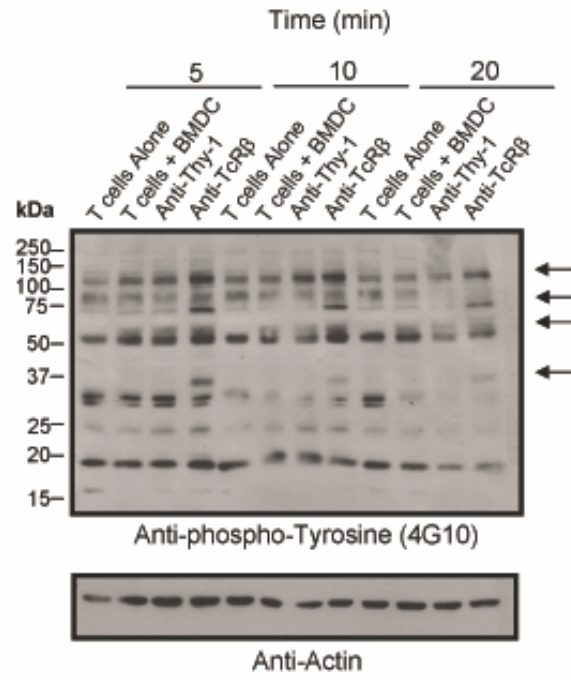


Figure 3.11. Thy-1 Signaling Induces Delayed Tyrosine Phosphorylation Compared to TcR Signaling. Highly purified CD3⁺ T cells (2.5×10^6 cells/well) with or without LPS-matured BMDCs (8×10^4 cells/well) were seeded into 24-well plates. T cells were then cultured in the presence or absence of 6 $\mu\text{g/ml}$ anti-Thy-1 mAb (clone G7), anti-TcR β mAb and/or the appropriate isotype control for short (A) or long time courses (B). Cell lysates were prepared and proteins with phosphorylated tyrosine residues were visualized by western blotting using a phospho-tyrosine specific mAb. Blots were then stripped and reprobbed with antibodies specific for actin (42 kDa) to confirm equal loading. Data are representative of three independent experiments. Arrows indicate bands which differ in TcR and Thy-1-stimulated T cell cultures.

A



B

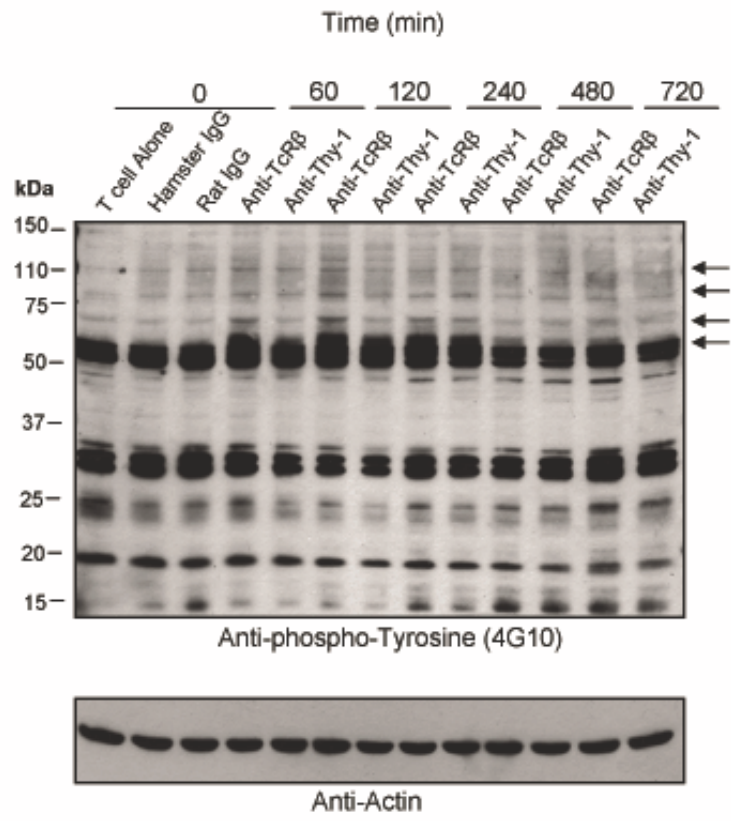
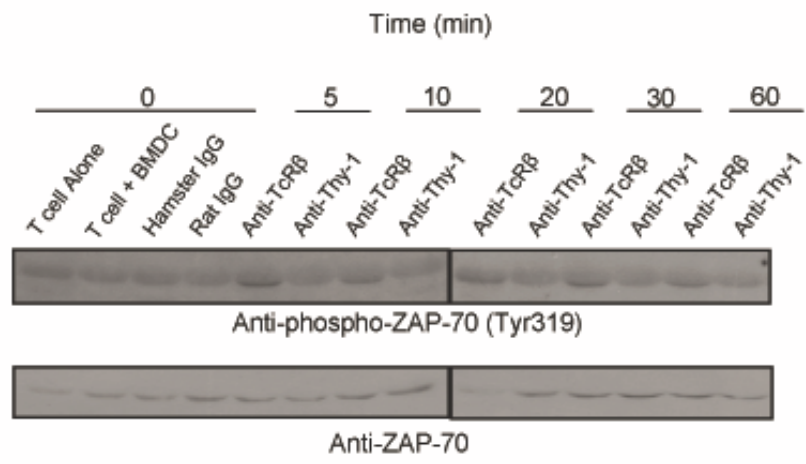
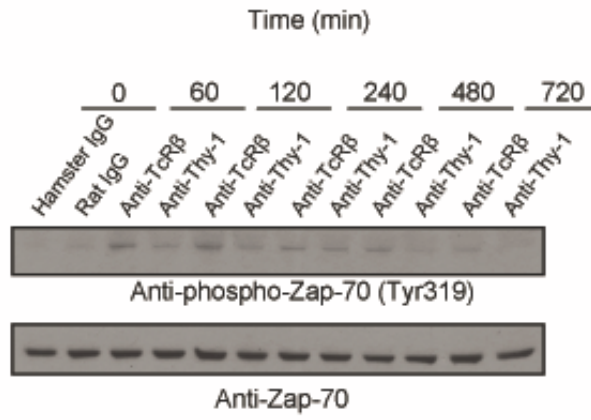


Figure 3.12. Thy-1 Signaling Induces Delayed ZAP-70 Phosphorylation Compared to TcR Signaling. Highly purified CD3⁺ T cells (2.5×10^6 cells/well) with or without LPS-matured BMDCs (8×10^4 cells/well) were seeded into 24-well plates. T cells were then cultured in the presence or absence of 6 μ g/ml anti-Thy-1 mAb (clone G7), anti-TcR β mAb and/or the appropriate isotype control for short (A) or long time courses (B). Cell lysates were prepared and levels of phospho-p56lck (Tyr505) and phospho-Zap-70 (Tyr³¹⁹) were assessed by western blotting. Blots were then stripped and reprobbed with antibodies specific for total Lck and total ZAP-70 to confirm equal loading. Data are representative of three independent experiments. (C) Optical density ratios were calculated by comparing the density of individual phospho-ZAP-70 bands from three independent experiments with the corresponding total ZAP-70 band. Data were normalized to isotype control (time = 0) and are shown as mean \pm SEM; ns denotes “not significant” as determined by the Student’s t-test.

A



B



C

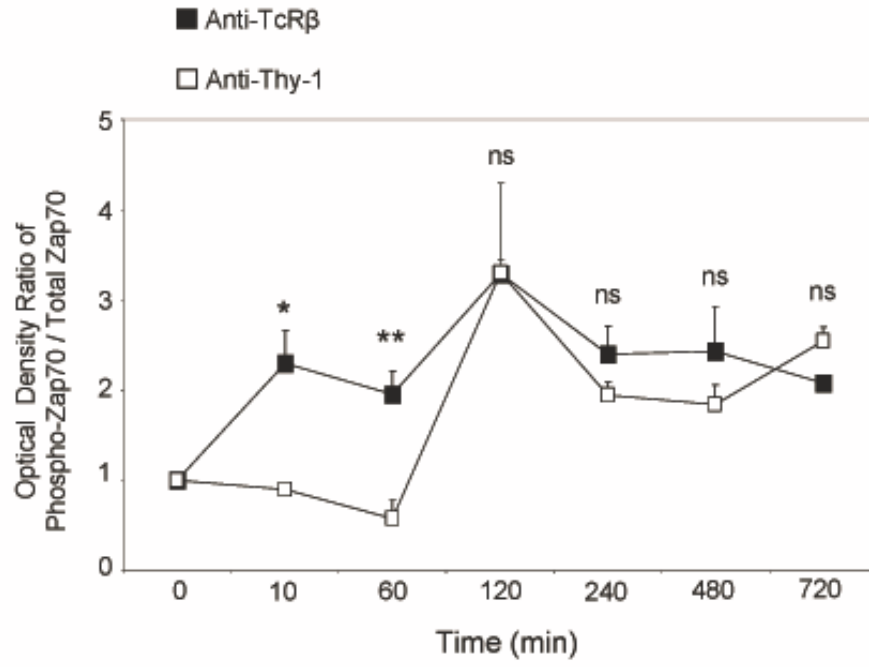
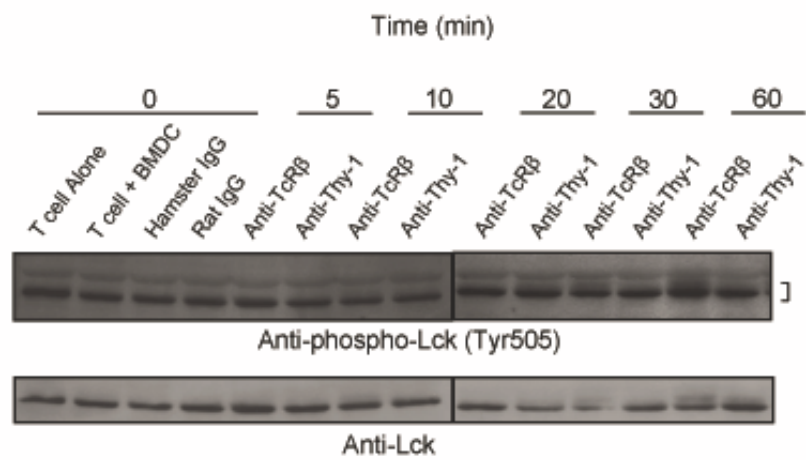


Figure 3.13. Thy-1 Signaling Induces Delayed Lck Serine Phosphorylation Compared to TcR Signaling in the Presence of Costimulation Provided by LPS-Matured Syngenic BMDCs. Highly purified CD3⁺ T cells (2.5×10^6 cells/well) with or without LPS-matured BMDCs (8×10^4 cells/well) were seeded into 24-well plates. T cells were then cultured in the presence or absence of 6 $\mu\text{g/ml}$ anti-Thy-1 mAb (clone G7), anti-TcR β mAb and/or the appropriate isotype control for short (A) or long time courses (B). Cells lysates were prepared and levels of phospho-lck (Tyr⁵⁰⁵) were assessed by western blotting. Blots were then stripped and reprobbed with antibodies specific for total lck to confirm equal loading. Data are representative of three independent experiments.

A



B

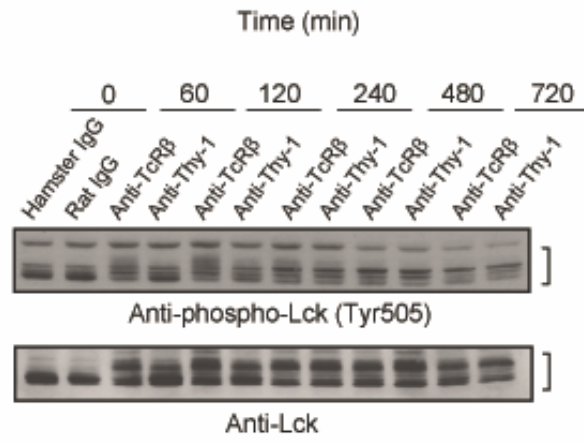
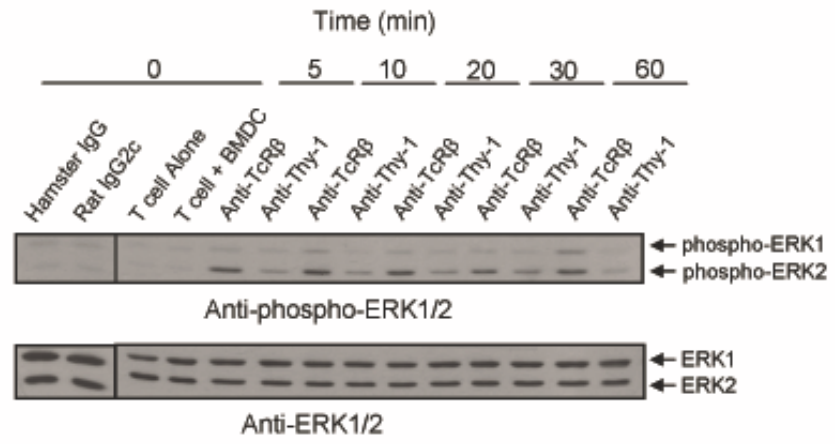
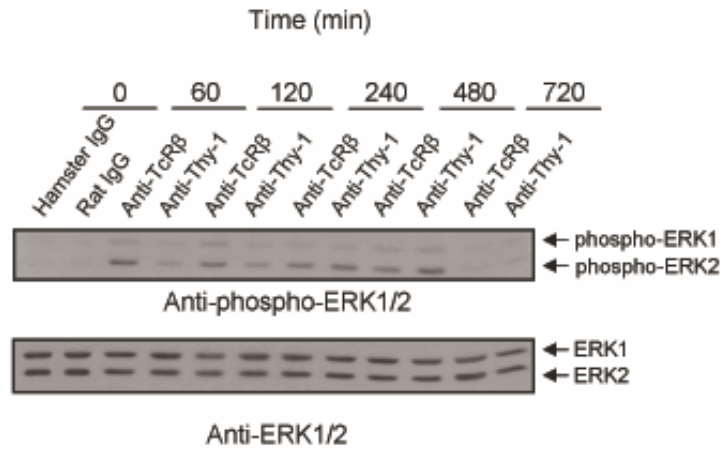


Figure 3.14. Thy-1 Signaling Induces Delayed ERK-1/2 Phosphorylation Compared to TcR Signaling. Highly purified CD3⁺ T cells (2.5×10^6 cells/well) with or without LPS-matured BMDCs (8×10^4 cells/well) were seeded into 24-well plates. T cells were then cultured in the presence or absence of 6 μ g/ml anti-Thy-1 mAb (clone G7), anti-TcR β mAb and/or the appropriate isotype control for short (A) or long time courses (B). Cell lysates were prepared and levels of phospho-ERK1/2 were assessed by western blotting. Blots were then stripped and reprobed with antibodies specific for total ERK1/2 to confirm equal loading. Data are representative of three independent experiments. Optical density ratios were calculated by comparing the density of individual (C) phospho-ERK-1/2 bands from three independent experiments with the corresponding total ERK-1/2, respectively. Data were normalized to isotype control (time = 0) and are shown as mean \pm SEM; ns denotes “not significant” as determined by the Student’s t-test.

A



B



C

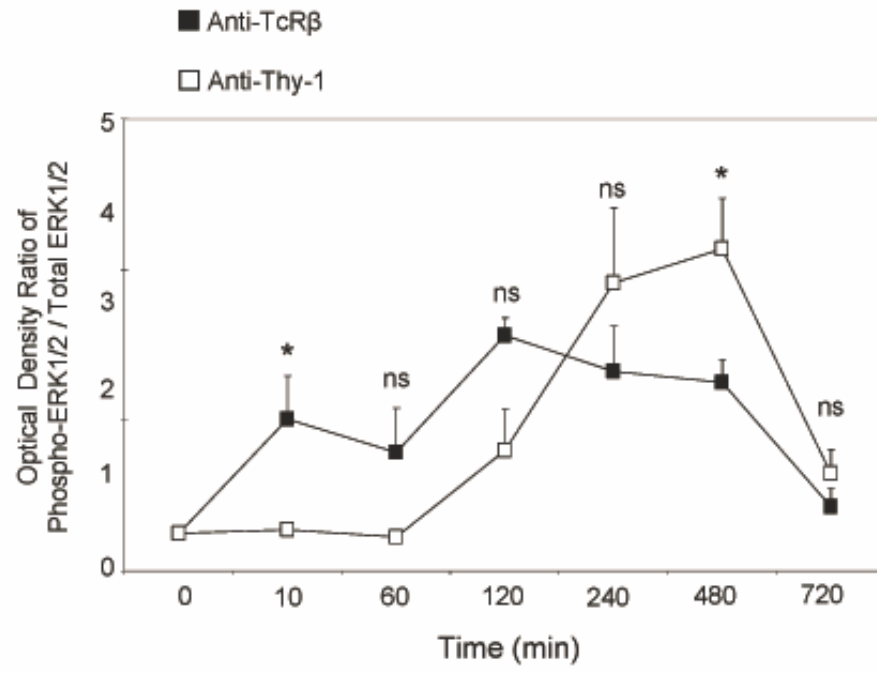


Figure 3.15. Thy-1 Stimulation Induces CTLs That Destroy Target Cells With Delayed Kinetics Compared to TcR Stimulation. Highly purified CD3⁺ T cells (2.5×10^6 cells/well) and LPS-matured BMDCs (8×10^4 cells/well) were seeded into 24-well plates. T cells were then cultured in the presence of 6 $\mu\text{g/ml}$ anti-Thy-1 mAb (clone G7) or anti-TcR β mAb for 72 h. The resulting CTLs were washed, counted, and resuspended in 96-well round bottom plates with [³H]TdR-labeled P815 target cells. (A) Cells were either plated at the indicated effector to target cell ratios and incubated for 4 h or (B) were plated at an effector to target ratio of 50:1 and incubated for the indicated times. B cells activated with 5 $\mu\text{g/ml}$ LPS for 72 h were also combined with P815 cells at the same effector to target ratio and as negative control. At the end point, DNA was harvested. Data are expressed as % specific DNA fragmentation \pm SD determined by comparison of cpm from combined cultures to P815 target cells alone. Background cpm for P815 target cells alone were $16,289 \pm 728$, $16,323 \pm 471$ and $15,637 \pm 288$ for 4, 12 and 24 h endpoints, respectively. Data are representative of three independent experiments; * denotes $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ as determined by the Bonferroni multiple comparisons test when compared to LPS-activated B cells.

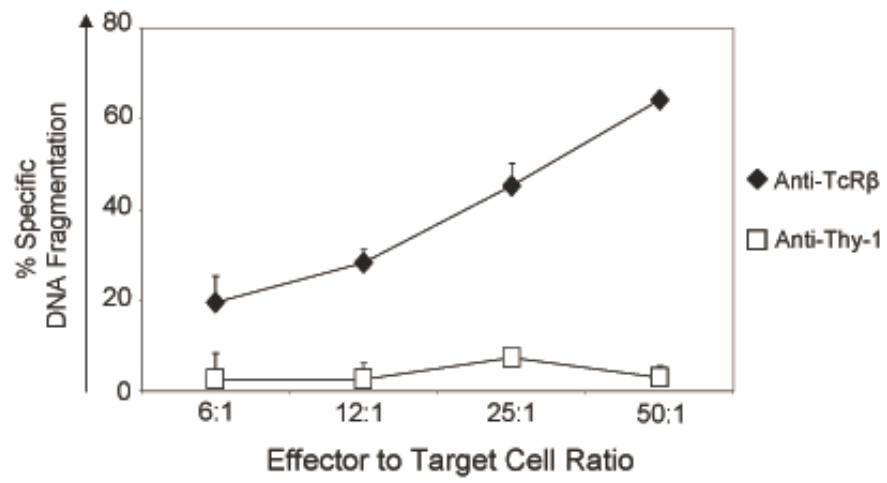
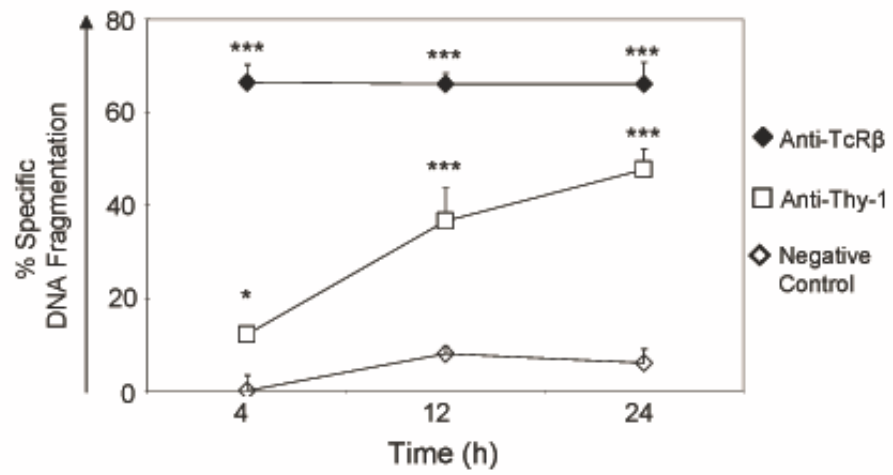
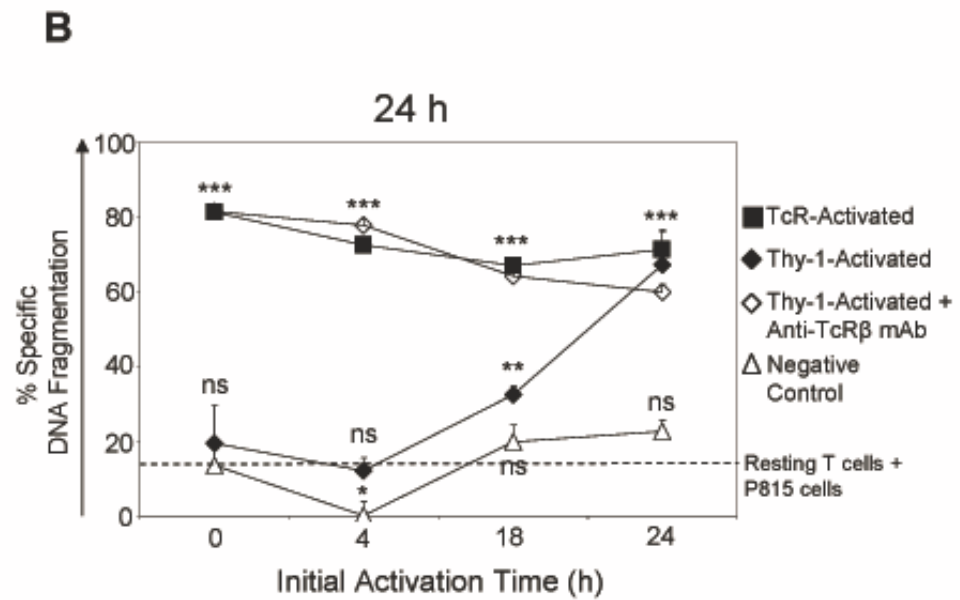
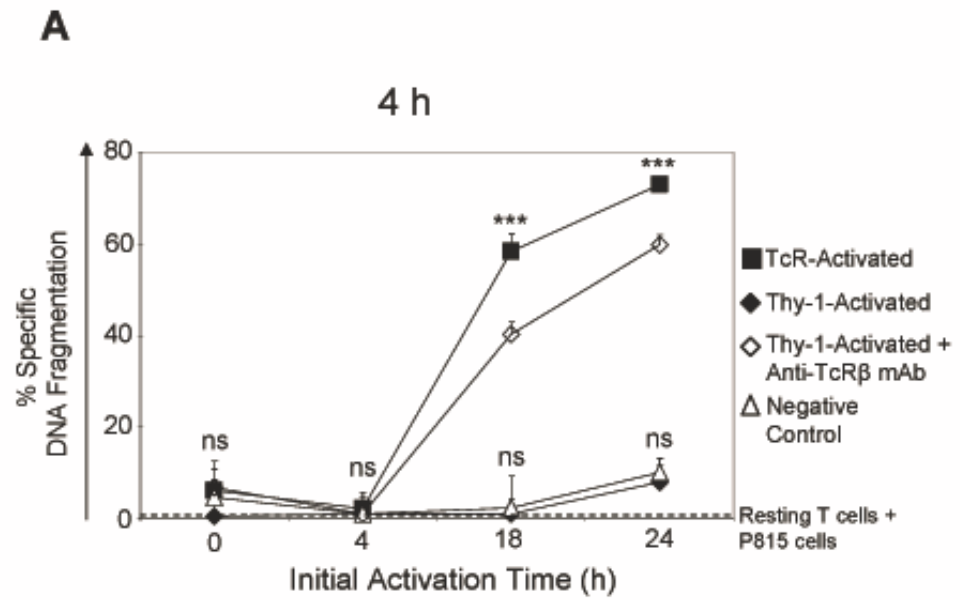
A**B**

Figure 3.16. Thy-1 Signaling Promotes Delayed Acquisition of Cytotoxic Effector Function Compared to TcR Signaling. Highly purified CD3⁺ T cells (2.5×10^6 cells/well) and LPS-matured BMDCs (8×10^4 cells/well) were seeded into 24-well plates. T cells were then cultured in the presence or absence of 6 $\mu\text{g/ml}$ anti-Thy-1 mAb (clone G7) or anti-TcR β mAb for the indicated times. The resulting CTLs were washed, counted, and resuspended in 96-well round bottom plates with [³H]TdR-labeled P815 target cells at the 50:1 effector to target cells with or without 1 $\mu\text{g/ml}$ anti-TcR β mAb. LPS-activated B cells were also combined with P815 cells at the same effector to target ratio and as negative control. Cells were incubated for either (A) 4 h or (B) 24 h and then intact DNA was harvested on fiberglass filter mats. Data are expressed as % Specific DNA fragmentation \pm SD as determined by comparison of cpm from combined cultures to P815 target cells alone. Background cpm for P815 target cells alone were $8,805 \pm 522$ and $7,825 \pm 448$ for 4 and 24 h cytotoxicity assays, respectively. Data are representative of three independent experiments; * denotes $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and ns denotes not significant as determined by the Bonferroni multiple comparisons test when compared to resting T cells and P815 cell cultures.



Chapter 4. Results: Anti-Thy-1 mAb-Mediated Signaling Induces Greater IL4 and IL-17A Production and Less IFN γ Production Than anti-TcR β mAb-mediated Signaling.

Although numerous T cell studies have been performed since the initial discovery of Thy-1, the exact role that this intriguing cell surface molecule plays in T cell biology remains unclear. An immense amount of evidence suggests that Thy-1 provides an activating signal to T cells. Thy-1 by itself can provide a TcR-like signal 1 and may also act as a costimulatory molecule that enhances TcR-mediated T cell activation (375, 451). There is also some evidence, however, that Thy-1 can provide negative regulatory signals under certain circumstances. For example, Thy-1-deficient thymocytes are hyperresponsive to TcR signals (403). Consistent with the ability of Thy-1 to provide a TcR-like activating signal, Thy-1 signaling can induce the differentiation of resting T cells into CTLs and also can provoke CTLs to destroy target cells (Figure 3.15)(375). In recent work, our lab has also demonstrated that Thy-1 signaling in the absence of costimulatory signals induces a T regulatory cell phenotype (422), implicating Thy-1 in regulating another type of T cell effector function. Considering the importance that Th cell subsets have in modulating the nature of immune responses through the production of distinct cytokine profiles, it is surprising that very little work has examined the role that Thy-1 plays in the regulation of T cell cytokine production and Th cell effector function.

The decision of a CD4⁺ T cell to differentiate into a particular Th subset when receiving activating signals depends upon numerous factors, including the cytokine environment, the nature and strength of the activating signaling, and costimulatory signals received from the pAPC (141, 142). In this section, I investigate the influence of Thy-1 signaling on T cell cytokine production and the polarization of CD4⁺ T cells into different Th cell phenotypes. The data presented in this chapter suggest that Thy-1 triggering with costimulation provided by BMDCs is sufficient to drive CD4⁺ T cell differentiation into cytokine-producing Th cells. Interestingly, in comparison to anti-TcR β mAb-mediated signaling, anti-Thy-1 mAb-mediated signaling favoured IL-17 and IL-4 production over IFN γ

production, suggesting a previously unidentified difference between TcR and Thy-1 signal transduction.

4.1 Anti-Thy-1 mAb-Mediated Signaling Induces More IL-17 and IL-4 mRNA and Less IFN γ mRNA in T cell Cultures than Anti-TcR β mAb-Mediated Signaling.

A number of signaling molecules influence the nature of T cell cytokine production. Signals through the CD28 and LFA-1 have been implicated in favouring a Th1 cytokine profile (452, 453), while other costimulatory molecules such as OX40 and ICOS are thought to promote a Th2 profile (454, 455). Since Thy-1 provides T cell activating signals, I asked whether Thy-1 also promotes a particular T cell cytokine profile. In order to study the influence of Thy-1 signaling on T cell cytokine production, I first examined the induction of cytokine mRNA by real-time PCR following the initial stimulation of resting T cells with anti-Thy-1 mAb and costimulation provided by LPS-matured BMDC. Since IFN γ , IL-4 and IL-17A are the signature cytokines of Th1, Th2 and Th17 cells, respectively, I chose to focus on the effect of Thy-1 signaling on the synthesis of these cytokines in T cell cultures. Consistent with Thy-1 providing a weaker activating signal, Thy-1-activated T cells expressed less IFN γ mRNA than TcR-activated T cells 24 h following activation (Figure 4.1). Most strikingly, Thy-1-stimulation induced approximately 2 times more IL-17A mRNA and 4 times more IL-4 mRNA than TcR-stimulation in T cell cultures. These results suggest that Thy-1 and TcR signal transduction pathways differ in their ability to promote the transcription of cytokines in T cells, and that Thy-1 signaling preferentially induces IL-4 and IL-17A gene transcription over IFN γ gene transcription.

4.2 Anti-Thy-1 mAb-Mediated Signaling Promotes Differential Cytokine Production Compared to Anti-TcR β mAb-Mediated Signaling.

To investigate the ability of Thy-1 to induce T cell cytokine production at the protein level, I first utilized Raybio® Mouse Cytokine Antibody Arrays (Raybiotech, Inc). This technology is useful because it allows for the detection of many different cytokines within one supernatant sample. The majority of the 22 cytokines detectable by the cytokine array were either produced at similar levels by anti-Thy-1- and anti-TcR β mAb-stimulated T cells or produced at such high

levels that the assay was saturated and any differences could not be determined (Figure 4.2A). IL-6 and IL-12 were present in supernatants from both Thy-1- and TcR-stimulated T cells. Since LPS-signaling is well known for inducing BMDCs to produce copious amounts of both IL-6 and IL-12, the source of these cytokines is likely to be the LPS-matured BMDCs (155, 456). Interestingly, several differences were observed in the cytokine profiles of Thy-1- and TcR-stimulated T cell cultures. Consistent with the mRNA levels, Thy-1 signaling did not provoke as much IFN γ production as TcR signaling. Moreover, Thy-1 signaling induced higher levels of both IL-4 and IL-17A (Figure 4.2B). Interestingly, there was also more IL-3 protein in the supernatants from Thy-1-activated T cell cultures. Since IL-3 is also produced by activated T cells, this suggests an additional difference between Thy-1 and TcR signal transduction during T cell activation.

To further confirm and quantify differences in Th cell cytokine production, I subsequently performed sandwich ELISAs for IL-2, IFN γ , IL-4 and IL-17A following 24 h of activation. Consistent with my previous observations, Thy-1-stimulated T cell cultures produced significantly less IL-2 (Figure 4.2C) and IFN γ (Figure 4.2D) than TcR-stimulated T cell cultures. Furthermore, Thy-1-stimulated T cell cultures produced significantly more IL-17A (Figure 4.2E) and IL-4 (Figure 4.2F) than TcR-stimulated T cell cultures. In these experiments, I also stimulated T cells with anti-TcR β and anti-Thy-1 mAb combined to determine whether Thy-1 signaling would affect anti-TcR β mAb-induced cytokine production. T cells receiving the combined signals produced similar levels of IFN γ and IL-2 as T cells activated with anti-TcR β mAb alone (Figures 4.2C and D), suggesting that Thy-1 signaling does not enhance anti-TcR β mAb-stimulated production of these cytokines. Although Thy-1 signaling alone induced two-fold more IL-4 production than TcR signaling, when T cells received Thy-1 and TcR signaling together, they produced low levels of IL-4 comparable to T cells receiving TcR signaling alone (Figure 4.2F). This finding suggests that the TcR may provide a signal that somehow blocks Thy-1-induced IL-4 production. Similar to Thy-1-stimulated T cells, those receiving both TcR and Thy-1 signaling produced more IL-17A than T cells stimulated through the TcR alone. These findings suggest that Thy-1

provides a signal that promotes IL-17A production that is not provided by the TcR.

4.3 Anti-Thy-1 mAb-Mediated Signaling Does Not Enhance Anti-TcR β mAb-Mediated Proliferation.

Several lines of evidence suggest that Thy-1 signaling is important in enhancing TcR-induced responses. Thy-1-deficient T cells have impaired IL-2 production and proliferation in response to anti-CD3 mAb stimulation and blocking Thy-1 with anti-Thy-1 mAb 30-H12 also inhibits anti-CD3 mAb-mediated T cell activation and CTL differentiation when costimulatory signals are provided by splenic accessory cells (376, 402). In addition, Thy-1 crosslinking enhances TcR-mediated activation of the LAT adaptor molecule (404). Having observed that anti-Thy-1 mAb-mediated signaling did not enhance anti-TcR β mAb-induced IL-2 and IFN γ production by T cells when costimulation was provided by LPS-matured BMDC, but did provide a signal that induced higher levels of IL-17A than anti-TcR β mAb-mediated signaling alone, I asked whether anti-Thy-1 mAb-mediated signaling would enhance anti-TcR β mAb-induced T cell proliferation in this system. Time course experiments using the [3 H]TdR incorporation assay showed that anti-Thy-1 mAbs did not significantly enhance anti-TcR β mAb-induced T cell proliferation when costimulation was provided by LPS-matured BMDCs and when the concentrations of both mAbs (6 μ g/ml) induced maximal mitogenic responses in T cell cultures when used alone (Figure 4.3A). Similarly, if suboptimal concentrations of anti-TcR β (0.001 and 0.01 μ g/ml) and anti-Thy-1 mAbs (0.1, 1, 3 μ g/ml) were combined, Thy-1 signaling did not enhance DNA synthesis in T cell cultures in comparison to stimulation with either mAb alone (Figure 4.3B). Consistently, neither suboptimal nor maximal concentrations of anti-Thy-1 mAb induced T cells that were activated with suboptimal concentrations of anti-TcR mAbs and costimulation provided by LPS-matured BMDCs to undergo more T cell divisions than anti-TcR β mAb and LPS-matured BMDCs alone (Figure 4.3C). Taken together, these data suggest that Thy-1 signaling does not enhance TcR-induced T cell proliferation when costimulation is provided by LPS-matured BMDCs.

4.4 Anti-Thy-1 mAb-Mediated Signaling Induces a Higher Ratio of Gata-3 to T-bet Expression than Anti-TcR β mAb-Mediated Signaling.

T-bet and Gata-3 are key lineage-specific transcription factors that regulate the distinct Th1 and Th2 phenotypes, respectively (157, 206). Consequently, T-bet and Gata-3 play a major role in regulating the expression of IFN γ and IL-4 in T cells. Having observed differential IFN γ and IL-4 production in anti-TcR β mAb- and anti-Thy-1 mAb-stimulated T cell cultures, I subsequently asked whether anti-Thy-1 mAb-mediated signaling promoted more Gata-3 and less T-bet expression in T cells than anti-TcR β mAb-mediated signaling. To examine this, I used an intracellular staining technique to determine transcription factor expression following T cell activation with either anti-TcR β or anti-Thy-1 mAbs. Combined cultures of T cells and LPS-matured BMDCs did not stain positive for either Gata-3 or T-bet expression at 0 h (Figure 4.4). Both anti-TcR β and Thy-1 mAb-mediated stimulation promoted expression of T-bet in T cell cultures by 12 h, which was diminished at 48 h. Consistent with the ability of Thy-1 to induce less IFN γ production in T cell cultures, anti-Thy-1 mAb-stimulated T cells not only expressed less T-bet, but a smaller percentage of T cells upregulated T-bet expression when compared to anti-TcR β mAb-stimulated T cells. Gata-3 expression, on the other hand, was only weakly upregulated by anti-TcR β or anti-Thy-1 mAb-mediated stimulation. Interestingly, at 12 h a small population of T cells expressed slightly higher levels of Gata-3 in anti-Thy-1 mAb-stimulated T cells compared to anti-TcR β mAb-stimulated T cells. This difference in expression, however, was lost by 24 h. Taken together, these results suggest that Thy-1 stimulation induces more Gata-3 and less T-bet expression in T cells than TcR-signaling and may explain the differential induction of IFN γ and IL-4 production.

4.5 Anti-Thy-1 mAb-Mediated Signaling Induces Higher Levels of ROR γ t Expression Compared to Anti-TcR β mAb-Mediated Signaling.

The key lineage-specific transcription factor that regulates the Th17 phenotype is ROR γ t (276). Figure 4.5 shows that anti-Thy-1 mAb-mediated signaling induced the upregulation of ROR γ t expression in T cell cultures while anti-TcR β mAb-mediated signaling did not. Anti-Thy-1 mAb induced the

expression ROR γ t protein, as determined by western blotting, as early as 12 h following T cell activation and ROR γ t expression began to diminish by 48 h. These results suggest that Thy-1 signaling activates a signaling pathway that leads to ROR γ t and may explain why Thy-1-signaling differentially promotes IL-17A production compared to signal transduction through the TcR/CD3 complex.

4.6 CD4⁺ T cells Express Lower Levels of Thy-1 On Their Surface than CD8⁺ T cells

All of my experiments thus far have used highly purified CD3⁺ T cells, which contain both CD4⁺ and CD8⁺ T cells. Both CD4⁺ and CD8⁺ T cells can produce cytokines in response to the T cell activating signals, although CD4⁺ generally express more. In fact, CD8⁺ T cells can differentiate into polarized subsets that express the same cytokine profiles as CD4⁺ T cell subsets and are designated Tc1, Tc2 and Tc17 cells (457). It is possible that the expression of different levels of Thy-1 could result in different responses by CD4⁺ and CD8⁺ T cells to the same amount of anti-Thy-1 mAb. To determine whether CD4⁺ and CD8⁺ T cells express similar levels of Thy-1 on their surface, I first isolated CD4⁺ and CD8⁺ T cells using Miltenyi Macs® kits. These isolations consistently resulted in purities of >90% for both T cell subsets (Figure 4.6A). I then labeled the CD4⁺ and CD8⁺ T cell populations with two different Thy-1 mAbs clones and quantified Thy-1 expression by flow cytometry. Consistently, both anti-Thy-1 mAbs labeled CD8⁺ T cells with higher fluorescence intensity than CD4⁺ T cells (Figure 4.6B). The average of the MCF of three independent experiments shows that the difference in Thy-1 expression of CD8⁺ and CD4⁺ T cells was significant (Figure 4.6C), indicating that CD8⁺ T cells express more Thy-1 on their surface than do CD4⁺ T cells.

4.7 CD4⁺ and CD8⁺ T cells Proliferate Similarly in Response to Anti-Thy-1 mAb-Mediated Signaling

I next compared the ability of Thy signaling to activate CD4⁺ and CD8⁺ T cells when costimulation was provided by LPS-matured BMDCs. Interestingly, although CD4⁺ and CD8⁺ T cells express different levels of Thy-1, both CD4⁺ and CD8⁺ T cell populations proliferated to similar levels in response to varying concentrations of anti-Thy-1 mAb, as measured by [³H]TdR-incorporation 72 h

following activation (Figure 4.7) Consistent with results obtained with unfractionated CD3⁺ populations of T cells, 6 µg/ml of anti-Thy-1 mAb-induced maximal levels of proliferation in both CD4⁺ and CD8⁺ T cells, and the proliferative response was weaker than that induced by anti-TcRβ mAb.

4.8 Thy-1 Signaling Promotes the Differentiation of Resting T cells into Th cells that Produce More IL-17 and IL-4 and Less IFN γ Than TcR-Induced Th cells Upon Restimulation.

Naïve CD4⁺ T cells differentiate into Th effector cells upon receiving TcR and costimulatory signals. Upon subsequent activation through the TcR, Th effector cells then produce distinct profiles of cytokines that direct the immune response to activate immune functions that are effective against particular types of pathogens. Having observed that Thy-1 signaling preferentially promotes the early production of IL-17 and IL-4 over IFN γ in highly purified CD3⁺ T cell cultures when compared to TcR signaling, I next asked if Thy-1-induced highly purified CD4⁺ T cells would produce similar patterns of Th cell cytokines upon subsequent activation. To examine this question, I stimulated highly purified CD4⁺ T cells with anti-TcRβ or anti-Thy-1 mAbs and costimulation provided by LPS-matured BMDCs for 6 days to promote differentiation into Th effector cells. LPS-matured BMDCs are known to favor Th1 differentiation through the production of high levels of IL-12 (458). It is not surprising then that TcR-induced Th cell cultures produced high levels of IFN γ and negligible levels of IL-4 and IL-17A when restimulated by anti-TcRβ mAbs and LPS-matured BMDCs (Figure 4.8A). Consistent with my previous findings, Thy-1-induced Th cells produced significant levels of both IL-4 and IL-17A but did not produce significantly higher levels of IFN γ when compared to T cells and BMDC alone. These findings provide further evidence that Thy-1 provides a signal that preferentially drives IL-4 and IL-17A production. Interestingly, if TcR-induced Th cells are instead restimulated with anti-Thy-1 mAb and LPS-matured BMDCs, they produce even higher levels of IFN γ than when stimulated with anti-TcR mAbs (Figure 4.8A). They also produce significant levels of IL-4 protein. These results suggest that the TcR may provide some signal that programs the T cells to produce IFN γ and allows a subsequent Thy-1 signal to activate the necessary pathway to promote

IFN γ production, as well as suggesting that Thy-1 has a unique ability to promote IL-4 production by T cells.

The activation threshold for Th effector cells is much lower than for naïve CD4⁺ T cells and as a result costimulation through CD28 is not necessary to activate T effector cells (459). To determine if Thy-1-induced Th cells would produce the same cytokine profile in the absence of costimulation, I next restimulated TcR- or Thy-1-induced Th cells with soluble anti-TcR β or anti-Thy-1 mAbs. TcR-induced T cells did not produce appreciable levels of any of the three cytokines in response to soluble anti-TcR β mAbs (Figure 4.8B), which was not surprising since anti-TcR β mAbs usually need to be cross-linked or immobilized to induce a mitogenic signal in T cells (460). Interestingly, the Thy-1-induced Th cells did produce significant levels of both IL-17A and IL-4 in response to restimulation with soluble anti-TcR β mAbs (Figure 4.8B). Consistent with my previous observations, Thy-1-induced T cells also did not produce appreciable levels of IFN γ , substantiating the idea that Thy-1 signaling preferentially promotes the expression of IL-17A and IL-4. Moreover, Thy-1-induced Th effector cells produced high levels of IL-17A and IL-4 but not IFN γ when restimulated with by soluble anti-Thy-1 mAbs. Intriguingly, TcR-induced Th cells again produced high levels of IFN γ and IL-4 when restimulated with soluble anti-Thy-1 mAbs. In some experiments the amount of IL-4 produced by TcR-induced Th cells upon restimulation with soluble anti-Thy-1 mAbs was equal to that of Thy-1-induced Th cells.

mAbs can be immobilized on polystyrene microspheres in order to simulate receptor interactions with ligands on another cell. I restimulated TcR- and Thy-1-induced Th cells with microspheres coated with either anti-TcR β or anti-Thy-1 mAbs in order to further examine the role of Thy-1 signaling on Th effector cell cytokine production. Neither TcR- nor Thy-1-induced Th cells produced appreciable cytokines in response to anti-Thy-1 mAb-coated microspheres (Figure 4.8C). This was probably not due to unsuccessful coating of the beads since the same microspheres induced resting T cells to express CD25 (data not shown). These results show that soluble anti-Thy-1 mAbs can

stimulate cytokine production in Th effector cells whereas immobilized anti-Thy-1 mAbs cannot. In contrast, anti-TcR β mAb-coated microspheres did induce cytokine production by both TcR- and Thy-1-induced Th cells. TcR-induced Th cells produced high levels of IFN γ and some IL-4 in response to anti-TcR β mAb-coated microbeads. Interestingly, not only did Thy-1-induced Th cells produce higher levels of IL-17A and IL-4 than TcR-induced Th cells when restimulated with anti-TcR β mAb-coated microbeads, but they also expressed IFN γ . In some experiments the levels of IFN γ were similar to TcR-induced Th cells (Figure 4.8C). In other experiments, however, the levels of IFN γ were significantly lower (Data not shown). The differences observed in IFN γ production maybe due to variation in the efficiency of coating beads with anti-TcR β mAb between different experiments. Immobilized anti-CD3 mAbs, which also cross-link the TcR complex can provide a very strong signal that can activate naïve T cells in the absence of costimulatory signals (460). Therefore, these results suggest that Thy-1-induced Th cells have the capacity to make IFN γ , however, they may require a strong TcR signal to do so. These results also further support the conclusion that the Thy-1 signal preferentially promotes IL-4 and IL-17A production in comparison to TcR signaling, since the Thy-1-induced Th cells expressed significantly higher levels of both of these cytokines upon restimulation.

4.9 Increasing the Number of BMDCs Does Not Allow Thy-1-induced Th cells to Produce IFN γ .

The observation that immobilized anti-TcR β mAbs provided Thy-1-induced Th effector cells with the necessary signal to provoke IFN γ production implies that a strong activating signal is required to induce Thy-1-induced Th effectors cells to produce IFN γ . Naïve T cells will proliferate and produce IL-2 in response to high density immobilized anti-CD3 mAb in the absence of costimulatory signals provided by accessory cells (460, 461). In contrast, weaker TcR signaling such as is provided by soluble or suboptimal levels of immobilized mAbs require costimulatory signals for productive T cell activation. Since the Th cells in my experiments were activated with LPS-matured BMDCs at a ~30:1 ratio (250,000 T cells : 8000 BMDC) with optimal levels of anti-TcR β mAb to induce a maximal mitogenic response, I asked whether increasing the ratio of BMDCs to Th cells in

order to provide higher levels of costimulation would provoke IFN γ production from Thy-1-induced Th cells. Figure 4.9 shows that although higher ratios of BMDC to Th cells provoked higher levels of IFN γ production by TcR-induced Th cells when stimulated with either anti-Thy-1 or anti-TcR β mAbs alone, higher ratios did not stimulate appreciable levels of IFN γ production by Thy-1-induced Th cells activated by either anti-Thy-1 or anti-TcR β mAbs.

4.10 Anti-Thy-1 mAb-Mediated Signaling Promotes the Development of Th1, Th2 and Th17 Effector Cell Phenotypes Under Polarizing Conditions.

The cytokine environment in which CD4⁺ T cells are activated has a strong influence on subsequent Th cell subset differentiation. The development of Th1, Th2 and Th17 cells is favored in environments that are rich in IL-12, IL-4 or TGF β and IL-6, respectively (152-154, 197, 270). In order to examine the role that Thy-1 might play in the polarization of Th cells when providing signal 1 in each of these different environments, I activated CD4⁺ T cells with either anti-TcR β or anti-Thy-1 mAbs in the presence of costimulation provided by LPS-matured BMDCs under Th1-, Th2- or Th17-polarizing conditions for 6 days. Th cells were rested and then restimulated for 24 h with 5 pg/ml phorbol 12-myristate 13-acetate (PMA) and 500 pg/ml ionomycin. PMA and ionomycin act together to promote T cell proliferation and cytokine production by activating signaling pathways downstream of the membrane proximal events that are initiated by TcR signal transduction. Ionomycin is a calcium ionophore that activates Ca⁺²-dependent signaling pathways, while PMA is an analog of DAG and activates PKC (462). PMA and ionomycin are often used to examine cytokine profiles of Th cells. As expected, TcR-induced Th cells that were activated under Th1-polarizing conditions (rmIL-12, anti-IL-4 mAb), Th2-polarizing conditions (rmIL-4, anti-IFN γ and anti-IL-12 mAb) or Th17-polarizing conditions (rmIL-6, rhTGF- β 1, anti-IL-4 and anti-IFN γ mAb) preferentially promoted high levels of the signature cytokines IFN γ , IL-4 and IL-17A, respectively, when restimulated with PMA and ionomycin (Figure 4.10). Thy-1-induced Th cells that were activated under Th1-, Th2- or Th17-polarizing conditions also produced high levels of IFN γ , IL-4 or IL-17A, respectively, when restimulated with PMA and ionomycin. Interestingly, Thy-1-induced Th cells consistently produced significantly more of each of the

signature cytokine when under the corresponding polarizing conditions than TcR-induced Th cells. Moreover, this difference was more extreme with IL-4 and IL-17A than IFN γ when under Th2-, Th17- and Th1-polarizing conditions, respectively.

Taken together, these results suggest that when the appropriate cytokine environment is present, Thy-1 can signal in the context of costimulation provided by BMDCs to promote the polarization of resting T cells into the different Th cell subsets. Anti-Thy-1 mAbs also appear to be better than anti-TcR β mAbs at inducing the polarization of CD4⁺ T cells, since Thy-1-induced polarized Th cells produce higher levels of the corresponding signature cytokine. Although Thy-1-induced Th1 cells did produce higher levels of IFN γ than TcR-induced Th1 cells, the difference was not as extreme as the IL-17 and IL-4 production seen in Thy-1-induced Th17 and Th2 cells. This is consistent with my previous observations that Thy-1 preferentially favors IL-17 and IL-4 production over IFN γ production.

4.11 Th1, Th2 or Th17 Effector Cells Express High Levels of Thy-1 on Their Surface.

Thy-1 expression on T cells can vary depending upon their stage of development (463). For example, murine thymocytes express higher levels of Thy-1 than do peripheral T cells. Whether Thy-1 expression varies on different Th subsets has never been examined. Following activation of T cells with either anti-TcR β or anti-Thy-1 mAbs and LPS-matured BMDCs under Th1-, Th2- or Th17-polarizing conditions, I stained the polarized Th cells with FITC-labeled anti-Thy-1 (clone 30-H12) mAb and then assessed Thy-1 expression by flow cytometry. Figure 4.11 shows that all TcR-induced Th subsets expressed similar levels of Thy-1. Although all Thy-1-induced Th subsets also expressed high levels of Thy-1, there were some differences. Thy-1-induced Th17 and Th1 cells consistently stained with lower fluorescence intensity than TcR-induced Th17 and Th1 cells. These differences, however, could be due to the blockade of 30-H12 mAb binding by residual anti-Thy-1 mAb (clone G7) on the surface of the cells (400).

4.12 Thy-1-Induced Th1 and Th2 Cells Express Less T-bet and Gata-3 Than TcR-Induced Th1 and Th2 Cells.

Having observed that CD4⁺ T cells activated with anti-Thy-1 mAbs and costimulation provided by LPS-matured BMDCs under different Th cell polarizing conditions produced more of the signature cytokines than did TcR-induced Th cell subsets when stimulated with PMA and ionomycin, I asked whether they also expressed more of the lineage-specific transcription factors, T-bet and Gata-3. As was expected, TcR-induced Th cells activated in Th1 polarizing conditions expressed high levels of T-bet, while TcR-induced Th cells activated under Th2- and Th17-polarizing conditions did not (Figure 4.12A). Similarly, Thy-1-induced Th cells activated under Th1-polarizing conditions also expressed T-bet, while those activated under Th2- and Th17-polarizing conditions did not. Interestingly, a lower percentage of T cells in the Thy-1-induced Th cells activated under Th1-polarizing conditions expressed T-bet in comparison to TcR-induced Th1 cells. Similar results were observed when Gata-3 expression was assessed. TcR-induced Th cells activated under Th2-polarizing conditions expressed high levels of Gata-3 whereas Th cells activated under Th1- and Th17-polarizing conditions did not (Figure 4.12B). Although Thy-1-induced Th cells activated under Th2-polarizing condition also preferentially expressed Gata-3, a lower percentage of these Th cells expressed Gata-3 in comparison to TcR-induced Th2 cells.

Taken together, these results provide further evidence that Thy-1 can provide signal 1 which is required for CD4⁺ T cells to differentiate into Th effector subsets. The observation that Thy-1-signaling induces a lower percentage of cells that express similar levels of T-bet and Gata-3 under Th1- and Th2-polarizing conditions, respectively, compared to TcR-signaling, suggests that the ability of Thy-1-induced Th1 and Th2 cells to produce more IFN γ and IL-4, respectively, than TcR-induced Th1 and Th2 cells is not due to an ability of Thy-1 signaling to induce higher levels of T-bet and Gata-3. Instead, these findings indicate that there is some other as yet unidentified difference in TcR and Thy-1 signaling pathways.

4.13 CD48 Stimulation Does Not Promote T Cell Proliferation in the Presence of Costimulation Provided by Syngeneic LPS-matured BMDC or PMA.

Although numerous other GPI-APs have also been implicated in providing activating signals to T cells, it is not clear what effect they may also have on T cell cytokine production. It is also unclear whether the ability to provide a signal 1-like signal during T cell activation is common to all GPI-AP or whether it is unique to Thy-1. CD48 is expressed on both human and murine T cells and has been shown to enhance TcR-driven T cell activation (464). CD48 is particularly relevant to study since the interaction between CD48 and its ligand CD2 enhance TcR-driven T cell activation in part by increasing IL-2 and IFN γ mRNA stability (463). To determine whether CD48 could also provide signal 1 during T cell activation when strong costimulation is provided by LPS-matured BMDCs, I activated resting T cells with mitogenic anti-CD48 mAb and examined cellular division by Oregon green staining. Figure 4.13 shows that concentrations as high as 6 μ g/ml of anti-CD48 mAb did not promote cellular division in resting T cells as late as 72 h after activation. In contrast, the same concentration of anti-TcR β promoted robust T cell division. Cross-linking of some GPI-APs can promote T cell activation when PMA is also present (400, 465). I therefore added PMA to T cell cultures to determine if it would enable CD48-stimulated T cells to proliferate in response to CD48 cross-linking. There was, however, no cellular division seen in T cells incubated with both anti-CD48 mAb and PMA in the presence of LPS-matured BMDCs. These results indicate that CD48 cannot substitute for the TcR signal when costimulation is provided by BMDCs during T cell activation.

4.14 Ly-6A/E Stimulation Promotes Moderate Levels of T cell Proliferation in the Presence of Costimulation Provided Syngeneic LPS-matured BMDCs and PMA.

Ly-6 describes a family of GPI-APs that are encoded for in the *Ly-6* multigene locus chromosome 15 of the murine genome (466). Ly-6A.2 and Ly-6E.1 are alternate allelic gene products of the *Ly-6a* locus that are strain-specific. Ly-6A.2 is expressed by C57BL/6 mice whereas Ly-6E.1 is expressed by BALB/C mice. Ly-6A.2 and Ly-6E.1 are thought to have similar function and have been designated Ly-6A/E. These molecules have similar expression patterns and are

expressed by peripheral T cells (466). Moreover, both molecules have been implicated in T cell activation. Cross-linking of Ly-6A/E with the mitogenic anti-Ly-6A/E mAb D7 induces proliferation in T cell hybridoma cells and resting T cells but only when in the presence of accessory cells and PMA (465). Highly purified T cells did not proliferate in response to high concentrations of anti-Ly-6A/E mAb even when strong costimulation was provided by LPS-matured BMDCs (Figure 4.14). The addition of PMA allowed anti-Ly-6A/E mAb to induce moderate amounts of T cell division when costimulation was provided by LPS-mature BMDCs. These results suggest that, unlike Thy-1, Ly-6A/E may not provide a sufficient signal that can substitute for the TcR signal in promoting T cell proliferation. Furthermore, they suggest that the Ly-6A/E signal may be deficient in the activation of DAG-dependent signaling pathways required to induce T cell proliferation that are provided by the TcR and Thy-1 signals, as the addition of PMA was required for Ly-6A/E-induced proliferation.

4.15 Ly6A/E Stimulation in the Presence of Costimulation Provided by LPS-matured BMDCs and PMA Does Not Induce the Production of Significant Amounts IL-2, IFN γ , IL-4 or IL-17A by Resting T cells.

The role that Ly6A/E may play in the regulation T cell cytokine production is unclear. T cell hybridoma cells produce IL-2 in response to Ly-6A/E signaling in the presence of splenic accessory cells (465). To my knowledge, the ability of primary resting T cells to produce IL-2 in response to Ly-6A/E stimulation has not been investigated. In fact, anti-Ly-6A/E mAbs actually inhibit TcR-driven IL-2 production (467). To determine whether Ly6A/E signaling can induce T cells to produce different cytokines, I measured IL-2, IFN γ , IL-4 and IL-17A production by ELISA following activation of T cells with anti-Ly6A/E mAbs and costimulation provided by LPS-matured BMDCs with or without PMA. T cells activated with anti-Ly-6A/E mAb did not produce appreciable levels of IL-2, IFN γ , IL-17A or IL-4 in the absence of PMA (Figures 4.15A-D). Combinations of anti-Ly-6A/E and PMA that were mitogenic also did not induce appreciable levels of IL-2, IFN γ , IL-17A or IL-4 in T cell cultures when costimulation was provided by LPS-matured BMDCs. In contrast, anti-Thy-1 mAbs induced higher levels of IL-4 and IL-17A production and lower levels of IFN γ than anti-TcR β mAbs. These results suggest

that signaling through Ly-6A/E is unable to induce Th cell cytokine production by resting T cells. Moreover, they suggest that Thy-1 is unique in its ability to preferentially induce IL-4 and IL-17A production, as this is not a common ability of all GPI-APs expressed on murine T cells.

4.16 Differential Effect of PMA on Anti-Thy-1 mAb- and Anti-TcR β mAb-Induced Cytokine Production.

Anti-Thy-1 mAb-induced T cell proliferation in the presence of splenic accessory cells is more sensitive to the pharmacological inhibition of PKC by calphostin C than is TcR-induced T cell proliferation (406). Synergy between Thy-1 and the PKC activator PMA in the induction of T cell proliferation and IL-2 production has also been observed in T cells activated with anti-Thy-1 mAbs in the presence of splenic accessory cells (468). These observations suggest that Thy-1 might not strongly induce PKC (406). In my hands, the addition of PMA had differential effects on anti-TcR β mAb- and anti-Thy-1 mAb-induced IL-2 and IFN γ production. Although, PMA enhanced both anti-TcR β mAb- and anti-Thy-1 mAb-induced IL-2 production, anti-Thy-1 mAb-induced IL-2 production was enhanced to a greater extent than was anti-TcR β mAb-induced IL-2 production (Figure 4.15A). Interestingly, PMA enhanced anti-Thy-1 mAb-induced IFN γ production, but modestly inhibited anti-TcR β mAb-induced IFN γ production (Figure 4.15B). PMA had no effect on either anti-TcR β mAb- or anti-Thy-1 mAb-induced IL-4 production (Figure 4.15D) and similarly inhibited IL-17A production (Figure 4.15C).

Figure 4.1. Thy-1 Signaling Induces More IL-17 and IL-4 mRNA and Less IFN γ mRNA than TcR Signaling. Highly purified CD3⁺ T cells (2.5×10^6 cells/well) with or without LPS-matured BMDCs (8×10^4 cells/well) were seeded into 24-well plates. T cells were then cultured in the presence or absence of 6 μ g/ml anti-Thy-1 mAb (clone G7), anti-TcR β mAb or appropriate isotype control for 24 h. Total RNA was isolated using the Trizol method and used to generate cDNA. Real-time PCR with primers specific for IFN γ , IL-17, IL-4 and RNA Pol II was performed. Relative expression of each cytokine mRNA was calculated using the standard curve method and normalized to the TcR-activated T cells. Data are the mean of at least three separate experiments \pm SEM.

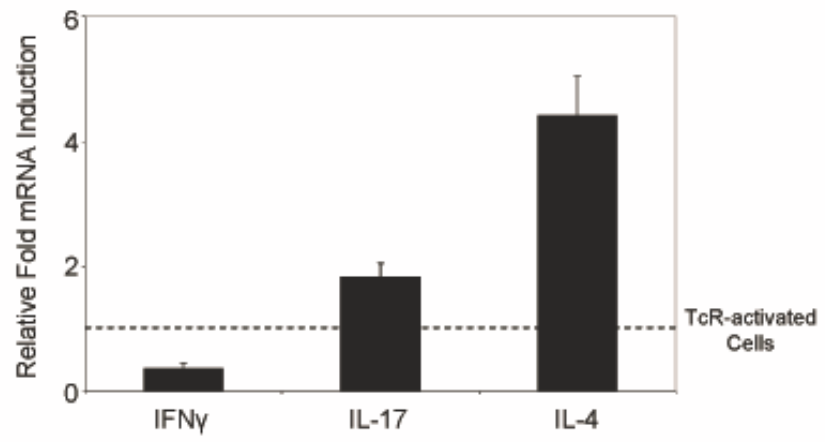
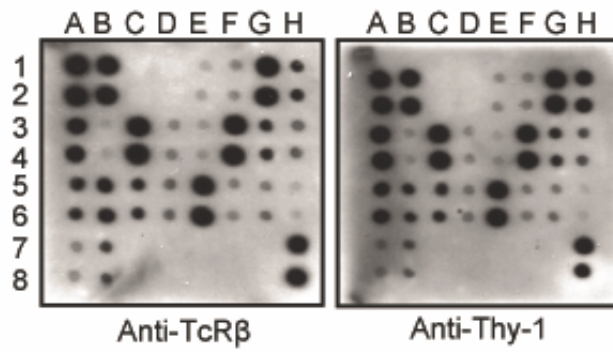


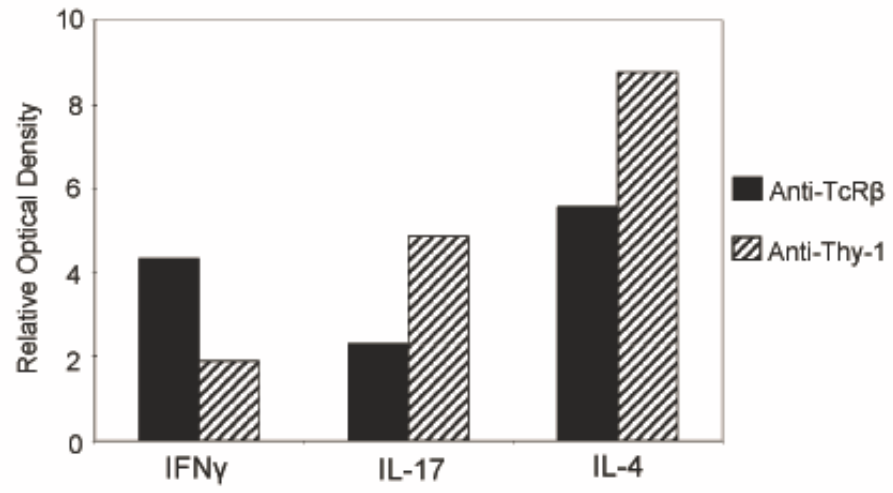
Figure 4.2. Thy-1 Signaling Promotes Differential Cytokine Production Compared to TcR Signaling. Highly purified CD3⁺ T cells (2.5×10^5 cells/well) with or without LPS-matured BMDCs (8×10^3 cells/well) were seeded in quadruplicate into 96-well round-bottom plates. T cells were then cultured in the presence of 6 $\mu\text{g/ml}$ anti-Thy-1 mAb (clone G7), anti-TcR β mAb or the appropriate isotype control for the 24 h. (A) Supernatants were isolated and analyzed for the production of 22 different cytokines using Raybio® Mouse Cytokine Arrays. (B) Densitometry was performed on the cytokine array and differences in relative optical density of T helper cell cytokines are shown. Data are from one experiment. To confirm the differences in cytokine production sandwich ELISAs for (C) IL-2 (D) IFN γ (E) IL-17A and (F) IL-4 were also performed on supernatants isolated from stimulated T cells. Data shown are the mean of at least three separate experiments \pm SEM; * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$, and ns denotes “not significant”, when compared to T cells activated with anti-TcR β mAb and LPS-matured BMDCs, as determined by the Bonferroni multiple comparisons test.

A

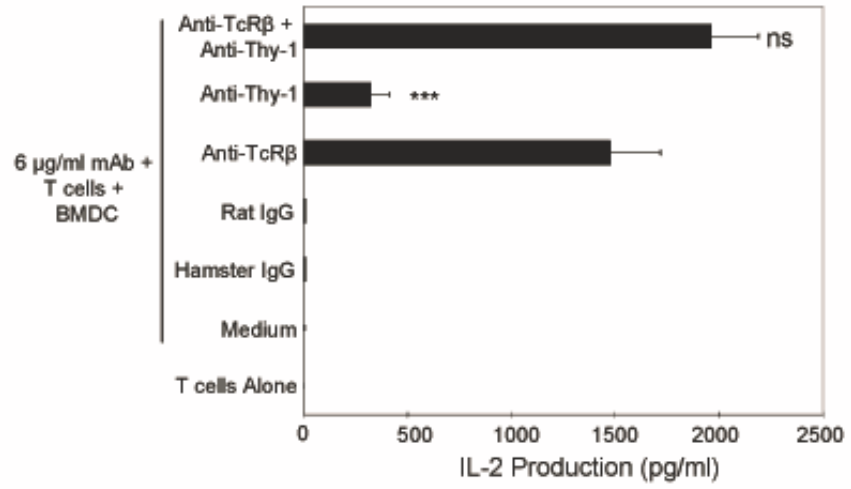
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2									
3	IL-4	IL-5	IL-6	IL-8	IL-10	IL-12 p40	IL-12p70	IL-13	
4									
5	IL-17	IFN- γ	MCP-1	MCP-5	RANTES	SCF	sTNFR1	TNF α	
6									
7	Thrombo- poietin	VEGF							
8								+	



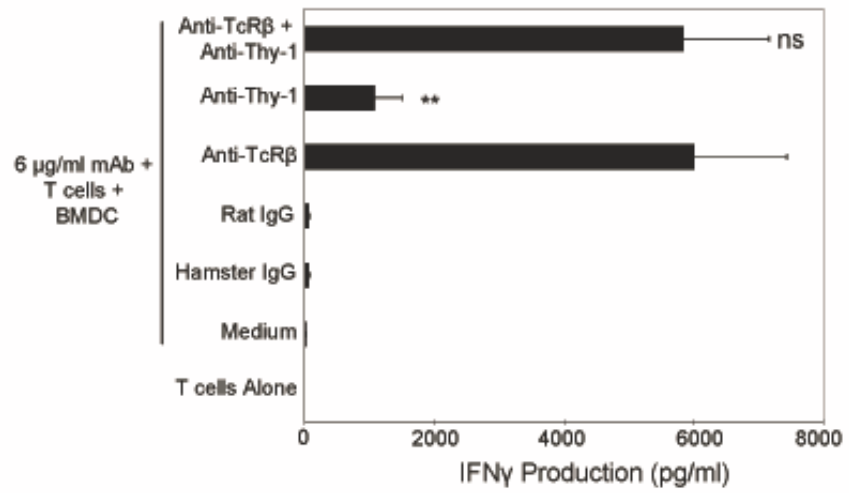
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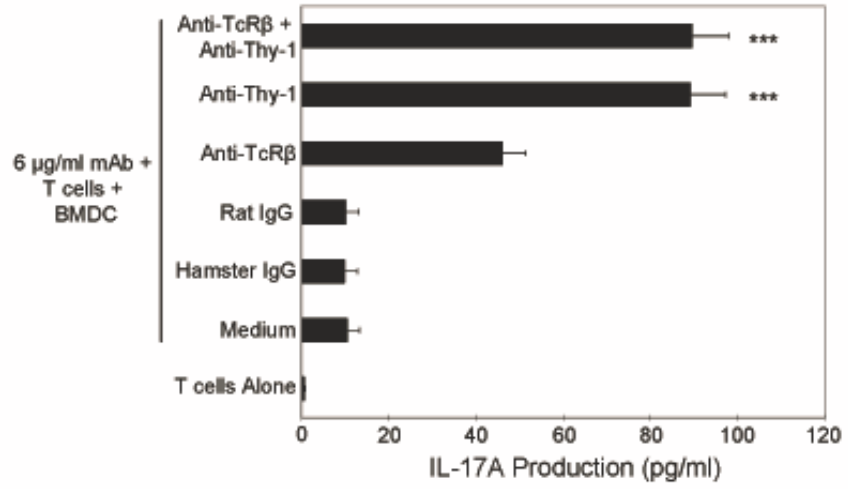
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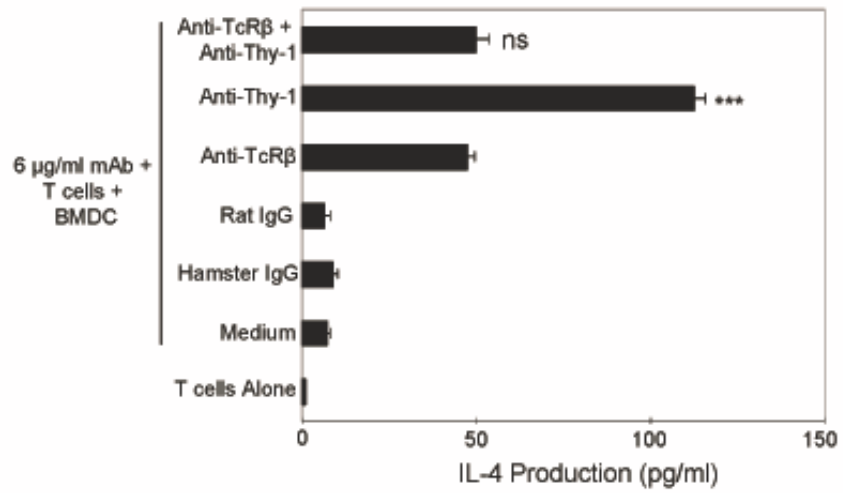
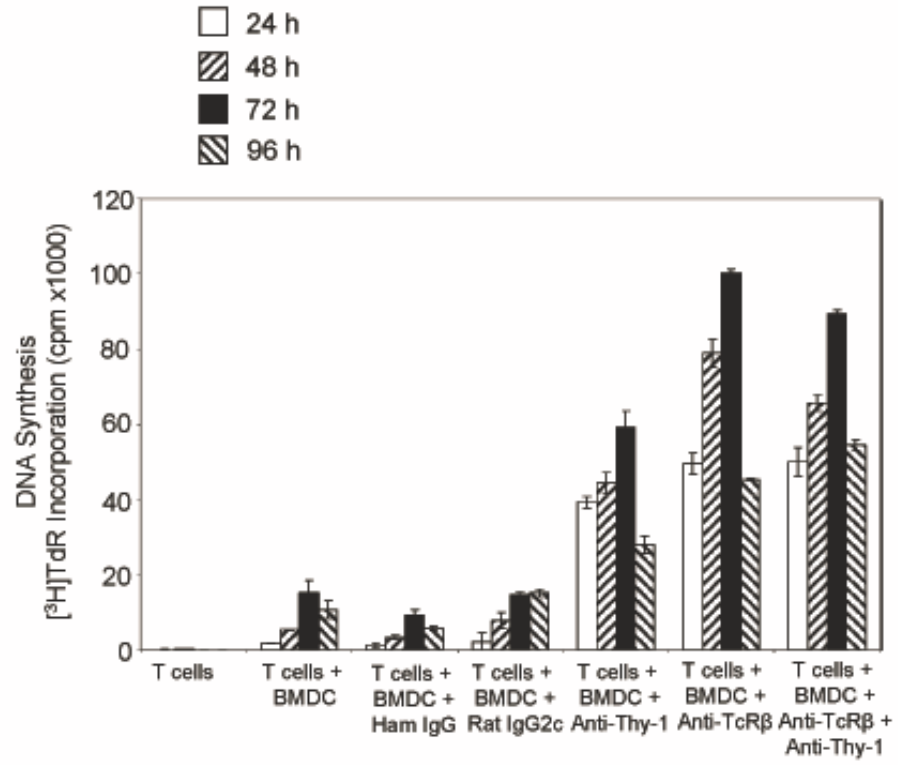


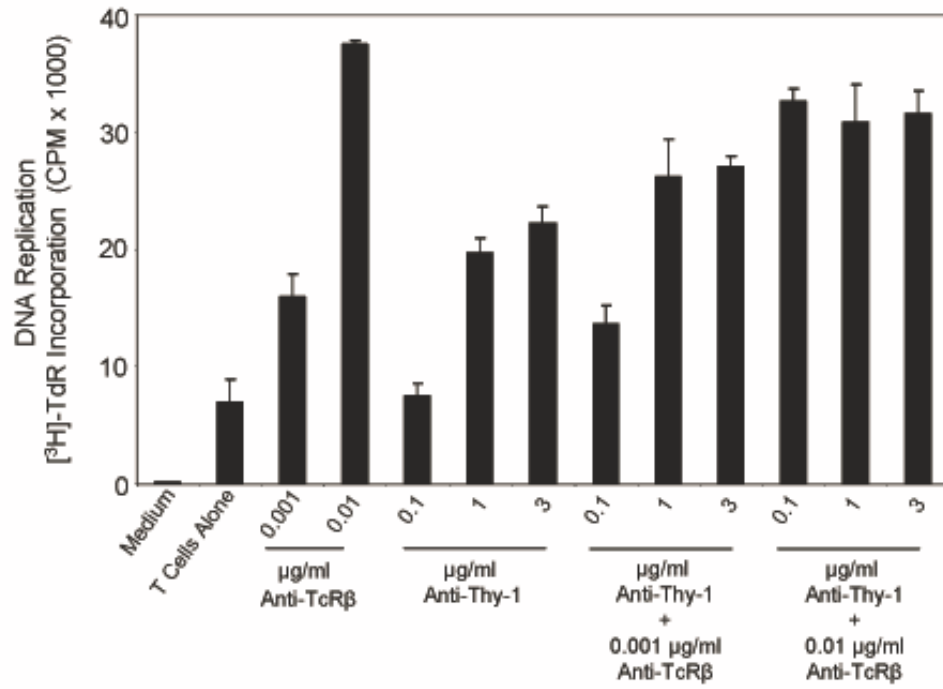
Figure 4.3. Thy-1 Signaling Does Not Enhance TcR-Induced Proliferation.

Highly purified CD3⁺ T cells (2.5×10^5 cells/well) with or without LPS-matured BMDCs (8×10^3 cells/well) were seeded in triplicate into 96-well round-bottom plates. (A) T cells were then cultured in the presence of 6 μ g/ml of anti-Thy-1 mAb, anti-TcR β mAb, anti-Thy-1 and anti-TcR β mAb combined or the appropriate isotype controls for the indicated times. (B) T cells were cultured in the presence of the indicated concentrations of anti-TcR β and anti-Thy-1 mAb, either alone or in combination for 72 h. Wells were pulsed with [³H]TdR 6 h before the endpoint at which time the cells were harvested and DNA synthesis was determined based on [³H]TdR incorporation. Data are means of 3 independent experiments \pm SEM. (C) Highly purified CD3⁺ T cells were labeled with 2.5 μ M Oregon Green and seeded (2.5×10^5 cells/well) in duplicate into 96-well round-bottom plates with or without LPS-matured BMDCs (8×10^3 cells/well) . T cells were cultured in the presence of the indicated concentrations of anti-TcR β and anti-Thy-1 mAb, either alone or in combination for 72 h. Wells were pooled and Oregon Green staining was measured by flow cytometry. The number of cellular divisions is indicated by decreasing fluorescence intensity. Data are representative of three separate experiments.

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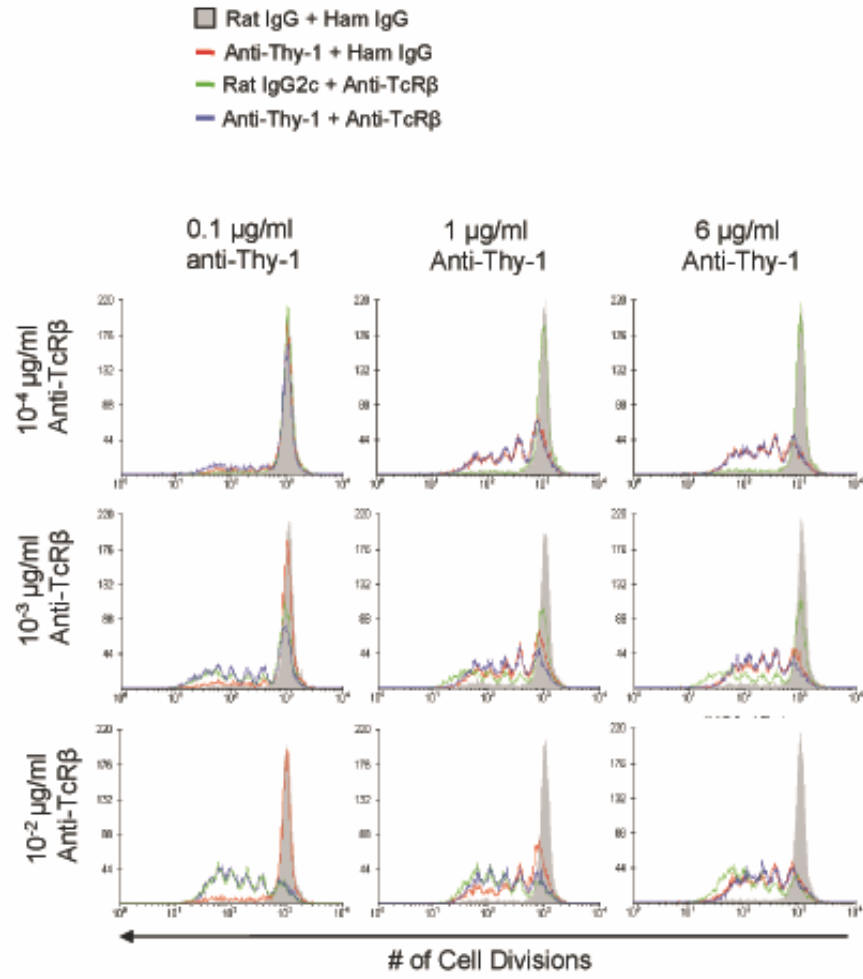


Figure 4.4. Thy-1 Signaling Induces A Higher Ratio of Gata-3 to T-bet Expression Than Does TcR Signaling. Highly purified CD3⁺ T cells (2.5×10^5 cells/well) were seeded in quadruplicate into 96-well round-bottom plates with or without LPS-matured BMDCs (8×10^3 cells/well). T cells were cultured in the presence of 6 $\mu\text{g/ml}$ anti-TcR β , anti-Thy-1 mAb or the appropriate isotype control for the indicated times. Cells were then fixed, permeabilized and stained with anti-Gata-3-PE, anti-T-bet-PE mAb or the appropriate PE-labeled isotype control. Gata-3 and T-bet expression were measured by flow cytometry. Data are representative of three separate experiments.

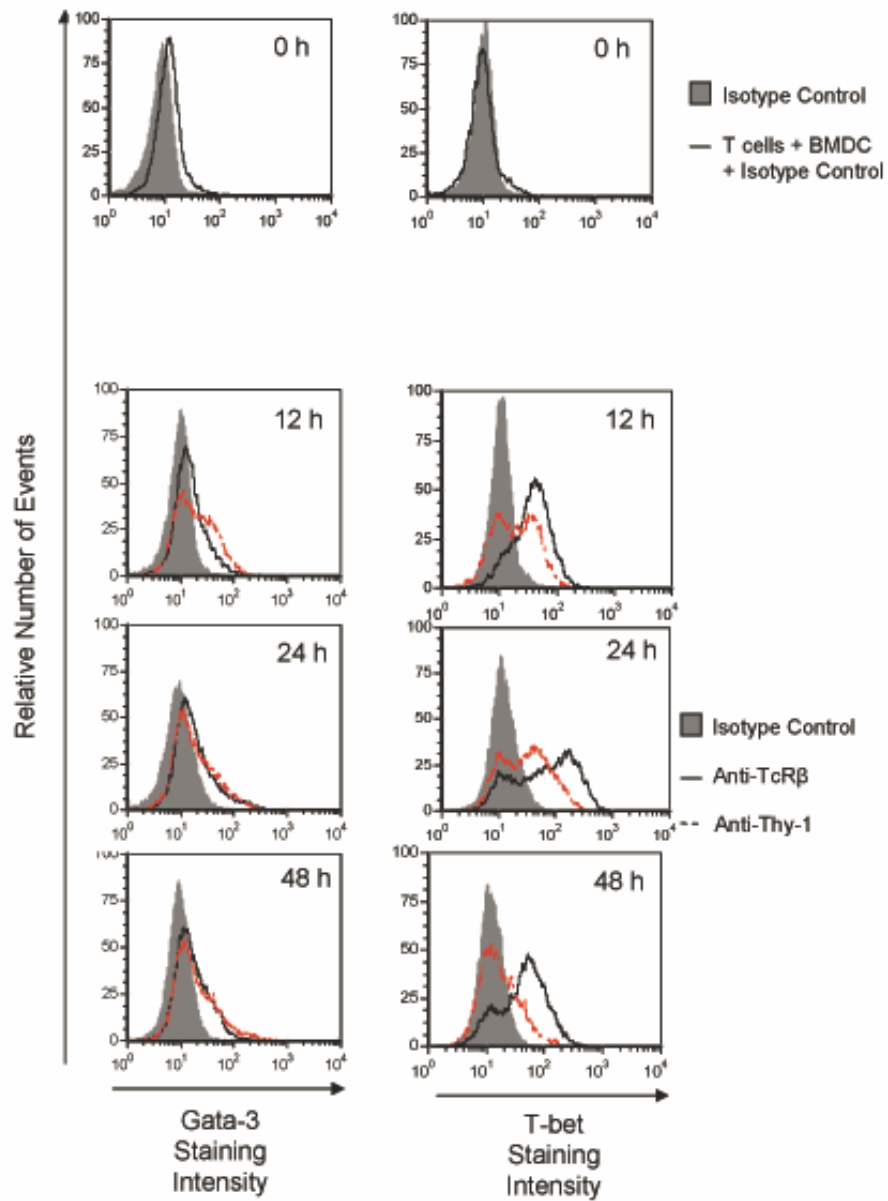


Figure 4.5. Thy-1 Signaling Induces Higher Levels of ROR γ t Expression In Comparison to TcR Signaling. (A) Highly purified CD3⁺ T cells (2.5×10^6 cells/well) with or without LPS-matured BMDCs (8×10^4 cells/well) were seeded into 24-well plates. T cells were then cultured in the presence or absence of 6 μ g/ml anti-Thy-1 mAb (clone G7), anti-TcR β mAb and/or the appropriate isotype control for the indicated times. Cells lysates were prepared and ROR γ (t) protein levels (58 kDa) were assessed by western blotting. Blots were then stripped and reprobed with antibodies specific for actin (42 kDa) to confirm equal loading. Data are representative of three independent experiments. (B) Optical density ratios were calculated by comparing the density of individual ROR γ t bands from three independent experiments with the corresponding actin band. Data are shown as mean \pm SEM; *** denotes $P < 0.001$ as determined by the Student's t-test.

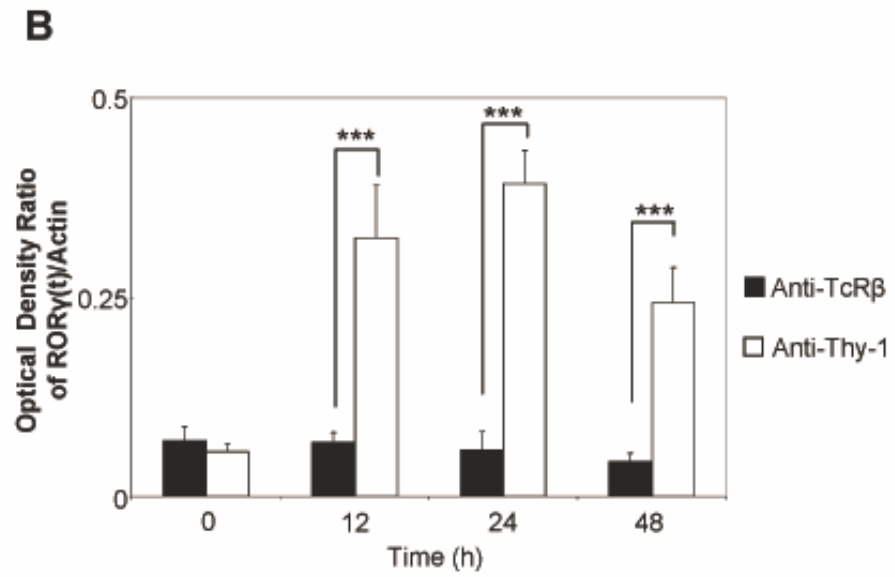
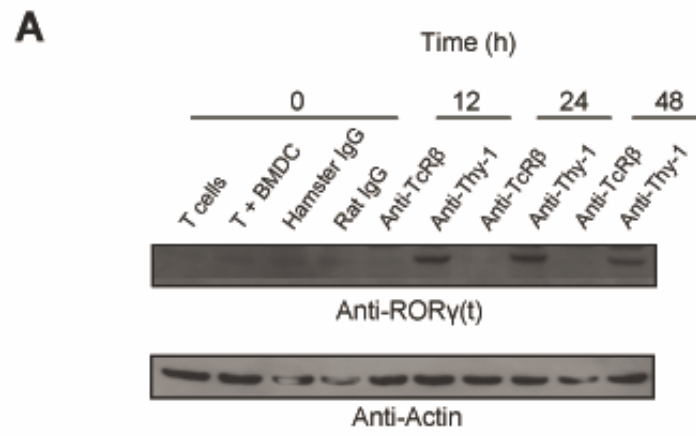
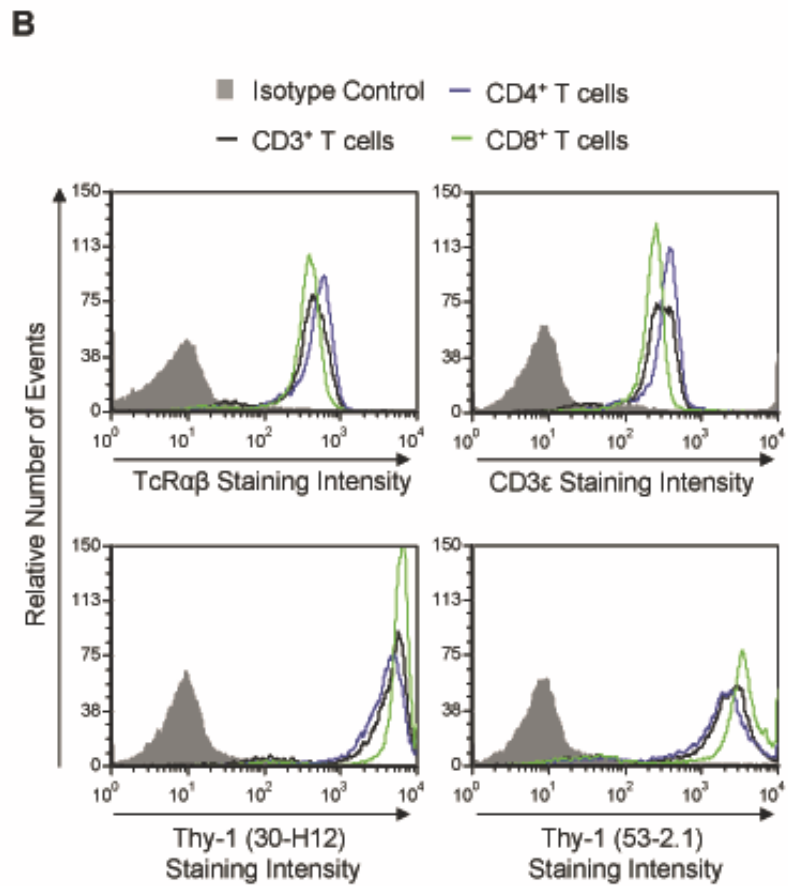
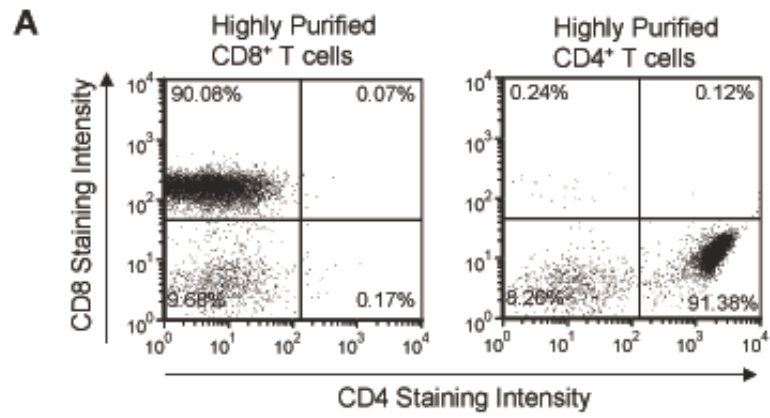


Figure 4.6. CD4⁺ T cells Express Lower Amounts of Thy-1 On Their Surface than CD8⁺ T cells. Highly purified CD3⁺ T cells, CD4⁺ T cells or CD8⁺ T cells were isolated from splenocytes using Miltenyi MACS negative selection kits for each T cell subset. (A) Highly purified CD4⁺ T cells or CD8⁺ T cells were co-stained with anti-CD4-FITC and anti-CD8-PE mAb or the appropriate isotype control and fixed with 1% paraformaldehyde. Cells were then analyzed by flow cytometry. Isotype controls showed less than 1% positive staining. (B) Cells were stained with anti-CD3 ϵ -FITC, anti-TcR $\alpha\beta$ -PE, anti-Thy-1-FITC (30-H12), anti-Thy-1-FITC (53-2.1) or the appropriate FITC- or PE-labeled isotype control. Cells were then analyzed by flow cytometry. (C) Data were expressed as the average MCF normalized to TcR expression on CD3⁺ T cells. Data are the mean of 3 independent experiments \pm SEM; * denotes $p < 0.05$, *** denotes $p < 0.001$ and ns denotes not significant as determined by the Bonferroni multiple comparisons test.



C

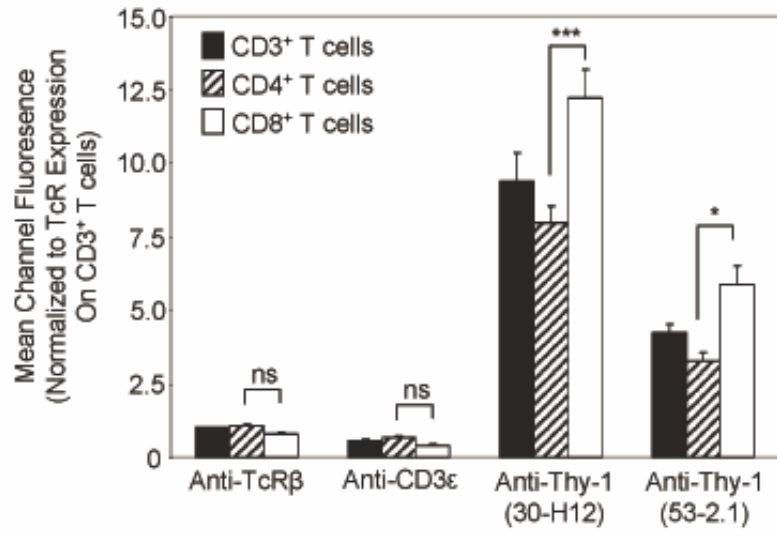


Figure 4.7. CD4⁺ and CD8⁺ T Cells Proliferate Similarly in Response to Thy-1 Signaling in the Presence of Costimulation Provided by LPS-Matured BMDCs. Highly purified CD4⁺ T cells or CD8⁺ T cells (2.5×10^5 cells/well) with or without LPS-matured BMDCs (8×10^3 cells/well) were seeded in triplicate into 96-well round-bottom plates. T cells were then cultured in the presence of the indicated concentrations of anti-Thy-1 mAb (clone G7), anti-TcR β mAb or isotype control for 72 h. Wells were pulsed with [³H]TdR 6 h before the endpoint at which time the cells were harvested and DNA synthesis was determined based on [³H]TdR incorporation. Background proliferation was controlled for by subtraction of experimental cpm from cpm of T cells and BMDC alone (7288.24 ± 1488.25 for CD8⁺ T cells and BMDC and 44157.44 ± 11919.57 for CD4⁺ T cells and BMDC) and are the mean of 3 independent experiments \pm SEM; ns denotes “not significant” as determined by the Bonferroni multiple comparisons test when the proliferation of CD4⁺ T cells was compared to that of CD8⁺ T cells that were activated by the same stimuli (anti-Thy-1 or anti-TcR β mAb).

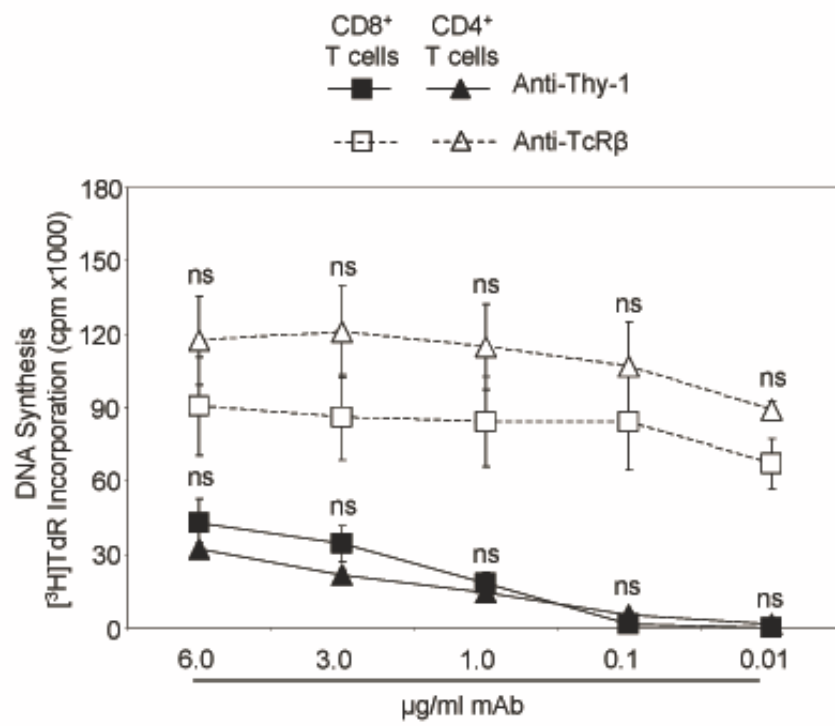
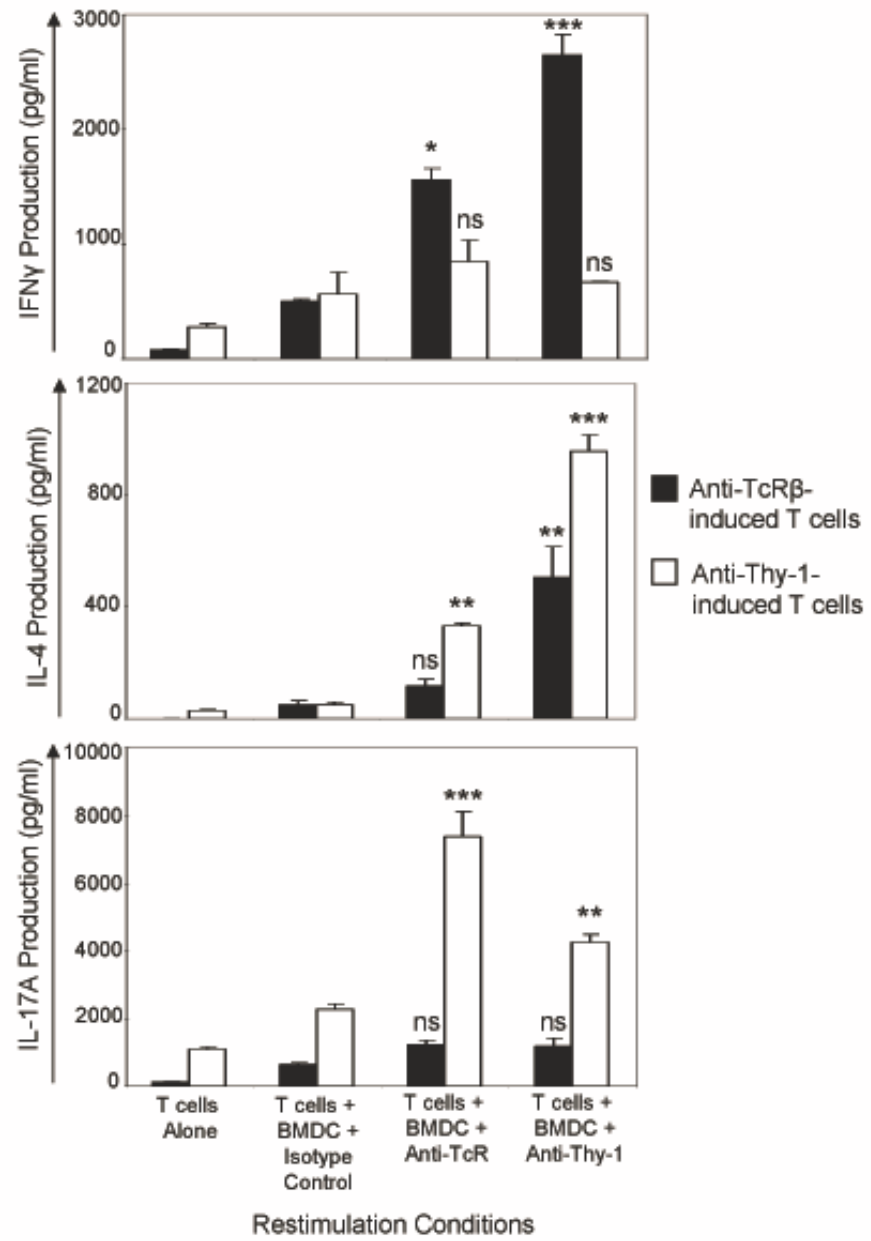
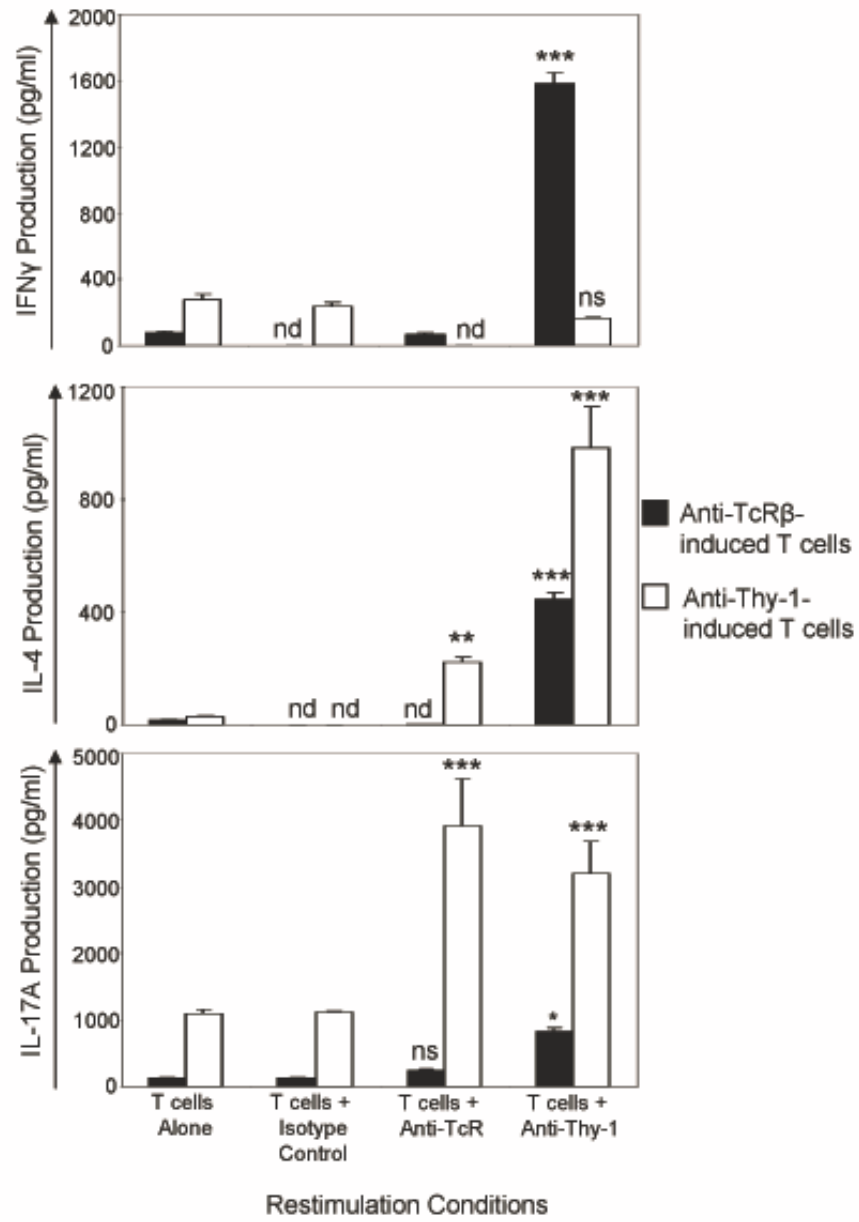


Figure 4.8. Restimulated Thy-1-Induced Th cells Produce More IL-17 and IL-4 and Less IFN γ Than TcR-Induced Th cells. Highly purified CD4⁺ T cells (1.25×10^6 cells/well) with or without LPS-matured BMDCs (4×10^4 cells/well) were seeded into 24-well plates. T cells were then cultured in the presence or absence of 6 μ g/ml anti-Thy-1 mAb (clone G7) or anti-TcR β mAb for 6 days. Th cells were then washed and allowed to rest for 6 h. T cell numbers were determined and equal numbers of viable cells were plated in 96 well flat-bottom plates. Th cells (2.5×10^5 cells/well) were reactivated with 6 μ g/ml anti-TcR β , anti-Thy-1 mAbs or isotype control that were either (A) in the presence of LPS-matured BMDCs (8×10^3 cells/well), (B) soluble or (C) coated on polystyrene microspheres for for 24 h. Supernatants were analyzed for cytokine production using IL-2, IFN γ , IL-17A and IL-4 ELISAs. Data are representative of two independent experiments \pm SD; * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$, ns denotes “not significant”, as determined by the Bonferroni multiple comparisons test when compared to isotype control or T cells alone. nd denotes “not detected”.

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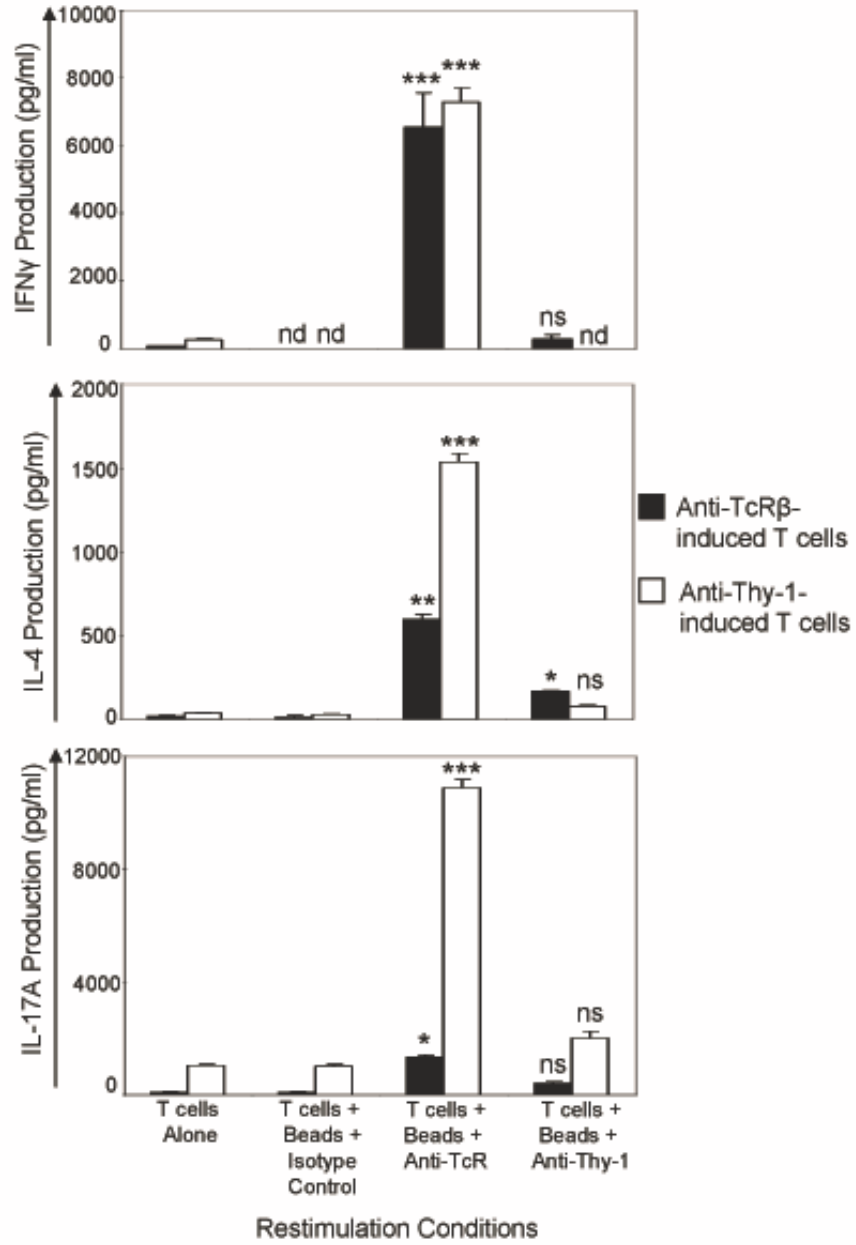


Figure 4.9. Increasing the Number of BMDCs Does Not Allow Thy-1-induced Th Cells to Produce IFN γ . Highly purified CD4⁺ T cells (1.25×10^6 cells/well) with or without LPS-matured BMDCs (4×10^4 cells/well) were seeded into 24-well plates. T cells were then cultured in the presence or absence of 6 μ g/ml anti-Thy-1 mAb (clone G7) or anti-TcR β mAb for 6 days. Th cells were then washed and allowed to rest for 6 h. Th cells numbers were determined and equal numbers of viable cells were plated in 96 well flat-bottom plates. Th cells were reactivated with 6 μ g/ml anti-TcR β or anti-Thy-1 mAbs that were in the presence of LPS-matured BMDCs at the indicated numbers for 24 h. Supernatants were analyzed for IFN γ cytokine production by ELISA. Data are representative of three independent experiments \pm SD.

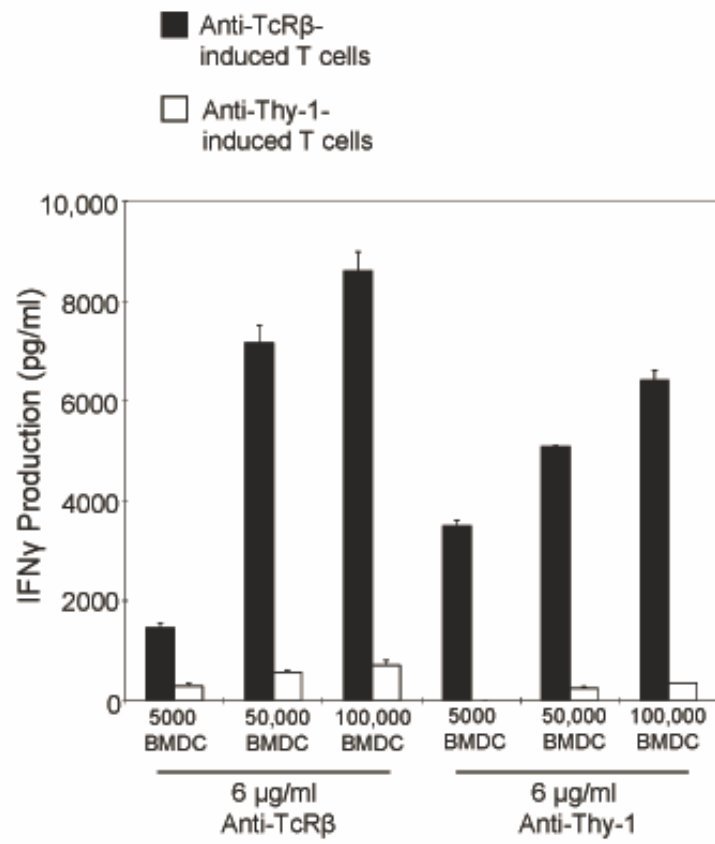


Figure 4.10. Thy-1 Signalling Promotes the Development of Th1, Th2 and Th17 Effector Cell Phenotypes Under Th Subset Polarizing Conditions. Highly purified CD4⁺ T cells (1.25×10^5 cells/well) with or without LPS-matured BMDCs (4×10^4 cells/well) were seeded in 24-well flat-bottom plates. T cells were then stimulated with 6 μ g/ml anti-Thy-1 mAb (clone G7) or anti-TcR β mAb under Th1 (rmIL-12, anti-IL-4 mAb), Th2 (rmIL-4, anti-IL-12 mAb, anti-IFN γ mAb) and Th17 (rmIL-6, rmTGF- β , anti-IFN γ mAb and anti-IL-4 mAb) polarizing conditions for 6 days. Th cells were washed, rested for 6 h and then the number of viable cells in each treatment were normalized by trypan blue counts and replated in 24-well flat-bottom plates. Polarized Th cells were then restimulated for 24 h with 5 μ g/ml PMA and 500 μ g/ml ionomycin. Supernatants were isolated and analyzed by ELISA for IFN γ , IL-17 and IL-4 production. Data shown are representative of three separate experiments \pm SD; * denotes $p < 0.05$, *** denotes $p < 0.001$, and ns denotes “not significant” as determined by Bonferroni multiple comparisons test.

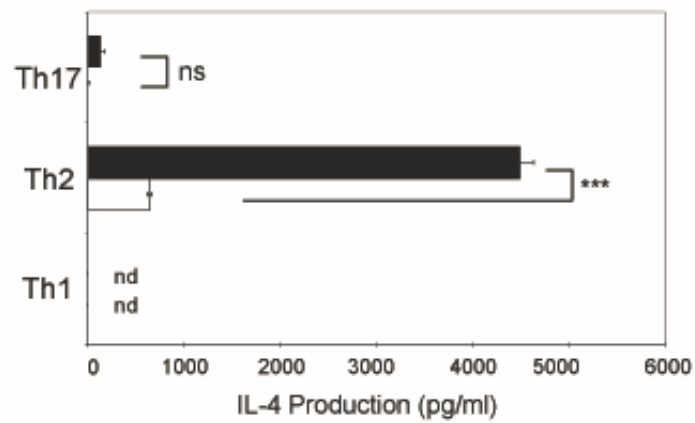
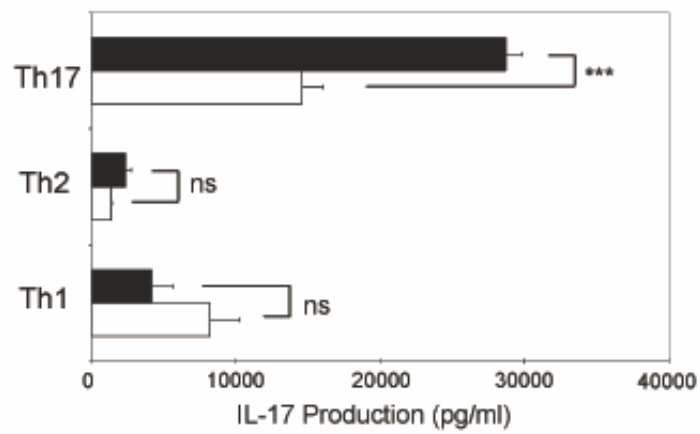
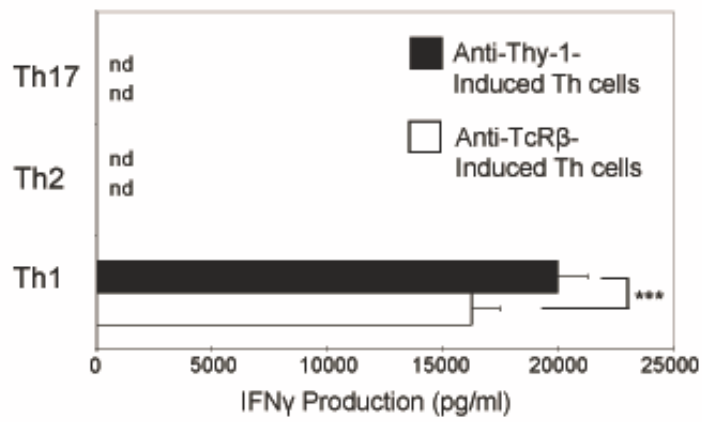


Figure 4.11. Th1, Th2 or Th17 Effector Cells Phenotypes Express High Levels of Surface Thy-1. Highly purified CD4⁺ T cells (1.25×10^6 cells/well) with or without LPS-matured BMDCs (4×10^4 cells/well) were seeded in 24-well flat-bottom plates. T cells were then stimulated with 6 μ g/ml anti-Thy-1 mAb (clone G7) or anti-TcR β mAb in the presence of medium alone or under Th1 (rmIL-12, anti-IL-4 mAb), Th2 (rmIL-4, anti-IL-12 mAb, anti-IFN γ mAb) and Th17 (rmIL-6, rmTGF- β , anti-IFN γ mAb and anti-IL-4 mAb) polarizing conditions for 5 days. On day 5, the number of viable cells in each treatment were normalized by trypan blue counts and replated in 24-well flat-bottom plates. Polarized Th cells were then restimulated for 24 h with 5 pg/ml PMA and 500 pg/ml ionomycin. Cells were stained with FITC-anti-Thy-1 mAb (30-H12) (open peak) or the appropriate FITC-labeled isotype control (closed peak) and Thy-1 staining intensity was assessed by flow cytometry. Data shown are representative of three separate experiments.

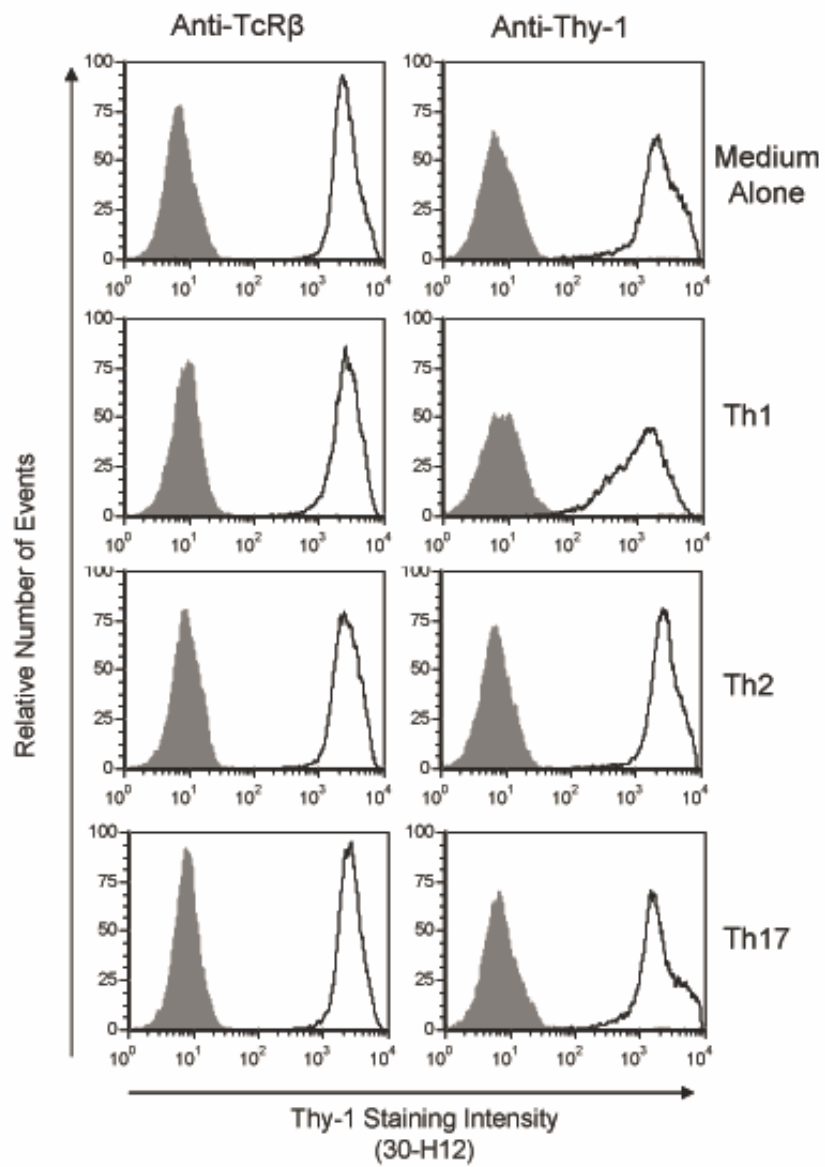
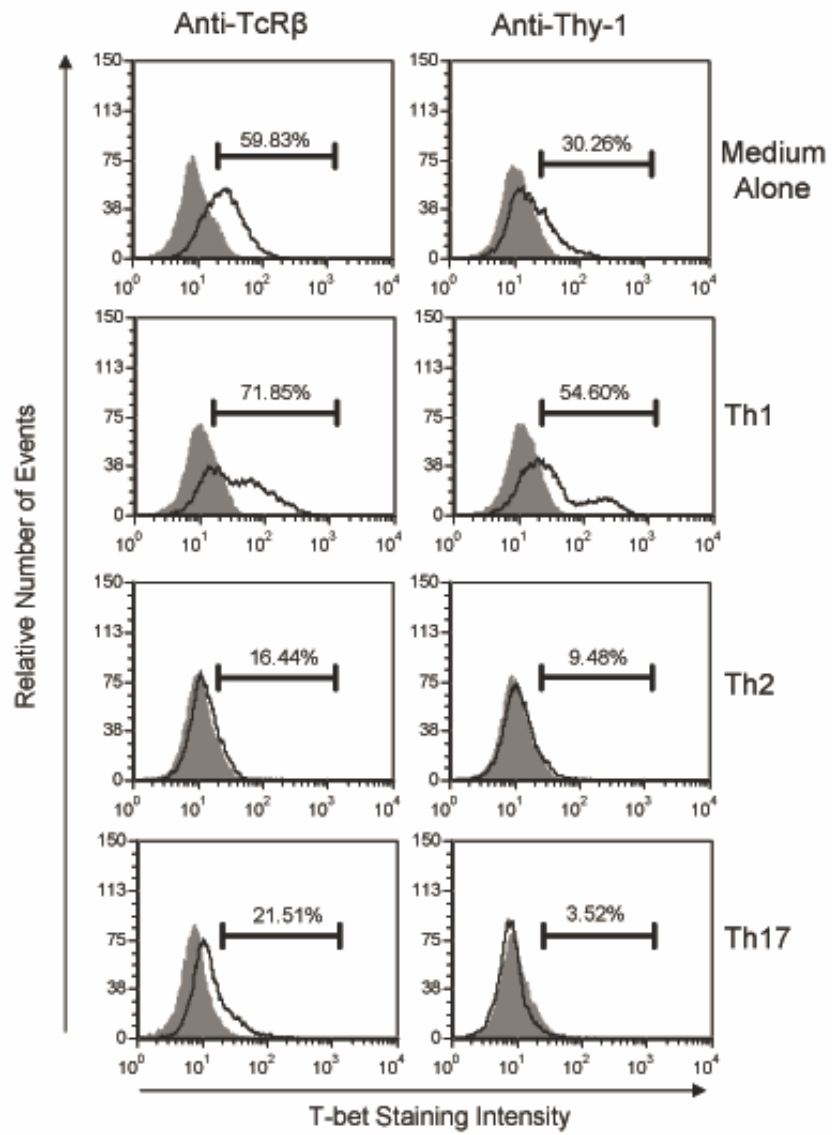


Figure 4.12. Thy-1-Induced Th1 and Th2 Cells Express Less T-bet and Gata-3 Than TcR-Induced Th1, Th2 and Th17 Cells. Highly purified CD4⁺ T cells (1.25×10^5 cells/well) with or without LPS-matured BMDCs (4×10^4 cells/well) were seeded in 24-well flat-bottom plates. T cells were then stimulated with 6 μ g/ml anti-Thy-1 mAb (clone G7) or anti-TcR β mAb in the presence of medium alone or under Th1 (rmIL-12, anti-IL-4 mAb), Th2 (rmIL-4, anti-IL-12 mAb, anti-IFN γ mAb) and Th17 (rmIL-6, rmTGF- β , anti-IFN γ mAb and anti-IL-4 mAb) polarizing conditions for 6 days. Cells were then fixed, permeabilized and stained intracellularly with (A) anti-T-bet-PE mAb or (B) anti-Gata-3-PE, (open peak) or the appropriate PE-labeled isotype control (closed peak). Gata-3 and T-bet expression were measured by flow cytometry. Data are representative of three separate experiments.

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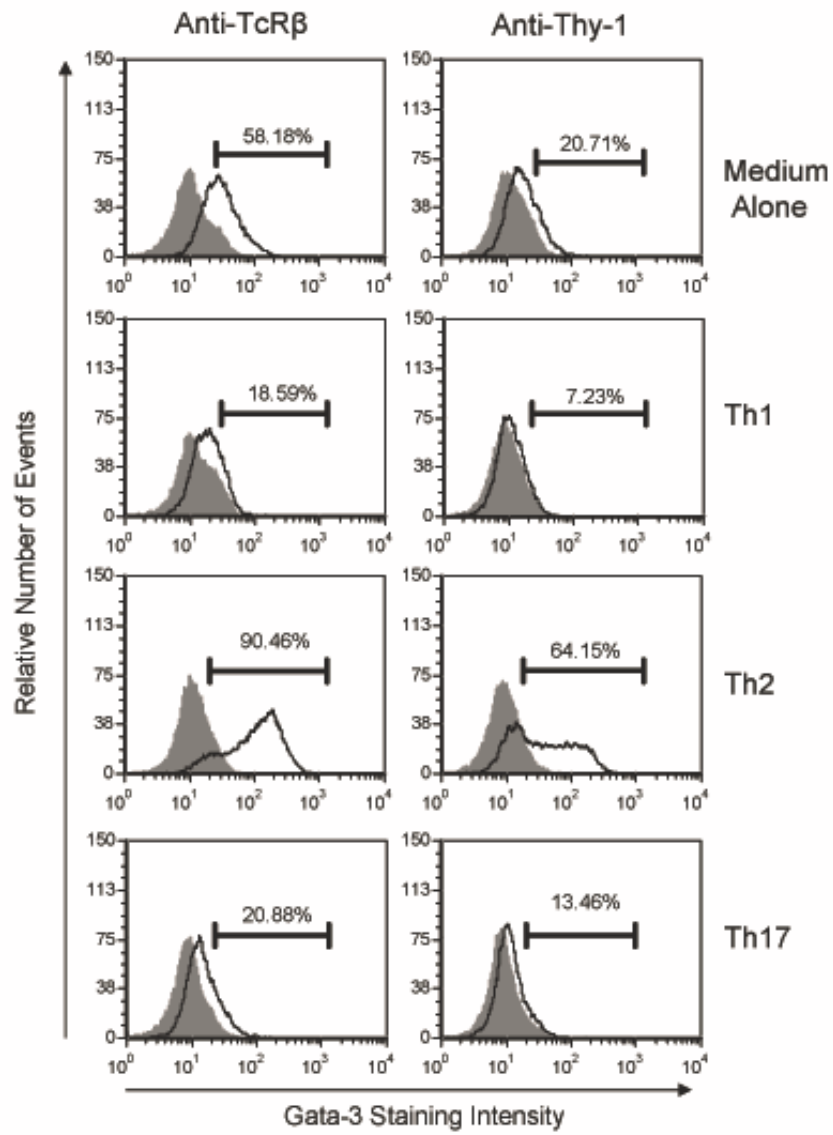
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Figure 4.13. CD48 Stimulation Does Not Promote T Cell Proliferation in the Presence of Costimulation Provided by Syngeneic LPS-matured BMDCs or PMA. Highly purified CD3⁺ T cells were labelled with 2.5 μM Oregon Green and seeded (2.5×10^5 cells/well) in duplicate into 96-well round-bottom plates with or without LPS-matured BMDCs (8×10^3 cells/well) . T cells were then cultured in the presence of the indicated concentrations of anti-CD48 mAb, anti-TcRβ mAb or the appropriate isotype control with or without PMA for 72 h. Wells were pooled and Oregon Green staining was measured by flow cytometry. Number of cellular divisions are indicated by decreasing fluorescence intensity. Data shown are representative of three separate experiments.

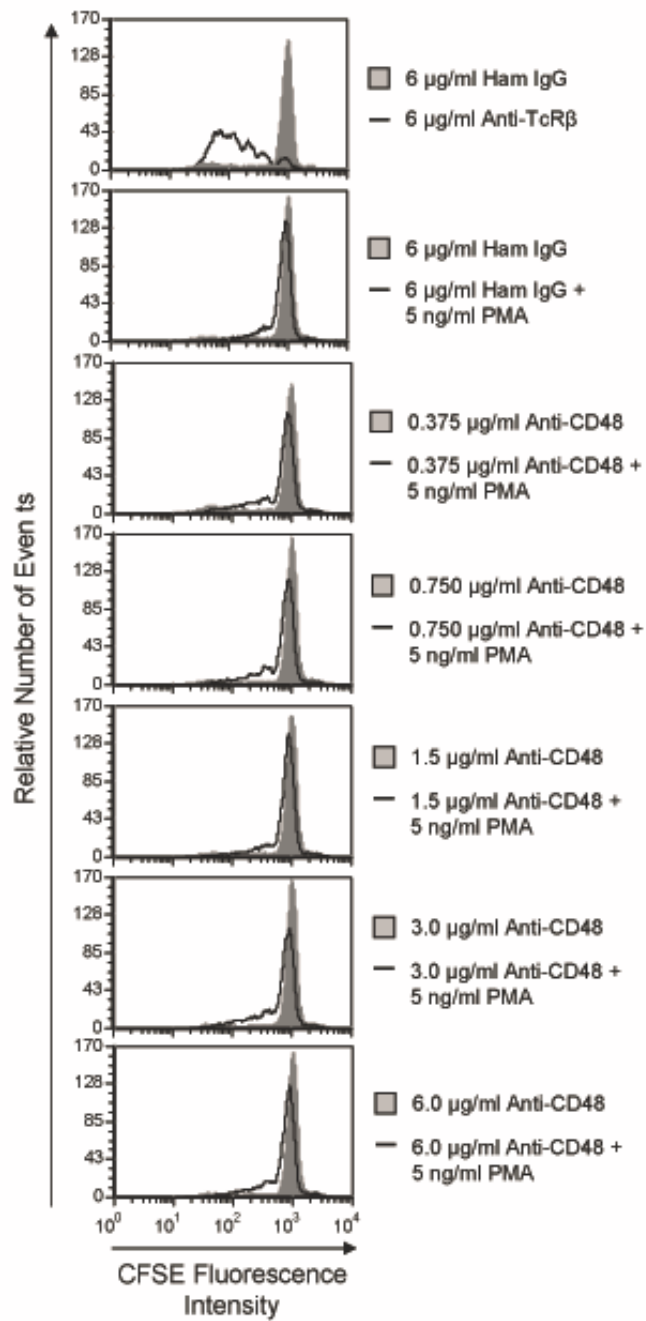


Figure 4.14. Ly6A/E Stimulation Promotes Marginal Activation in the Presence of Costimulation Provided Syngeneic LPS-matured BMDCs and PMA. Highly purified CD3⁺ T cells (2.5×10^5 cells/well) were labelled with 2.5 μ M Oregon Green and seeded in duplicate into 96-well round-bottom plates with or without LPS-matured BMDCs (8×10^3 cells/well) . T cells were then cultured in the presence of the indicated concentrations of anti-Ly6A/E mAb (D7) or the appropriate isotype control with or without PMA for the indicated times. Wells were pooled and Oregon Green staining was measured by flow cytometry. The number of cellular divisions are indicated by decreasing fluorescence intensity. Data shown are representative of three separate experiments.

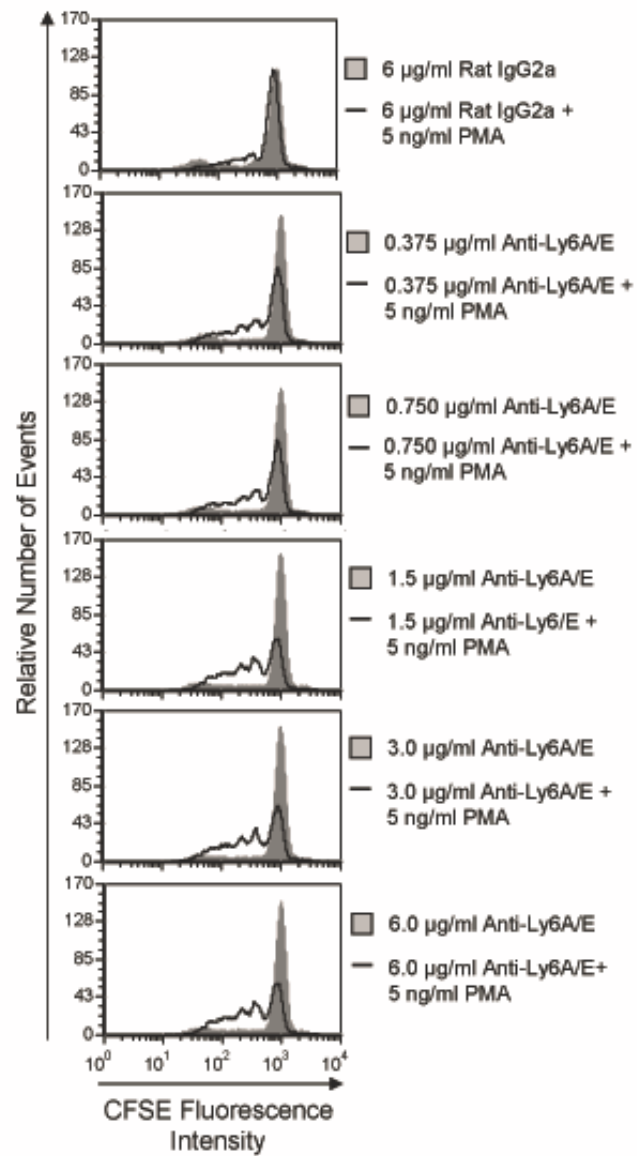
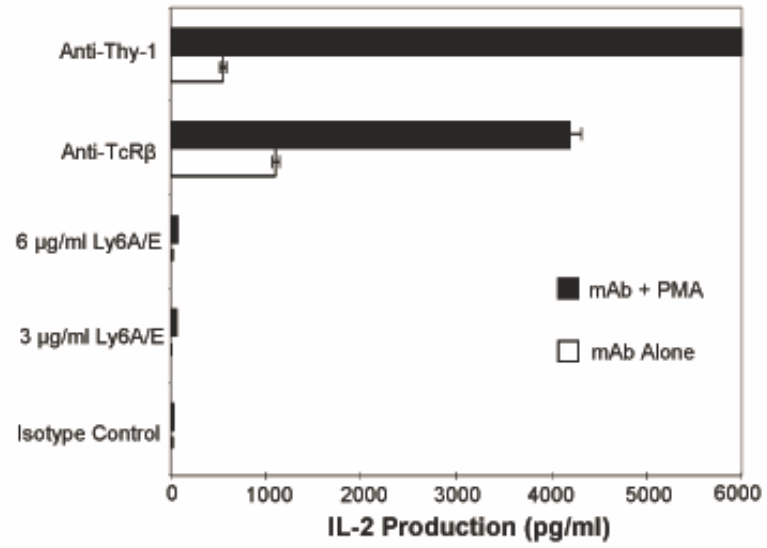
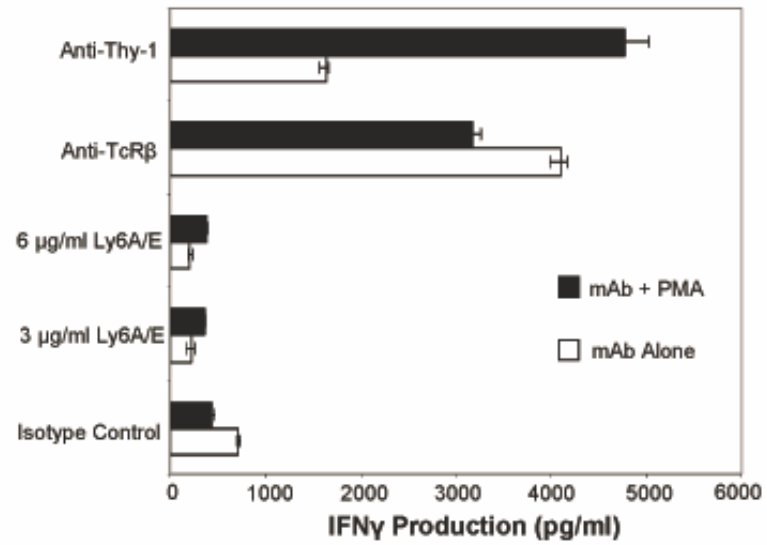


Figure 4.15. Ly6A/E Stimulation in the Presence of Costimulation Provided by LPS-matured BMDCs and PMA Does Not Induce Significant IL-2, IFN γ , IL-4 or IL-17A Production by Resting T cells. Highly purified CD3⁺ T cells (2.5×10^5 cells/well) with or without LPS-matured BMDCs (8×10^3 cells/well) were seeded in into 96-well round-bottom plates. T cells were then cultured in the presence of 6 μ g/ml anti-Thy-1 mAb (clone G7), anti-TcR β mAb , anti-Ly-6A/E mAb or the appropriate isotype control for the 24 h. Supernatants were isolated and analyzed for the production of (A) IL-2 (B) IFN γ (C) IL-17A and (D) IL-4 by ELISA. Data shown are representative of two separate experiments +/- SD.

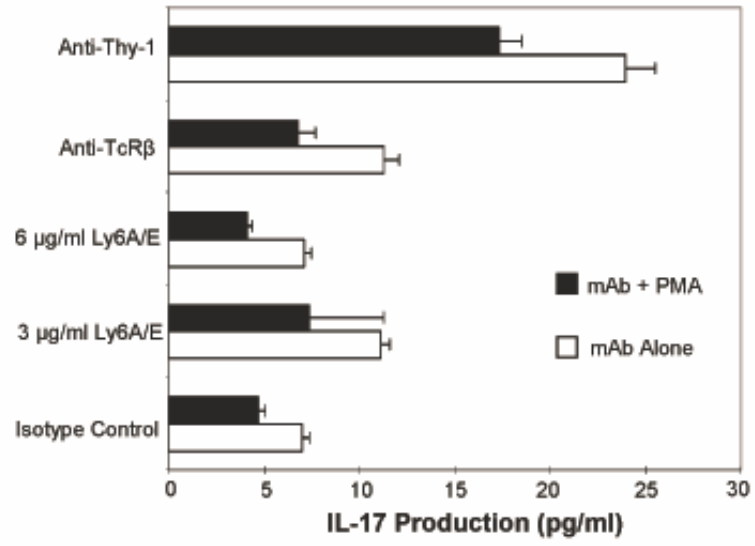
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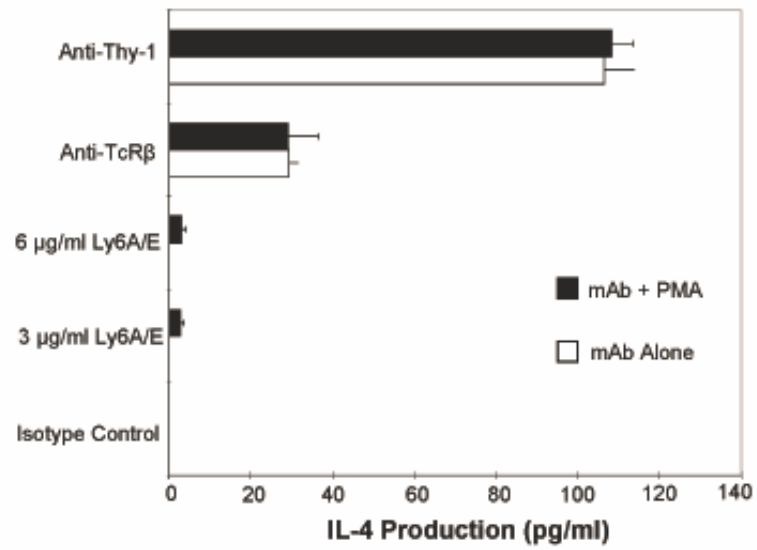
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Chapter 5. Discussion

5.1 Anti-Thy-1 mAb-Mediated Signaling is Weaker and has Delayed Signaling Kinetics Compared to Anti-TcR β mAb-Mediated Signaling.

Although a compelling body of evidence suggests that Thy-1 triggering results in the activation of a signal transduction pathway that is very similar to that activated when the TcR is triggered on T cells, there is also evidence that differences exist between the signaling pathways activated by the TcR and Thy-1. For example, although Thy-1 signaling can induce the differentiation of CTLs, the Thy-1 signal is not sufficient to trigger granule-mediated exocytosis, suggesting that Thy-1 may not provide a complete TcR-like signal (375). Moreover, pharmacological inhibition of p38 MAPK has differential effects on IL-2 production induced by TcR and Thy-1 signaling: Anti-CD3/anti-CD28 mAb-induced IL-2 production was decreased in murine T cells when p38 MAPK was inhibited (469), while anti-Thy-1/anti-CD28 mAb-induced IL-2 production was strikingly enhanced (405). These observations suggest that the p38 MAPK pathway may play different roles in TcR- and Thy-1-mediated T cell activation.

The strength and kinetics of signal transduction pathways can have a dramatic influence on the functional outcomes provoked when a cell-surface receptor is triggered. For example, the strength and duration of intracellular Ca⁺² flux and the ERK signaling pathway triggered by the TcR during thymocyte differentiation is crucial to the decision between CD4 or CD8 lineage commitment (431). It is therefore surprising that a direct comparison of TcR and Thy-1 signaling kinetics in primary T cells has never been performed. Consequently, I conducted a study comparing Thy-1 and TcR signaling focusing on the kinetics of the activation of key signaling molecules in order to further elucidate similarities and differences between these two important T cell signaling pathways.

5.1.1 Anti-Thy-1 mAb Triggers a Weaker T cell Activating Signal than Anti-TcR β mAb

I chose to compare TcR and Thy-1 signaling kinetics in a system where costimulation was provided by LPS-matured syngeneic BDMCs for several reasons. DCs are arguably the most important pAPC for initiating naive T cell responses. In response to maturation signals such as that produced by LPS, DCs

express many different ligands, including B7-1 and B7-2, that are important in providing costimulatory signals to T cells (423). If Thy-1 is involved in providing signal 1 during T cell activation *in vivo*, it is possible that DCs could be involved in providing costimulatory signals to T cells receiving Thy-1-activating signals. Moreover, the system has already been established in our lab, as anti-Thy-1 mAb has previously been shown to induce high levels of proliferation in resting T cells when costimulation is provided by LPS-matured BMDCs (375). Not surprisingly, anti-Thy-1 mAb-induced T cell proliferation in the presence of LPS-matured BMDCs is dependent upon B7-1- and B7-2-mediated costimulation. Although a direct comparison has not been done between macrophages and DCs, LPS-activated B cells are inferior in their ability to enhance anti-Thy-1 mAb-induced T cell activation in comparison to LPS-matured BMDCs (375).

Dose response assays were first performed to determine the optimal concentrations of anti-TcR β and anti-Thy-1 mAbs to use in my study. Maximal DNA synthesis was consistently obtained with 6 μ g/ml of anti-Thy-1 mAb. This result was consistent with another study by Haeryfar *et al.* (376), in which 6 μ g/ml of anti-Thy-1 mAb induced a maximal mitogenic response in T cells activated in the presence of costimulation provided by splenic accessory cells. Interestingly, DNA synthesis initiated by concentrations of anti-Thy-1 mAb that induced a maximal response was significantly lower than DNA synthesis initiated by concentrations of anti-TcR mAb that induced a maximal response. Consistently, analysis of T cell division by CFSE staining revealed less T cell division induced by anti-Thy-1 mAb than by anti-TcR β mAb. Moreover, time course assays in which cells were counted showed significantly lower numbers of total cells in wells that were activated with anti-Thy-1 mAb compared to those activated with anti-TcR β mAb. Taken together, these results show that anti-Thy-1 mAb induce a weaker activating signal in T cells than anti-TcR β mAbs.

Time course assays revealed that although anti-Thy-1 mAb induced less T cell proliferation than anti-TcR β mAb, the two stimuli induced similar kinetics of T cell proliferation that peaked at 72 h after activation and diminished by 96 h. Conversely, increases in total number of cells following both Thy-1- and TcR-

mediated T cell activation peaked by 96 h. These differences were due to the nature of the two assays used to examine proliferative responses. The [³H]TdR incorporation assay measures only cells proliferating in the last 6 h of culture. Therefore the diminished level of [³H]TdR at 96 h suggests that the T cells are not proliferating as much in the last 90 h of culture. However, there are more cells in culture at 96 h because of the earlier proliferative response. The observation that anti-Thy-1 mAb induces peak proliferation at 72 h was consistent with another study showing peak proliferation at the same time point when costimulation is instead provided by splenic accessory cells (400). Interestingly, if T cells are activated with polystyrene microbeads coated with anti-Thy-1 mAb and anti-CD28 mAb peak proliferation is observed at 48 h and diminishes by 72 h (405). Although a parallel study has not yet been performed, the peak T cell proliferation induced by anti-Thy-1/anti-CD28 mAb-coated microbeads is consistently lower than the peak T cell proliferation induced by anti-Thy-1 mAb in the presence of LPS-matured BMDCs when individual experiments are compared (data not shown). The difference seen in strength and kinetics between these two systems could be due to the fact that LPS-matured BMDCs can provide more costimulatory signals than just the CD28 signal (4), which could lead to a stronger enhancement of the Thy-1 signal and a more sustained mitogenic response. It would therefore be interesting to determine whether other costimulatory molecules can play a role in enhancing the strength of the Thy-1 signal. Alternatively, the difference may be due to the nature of how the signals in each system are produced. The rearrangement of signaling molecules and their respective ligands on the surface of both T cells and pAPCs upon interaction is an important event during T cell activation that is thought to be required for sustained signaling (470). The immobilization of mAb on microbeads used to imitate ligands on the surface pAPCs likely does not allow for this rearrangement and may therefore explain lower levels of T cell proliferation. It is also difficult to control the amount and proportion of mAb that coat the microbeads, consequently the difference in strength and kinetics between the two systems

could simply be due to the fact that optimal levels of Thy-1 and CD28 signaling are not being initiated to promote a maximal response.

An important control mechanism of T cell immune responses is the induction of AICD (134). In response to TcR-driven activation, T cells upregulate their expression of FasL, which upon interaction with constitutively expressed Fas on other T cells leads to fratricide and thus prevents excessive T cell expansion. Since [³H]TdR incorporation assays do not differentiate between decreased DNA synthesis and increased DNA fragmentation within a cell culture, it was possible that the lower amounts of [³H]TdR incorporation seen within the anti-Thy-1 mAb-activated T cell cultures compared to the anti-TcR β mAb-activated T cell cultures could have been the result of increased T cell death via AICD. I demonstrated, however, that this was not the case since time course experiments using Annexin V/PI staining revealed similar levels of cell death within anti-Thy-1 mAb- and anti-TcR β mAb-activated T cell cultures.

Survivin is an IAP that is upregulated upon TcR-driven T cell activation and has been implicated in both T cell survival and proliferation (439). I showed that survivin is also upregulated in response to anti-Thy-1 mAb-mediated T cell activation. Time course assays showed that anti-TcR β mAb- and anti-Thy-1 mAb-induced signaling resulted in similar levels of survivin expression with comparable kinetics. Consistent with previous studies (438), survivin expression was not observed until 24 h following T cell activation. Survivin has been shown to be selectively expressed in proliferating T cells (438). The observation that survivin expression diminished by 48 h was therefore most likely due to the fact that a lower percentage of cells was proliferating at later time points. A role for survivin in T cell survival has been proposed based on evidence that survivin expression correlates with decreased T cell apoptosis in expanding T cells (471). Moreover, forced expression of survivin increases the survival of activated T cell populations (472). Given the role that survivin may play in T cell survival, my results suggest that Thy-1 and TcR signaling may be equal in their ability to induce survivin expression and thus survivin-mediated T cell survival. However, survivin expression is largely regulated by costimulatory signals since T cells deficient in

either CD28 or OX40, a costimulatory molecule that is expressed on T cells several hours after their initial activation, are defective in their subsequent ability to upregulate survivin expression in response to T cell activation and do not survive long-term in culture (472). It is therefore possible that the similar levels of survivin observed in anti-TcR β mAb- and anti-Thy-1 mAb-activated T cells is due to equal amounts of costimulatory signals provided by the LPS-matured BMDCs rather than an inherent similarity in the strength and kinetics of the TcR and Thy-1 signal. Survivin may also promote T cell division by regulating the G1/S transition (472). Since survivin is similarly upregulated by anti-TcR β mAb- and anti-Thy-1 mAb-mediated T cell activation, it is unlikely that the difference in T cell proliferation is due to any differential activation of this regulatory molecule.

My observation that anti-Thy-1 mAb-mediated signaling induces lower levels of cyclin D3 protein than anti-TcR β mAb-mediated signaling may explain the weaker mitogenic response of T cells to anti-Thy-1 mAb stimulation. Cyclin D3 is expressed early in the G1 phase of the cell cycle and forms holoenzymes with CDK4 and/or CDK6 to activate their catalytic function (440). Cyclin D-CDK4/6 complexes regulate the transition through the early G1 phase by partially inactivating the retinoblastoma (Rb) proteins through phosphorylation. Phosphorylated Rb releases the transcription factor E2F, which promotes the expression of E-type cyclins, which then bind to and activate CDK2, inactivating Rb proteins and resulting in a positive feedback loop. Accumulation of the Cyclin E-CDK2 complexes in late G1 phase triggers the transition into S phase. Beyond this restriction point, cells are fully committed to undergo the entire cell cycle. Because of the key role that cyclin D3 plays in the transition through the G1 phase in T cells (473), the lower amounts of cyclin D3 observed in anti-Thy-1 mAb-stimulated T cells likely explains why they proliferate less than anti-TcR β mAb-stimulated T cells. In a recent paper, Lupino *et al.* show that cyclin D3 expression is regulated by IKK during TcR/CD28-mediated T cell activation in an IL-2-independent manner (474). It is therefore possible that anti-Thy-1 mAb-mediated signaling induces less IKK activation than anti-TcR β mAb-mediated signaling, leading to lower levels of cyclin D3 expression.

Consistent with anti-Thy-1 mAb triggering a weaker T cell-activating signal than anti-TcR β mAb, I also showed that anti-Thy-1 mAb-activated T cells express less CD25 and produce lower levels of IL-2 than anti-TcR β mAb-activated T cells. Resting T cells express the intermediate affinity IL-2R, which is a heterodimer composed of the IL-2R β and γ_c chains, but do not express CD25 and consequently lack the high affinity IL-2R, which is a trimer that is composed of CD25, IL-2R β and the γ_c chain (443). CD25 expression is upregulated on the surface of T cells in response to TcR-mediated T cell activation and is required for optimal T cell expansion and differentiation. Consistent with an ability of Thy-1 to provide a TcR-like signal when costimulation is also provided, CD25 expression is induced in T cells activated with anti-Thy-1 mAb in the presence of splenic accessory cells or with anti-Thy-1/anti-CD28-coated microbeads (400, 405). My observation that not only a lower percentage of T cells stain positive for CD25 expression but also express lower levels of CD25 following anti-Thy-1 mAb-mediated T cell activation compared to anti-TcR β mAb-mediated T cell activation, suggests that the Thy-1 signal is weaker than the TcR signal in promoting CD25 upregulation. It is notable that CD25 expression peaked at 48 h by western blotting, whereas CD25 expression by flow cytometry peaked by 72 h. This discrepancy could be due to the presence of protein from dead and dying cells in lysates, which would have been gated out on the flow cytometry. CD25 expression in response to TcR-mediated activation is largely due to new gene transcription regulated by transcription factors that include NF κ B, AP-1, NFAT, and Elf-1, which bind to six positive regulatory regions in the *cd25* gene (475). It is therefore likely that the anti-Thy-1 mAb is weaker than anti-TcR β mAb in its ability to activate one or more of these transcription factors in the presence of costimulation provided by LPS-matured BMDCs. Cross-linking of Thy-1 on highly-purified resting T cells with immobilized anti-Thy-1 mAb in the absence of costimulation promotes significant CD25 expression but does not promote [3 H]TdR incorporation or IL-2 production (429). The Thy-1 signal alone may thus be sufficient to activate at least one of the above transcription factors, allowing for some new *cd25* gene transcription. Pharmacological inhibition of PTKs, ERK1/2,

JNK, PLC, PKC, calcinerurin, PI3K and PKA significantly suppresses anti-Thy-1 mAb-mediated CD25 expression (429). Since these signaling molecules are involved in the activation of NF κ B, AP-1 and NFAT, anti-Thy-1 mAb-mediated signaling in the absence of costimulation may activate all of these transcription factors to some degree.

Since I found significantly lower levels of IL-2 protein in supernatants from T cell cultures activated with anti-Thy-1 mAb compared to anti-TcR β mAb, it was possible that anti-Thy-1 mAb-mediated signaling induced less T cell proliferation and CD25 expression than anti-TcR β mAb-mediated signaling merely due to an inability to induce high levels of IL-2 production. IL-2 signaling positively regulates IL-2 responsiveness in T cells by promoting CD25 expression. The *cd25* gene contains positive regulatory regions that bind to the transcription factor STAT5a, which is activated by IL-2 receptor signaling (476, 477). Mice deficient in STAT5a therefore have severely diminished CD25 expression in response to T cell activation and decreased proliferation in response to IL-2 (444). Moreover, IL-2 receptor signaling is also involved in cyclin D3 expression (478), and thus weaker IL-2 signaling could also explain my observation that anti-Thy-1 mAb induce less cyclin D3 expression than anti-TcR β mAb. Addition of up to 100 U/ml exogenous IL-2, however, did not significantly increase anti-Thy-1 mAb-mediated T cell proliferation, suggesting that this is not the case and that the anti-Thy-1 mAb is weaker in its ability to promote T cell proliferation due to an IL-2-independent mechanism. In contrast, Conrad *et al.* (405) show that the addition of 50 U/ml IL-2 significantly enhances both anti-Thy-1 mAb- and anti-Thy-1/anti-CD28 mAb-coated microsphere-induced T cell proliferation. The difference between these observations and my results could be due to the nature of costimulation that was provided to the T cells. Since immobilized anti-Thy-1 mAb in the absence of costimulatory signals can induce CD25 expression and thus IL-2 responsiveness in T cells (429), it is not surprising that the addition of exogenous IL-2 would enhance T cell proliferation in these cultures. Costimulation provided by only anti-CD28 mAb coimmobilized with anti-Thy-1 mAb is weaker than costimulation provided by LPS-matured BMDCs, and thus likely does not induce as much IL-2

production. Consequently, the former system may be much more sensitive than the latter system to that addition of exogenous IL-2.

5.1.2 Anti-Thy-1 mAb-Mediated Signal Transduction Has Delayed Kinetics in Comparison to Anti-TcR β mAb-Mediated Signal Transduction.

The observation that anti-Thy-1 mAb was weaker in its ability to induce several hallmark outcomes of T cell activation, including DNA synthesis, T cell division, cyclin D3 expression, CD25 expression, and IL-2 production, than anti-TcR β mAb led me to hypothesize that the signal generated by anti-Thy-1 mAb would be overall weaker than the signal generated by anti-TcR β mAb when costimulation is provided by LPS-matured BMDCs. Upon examination of key signaling molecules, including PTK, ZAP-70 and ERK1/2, involved in the TcR and Thy-1 signal transduction pathway; however, I found that the appearance of detectable levels of the phosphorylated active forms of these signaling molecules was strikingly delayed in anti-Thy-1 mAb-stimulated T cells compared to anti-TcR β mAb-stimulated T cells. Although the possibility that anti-Thy-1 mAb-stimulation actually activated these molecules at earlier time points at low levels not detectable by western blotting cannot be ruled out, these findings are consistent with anti-Thy-1 mAb generating a signal that has delayed kinetics compared to anti-TcR mAb-induced signaling when costimulatory signals are provided by LPS-matured BMDCs. Moreover, the observation that anti-Thy-1 mAb-induced the phosphorylation of ZAP-70 and ERK1/2 to levels that were comparable to the maximal levels of phosphorylation induced by anti-TcR β -mAb, although much later, is consistent with the signal generated by anti-Thy-1 mAb being delayed and not just weaker overall in comparison to anti-TcR β mAb-induced signaling.

The activation of PTKs is one of the earliest events in TcR-mediated signal transduction. Consistently, I found that the Tyr phosphorylation of several proteins was increased in lysates isolated from T cells activated with anti-TcR β mAb and costimulation provided by LPS-matured BMDCs for as little as 5 min. In contrast, increased Tyr phosphorylation was not observed in lysates isolated from T cells activated with anti-Thy-1 mAb until 1 h following initial activation and did not reach peak levels until 4 h after stimulation, suggesting that anti-Thy-1 mAb-

induced signaling activates PTKs with delayed kinetics compared to the anti-TcR mAb signaling. Several PTKs have been implicated in Thy-1-mediated signal transduction in T cells. Herbimycin A, a selective inhibitor of PTKs, prevents both Thy-1-induced T cell proliferation and CD25 expression, suggesting an important role of PTKs in Thy-1-mediated T cell activation (406, 429). The Src family kinases lck and fyn both appear to be activated in response to TcR- and Thy-1-mediated T cell activation (417). Both lck and fyn colocalize to lipid rafts and coimmunoprecipitate with Thy-1 (409, 418, 445, 479), suggesting a possible physical association between Thy-1 and these PTKs. Interestingly, fyn may play a more important role in anti-Thy-1 mAb-mediated T cell activation than in anti-TcR β -mediated T cell activation. Lancki *et al.* (418) showed that fyn^{-/-} T cell clones have severely diminished proliferation and IL-2 production in response to anti-Thy-1 mAb-mediated T cell activation, but normal responses to TcR triggering. It is also possible that fyn plays a more dominant role than lck in Thy-1 signaling in T cells, whereas the opposite is true for TcR signaling. Thomas and Samelson (409) demonstrated that 25% of total fyn protein in T cells co-immunoprecipitates with Thy-1, whereas only 4% of total lck protein co-immunoprecipitates with Thy-1. These observations suggest that Thy-1 may preferentially associate with fyn. Although Thy-1 signaling has not been examined in lck-deficient T cells, TcR signaling is severely impaired. If fyn is indeed more important than lck in Thy-1-mediated T cell activation, it would be expected that Thy-1-induced T cell responses would not be as compromised in lck-deficient T cells as in fyn-deficient T cells.

The mechanism by which lck and fyn are activated during Thy-1 signaling is not clear. Both proteins are localized to the inner leaflet of the plasma membrane within lipid rafts due to the myristoylation and palmitoylation of amino acid residues in their N-terminus (480, 481). It is possible that lck and fyn functionally associate with Thy-1 through an interaction with other proteins that transverse the membrane. Thy-1 co-immunoprecipitates with several transmembrane proteins expressed in T cells, including p100, CD45 and PAG, which have all been implicated in modulating Src family kinase activity (411-413).

The activity of lck and fyn is regulated primarily by two Tyr residues. An inhibitory Tyr residue (Tyr⁵⁰⁵ and Tyr⁵²⁸, respectively) located in the C-terminus promotes a closed, inactive conformation when it is phosphorylated by C-terminal src kinase (Csk) (482, 483), a protein that is recruited to the membrane by PAG (484). An activating Tyr residue located in the activation loop of the kinase domain enhances enzymatic activity when it is phosphorylated (12). It is commonly thought that during TcR signaling, CD45 dephosphorylates the inhibitory residue which allows for lck activation via autophosphorylation of the activating Tyr residue (11, 12). In my hands, however, neither anti-TcR β mAb nor anti-Thy-1 mAb caused detectable dephosphorylation of lck Tyr⁵⁰⁵ in T cells when costimulation was provided by LPS-matured BMDCs. This observation is consistent with a study by Dong *et al.* (485) in which Tyr phosphorylation of lck and fyn at both the inhibitory and activating Tyr residues did not change in response to anti-CD3 mAb-mediated T cell activation. In a recent study, Nika *et al.* (486) showed that a large fraction of lck and fyn is constitutively activated in naïve T cells and that the activation status does not change with TcR triggering. This finding was subsequently confirmed in the study by Dong *et al.* (485). These observations have led to a new model for the initiation of TcR signaling, in which lck and fyn exist in a preactivated pool that is somehow sequestered away from the TcR complex until their recruitment is stimulated by TcR ligation (486). Accessibility of lck and fyn to TcR ζ chain and CD3 ITAMs may result from a conformational change triggered by the ligation of the TcR that is required to free them from the inner leaflet of the plasma membrane (21). Alternatively, lck and fyn may be sequestered in membrane microdomains away from nonengaged TcRs, which may be aggregated together upon TcR triggering (487). Interestingly, Thy-1 signaling is partially dependent upon the expression of the complete TcR complex (407). Consequently, it has been suggested that Thy-1 somehow signals through the TcR receptor. Consistent with this hypothesis, Thy-1 signaling induces TcR ζ chain phosphorylation (488), and I show that Thy-1 signaling promotes the phosphorylation of Zap-70. Since Thy-1 associates with fyn and lck in lipid rafts, it is possible that cross-linking Thy-1 promotes lipid raft

aggregation, which brings the constitutively active fyn and lck in to close proximity to the ITAMs in the TcR ζ chain and CD3 chains. It is difficult to imagine how Thy-1 cross-linking could trigger a conformation change within the TcR complex that would release the TcR ζ and CD3 ITAMs from the inner leaflet of the membrane unless they were intimately associated. All studies to date, however, have not been able to co-immunoprecipitate Thy-1 and the TcR, suggesting that these molecules are not physically associated (411). Furthermore, the TcR ζ chains are partially phosphorylated in resting T cells (10), likely due to the presence of the constitutively active lck and fyn (485). Although this observation suggests that a conformation change may not be necessary for TcR ζ chain ITAM phosphorylation, it is possible that such a conformational change simply makes it easier for the ITAMs to be fully phosphorylated by lck and fyn and thus is required for optimal levels of TcR-mediated signaling. Therefore, a potential explanation for why the Thy-1 signal has delayed kinetics compared the TcR signal is that crosslinking of Thy-1 with mAb does not induce a conformation change in the TcR complex. As a result, the TcR ζ chain and CD3 chains may be less accessible and as a result it is more difficult for Thy-1-activated lck and fyn to phosphorylate the ITAMs. Consequently, it takes longer for the activating signal to reach the necessary threshold to trigger downstream signaling pathways.

I observed the appearance of slower migrating forms of lck in both anti-TcR mAb- and anti-Thy-1 mAb-activated T cells. These banding patterns have been previously seen in activated T cells and have been shown to be due to the phosphorylation of several serine residues on lck (446, 447, 489). One such serine phosphorylation event (Ser⁵⁹) is mediated by ERK (447). The exact role of this modification in lck function and TcR signaling is currently unclear. ERK-mediated lck phosphorylation might be a negative regulator of lck activity and thus may play a role in the down-regulation of TcR signaling. Consistent with this, Watts *et al.* (447) showed that lck protein phosphorylated at Ser⁵⁹ has decreased kinase activity compared to lck protein that is not phosphorylated at this residue. Conversely, ERK-dependent phosphorylation of lck may prevent the recruitment of the phosphatase SHP-1 to the TcR complex, leading to sustained TcR

signaling. Ser⁵⁹ is located within the N-terminus of Ick and regulates the ability of Ick to interact with different phospho-proteins via the SH2 domain (490). Consequently, phosphorylation of Ser⁵⁹ can affect the ability of Ick to bind to several of its substrates. In particular, Ser⁵⁹ phosphorylation decreases the ability of Ick to bind to and recruit phosphorylated SHP-1 to the TcR complex. Since phosphorylated SHP-1 mediates dephosphorylation of Ick Tyr³⁹⁴, modification of Ick Ser⁵⁹ prevents the inactivation of Ick.

Although my findings do not shed any light on the ability of Thy-1 signaling to initiate Ick-dependent pathways, they are consistent with the activation of downstream signaling pathways, such as the ERK pathway, which could modify Ick through serine phosphorylation by Thy-1 signaling in a fashion similar to TcR signaling. Indeed, it is clear that the ERK pathway plays a major role in Thy-1 signaling. Pharmacological inhibition of the ERK pathway with the MAPK kinase-1 (MEK-1) inhibitor PD98059 severely diminishes anti-Thy-1 mAb-mediated T cell proliferation when costimulation is provided by either splenic accessory cells or co-immobilized anti-CD28 mAb on microbeads (405, 406). Additionally, inhibition of MEK-1 prevents CD25 expression in T cells activated with anti-Thy-1 mAb in the absence of costimulatory signals (429). Moreover, Conrad *et al.* (405) showed that ERK1/2 is phosphorylated in T cells activated with anti-Thy-1/anti-CD28 mAb-coated microbeads. Consistently, I also show that ERK1/2 is phosphorylated in response to anti-Thy-1 mAb-stimulation and costimulation provided by LPS-matured BMDCs. In anti-TcR β mAb-activated T cells, ERK1/2 phosphorylation occurred prior to the appearance of the slower mobility Ick bands, consistent with ERK1/2-dependent serine phosphorylation of Ick. In contrast, slower mobility Ick bands were observed prior to the appearance of detectable ERK1/2 phosphorylation in response to anti-Thy-1 mAb-mediated T cell activation. These findings suggest that some other kinase may be involved in the serine phosphorylation of Ick. Although the physiological significance not known, Cdc2 has been shown to phosphorylate Ick at Ser⁵⁹ during mitosis (491). It is unlikely, however, that the appearance of the slower mobility Ick bands following only 1 h of stimulation with anti-Thy-1 mAb are due to Cdc2 activity,

since Thy-1 stimulated cells do not undergo a first round of division until at least 24 h following their activation.

5.1.3 Anti-Thy-1 mAb-Mediated Signaling Results in the Delayed Acquisition of CTL Effector Function as well as Delayed Destruction of Target Cells.

An important outcome of TcR signaling in the presence of costimulatory signals is the differentiation of naïve T cells into CTLs, which are critical for the destruction of virus-infected or neoplastic host cells. CTLs destroy target cells by two main mechanisms; granule-mediated exocytosis and the Fas-mediated death receptor pathway (115, 116). Thy-1 has also been implicated in promoting the CTL differentiation. Haeryfar *et al.* (375) showed that anti-Thy-1 mAb and costimulation provided by LPS-matured BMDCs induces the expression of CTL effector molecules, granzyme B, perforin and FasL in primary T cells. The ability of Thy-1 to trigger CTLs to deliver a lethal hit to target cells is more controversial. Haeryar *et al.* showed that anti-Thy-1 mAb-induced CTLs could form stable conjugates with target cells but could not polarize their lytic granules toward the site of contact, suggesting that the Thy-1 signal is not sufficient to trigger granule-mediated cytotoxicity. In the same study, anti-Thy-1 mAb-induced CTLs did not destroy Fas-sensitive target cells, suggesting that the Thy-1 signal is also not sufficient to trigger the Fas-mediated pathway of cytotoxicity. However, in a conflicting study, Kojima *et al.* (419) provided evidence that the anti-Thy-1 mAb selectively triggers Fas-mediated cytotoxicity, but not granule-mediated cytotoxicity.

Having observed that anti-Thy-1 mAb generates a TcR-like signal with delayed kinetics, I asked whether anti-Thy-1 mAb-induced CTLs may actually be able to destroy target cells with delayed kinetics compared to anti-TcR mAb-induced CTLs. My results confirm our lab's previous findings that, unlike anti-TcR mAb-induced CTLs, anti-Thy-1 mAb-induced CTLs are unable to destroy P815 target cells within a standard 4 h ⁵¹Cr release assay. I show for the first time, however, that anti-Thy-1 mAb-induced CTLs can destroy P815 target cells if co-cultured together for 12 h. This finding suggests that anti-Thy-1 mAb can trigger the lethal hit by CTLs but with delayed kinetics compared to the anti-TcR mAb. I

also demonstrated that resting T cells required a much longer duration of anti-Thy-1 mAb- than anti-TcR mAb-mediated signaling in order to acquire the ability to destroy P815 target cells. This finding is also consistent with the delayed kinetics of anti-Thy-1 mAb-mediated T cell signaling that I observed, and suggests that anti-Thy-1 mAb-mediated T cell differentiation into CTLs is delayed compared to anti-TcR mAb-mediated T cell differentiation. It is important to note, however, that there are also alternative explanations for these data. Since CTL triggering in redirected lysis assays is dependent upon the cross-linking of the anti-TcR β mAb or anti-Thy-1 mAb by Fc γ Rs that are expressed on the target cell, it is possible that differences in the affinities of anti-TcR β and anti-Thy-1 mAbs for Fc γ R could account for delayed triggering of cytotoxic effector function. Moreover, it is possible that the observed cytotoxicity could be due to nonspecific death of CTLs and release of toxic granule contents, since survival signals such as IL-2 were not provided in culture. It would therefore be worthwhile to determine if Thy-1-induced CTLs can still destroy target cells when exogenous rIL-2 is added to culture.

The mechanism by which the anti-Thy-1 mAb-induced CTLs destroyed the P815 target cells in my study is not clear. Haeryar *et al.* (375) showed that anti-Thy-1 mAb- and anti-CD3 mAb-induced CTLs express similar levels of granzyme B, perforin and FasL mRNA 72 h following activation, suggesting that anti-Thy-1 mAb-induced CTLs are armed with the necessary effector molecules required to mediate either pathway of cytotoxicity. It is important to note, however, that the level of mRNA expression is not always indicative of the amount protein found within a particular cell-type due to post-translational regulation. It is therefore possible that although anti-Thy-1 mAb-induced and anti-TcR β mAb-induced CTLs express similar levels of granzyme B, perforin and FasL mRNA, they may not express the same amount of these effector proteins. Nevertheless, anti-Thy-1 mAb-induced CTLs must express some of these molecules since they can be redirected with anti-CD3 mAb to destroy P815 target cells in 4 h ⁵¹Cr release assays at similar levels as anti-CD3 mAb-induced CTLs (375). P815 cells express very low levels of Fas and are less sensitive to Fas-mediated cytotoxicity

(492). Consequently, it is less likely that Thy-1-induced cytotoxicity is due to this pathway of cytotoxicity. On the other hand, the strength of the TcR signal can determine the relative contribution of granule- or Fas-mediated pathways to CTL-mediated cytotoxicity. CTLs express preformed FasL stored in vesicles that are separate from cytolytic granules (131). These vesicles require only low levels of TcR signaling in order for mobilization and delivery of FasL to the plasma membrane. (493). In contrast, strong TcR signaling is required for the mobilization of cytolytic granules, suggesting that the signaling threshold for the initiation of Fas-mediated cytotoxicity in CTLs is much lower in comparison to granule-mediated cytotoxicity. Since anti-Thy-1 mAb induces weaker and delayed signaling compared anti-TcR mAb and P815 cells do express very low levels of Fas (492), it is possible that anti-Thy-1 mAb may preferentially induce Fas-mediated cytotoxicity. This would be consistent with a study in which anti-Thy-1 mAb induced CTL clones to destroy target cells by Fas- but not granule-mediated cytotoxicity (419). In this study, however, the CTL clones redirected with anti-Thy-1 mAb were able to destroy target cells within 4 h, only at a reduced level compared to CTL clones redirected with anti-CD3 mAb. Since the CTL clones would have presumably been primed with a TcR signal, they may have different activation requirements than primary T cells that are primed with a Thy-1 signal and may destroy target cells by a different mechanism than Thy-1-induced CTLs.

It is unclear whether delayed acquisition of cytotoxic effector function in anti-Thy-1 mAb-induced CTLs is due to delayed expression perforin, granzyme B and/or FasL. Because I found that the activation of several key signaling molecules involved in TcR signal transduction is delayed in anti-Thy-1 mAb-mediated T cell activation, it is certainly possible that the expression of cytotoxic molecules is also delayed. It is important to note, however, that the delayed signaling kinetics of anti-Thy-1 mAb-mediated T cell activation did not appear to result in delayed kinetics of several important indicators of T cell activation. Although T cell proliferation, CD25 expression and IL-2 production were weaker in anti-Thy-1 mAb- vs. anti-TcR mAb-activated T cells, the kinetics of these three important outcomes of T cell activation were similar. Moreover, there was no

difference in the protein level or kinetics of survivin and CDK6 expression in response to the two different activating stimuli.

It remains to be determined why the kinetics of anti-Thy-1 mAb-mediated signal transduction was delayed in this system compared to anti-TcR β mAb-mediated signal transduction. It is possible that the difference in signaling is due to a difference in the affinity of the mAbs used in this study. However, T cells express many more Thy-1 molecules than TcR molecules on their surface (approx. 200,000 vs. 2,000 molecules per cell, respectively) (382, 494). Consistent with this, two different clones (30-H12 and 53-2.1) of fluorochrome-conjugated anti-Thy-1 mAb stained T cells with significantly higher MCF than either fluorochrome-conjugated anti-TcR β or anti-CD3 ϵ mAb (Figure 4.7). Moreover, the T cells were stimulated with equimolar concentrations of anti-Thy-1 mAb and anti-TcR β , which induced a maximal mitogenic response. Thus, it is probable that a higher number of Thy-1 molecules were engaged compared to TcR ligation, and leading me to conclude that the Thy-1 signal is inherently weaker than the TcR signal. A weaker signal explains the delayed appearance of activated signaling molecules, since it would take longer to accumulate enough of the activated signaling molecules to become detectable. Ag with low affinity for the TcR also induces delayed ERK1/2 activation in comparison to Ag with high affinity (430), suggesting that the anti-Thy-1 mAb-induced signal may be similar to a weak TcR signal. Alternatively, it is possible that the signal generated by anti-Thy-1 mAb was delayed due to the mechanics of Thy-1 signaling vs. TcR signaling. Since Thy-1 may signal through the TcR, it may take longer for Thy-1 to initiate TcR ζ chain phosphorylation than due to direct stimulation through the TcR. It is also possible that anti-Thy-1 mAb may activate more phosphatases, like SHP-1, than anti-TcR β -mAb, which may prevent early Tyr phosphorylation.

In summary, my data demonstrate that anti-Thy-1 mAb-mediated signal transduction in the presence of costimulation provided by LPS-matured BMDCs is delayed and of lesser strength in comparison to strong TcR signaling induced by anti-TcR β mAb cross-linking. The delayed signaling kinetics resulted in weaker T cell proliferation, CD25 expression and IL-2 production. Moreover, the

delayed signaling kinetics resulted in the delayed acquisition of CTL effector function by primary T cells, as well as delayed destruction of target cells. These findings suggest the Thy-1 signal is sufficient to trigger cytotoxic effector function and are consistent with the notion that Thy-1 ligation provides a signal similar to low affinity Ag/TcR interactions.

5.2 Anti-Thy-1 mAb-Mediated Signaling Promotes the Development of Th Cells that Preferentially Produce IL-4 and IL-17.

Th cells play a central role in the adaptive immune response by shaping its nature through the production of distinct cytokine profiles. The three main types of Th cell subsets; Th1, Th2 and Th17 cells, produce high levels of IFN γ , IL-4 and IL-17, respectively, and modulate the immune response so that it is optimal for the elimination of particular types of pathogens (141). In addition to host defense, each Th subset has been implicated in different types of immune-mediated pathogenesis. Th1 and Th17 cell subsets are involved in autoimmune diseases such as MS, EAE and Crohn's disease (148-151), whereas the Th2 cell subset is involved in allergy and asthma. In order to further understand the role that Thy-1 signaling may play in Th cell differentiation and effector function, I explored the role that Thy-1 has in promoting T cell cytokine production and the differentiation of resting CD4⁺ T cells into cytokine-producing Th effector cells.

I demonstrated that maximal anti-Thy-1 mAb-mediated signaling preferentially promotes IL-4 and IL-17 production over IFN γ production, when compared to anti-TcR β mAb-mediated signaling. Anti-Thy-1 mAb-stimulated T cells expressed higher levels of IL-4 and IL-17 mRNA and produced more IL-4 and IL-17 protein than anti-TcR β mAb-stimulated T cells following their initial activation in the presence of costimulation provided by LPS-matured BMDCs. In contrast, anti-Thy-1 mAb-stimulated T cells expressed lower levels of IFN γ mRNA and produced less IFN γ protein in comparison to anti-TcR β mAb-stimulated T cells. These findings suggest that the signal generated by crosslinking Thy-1 with mAb somehow differs from the signal generated by TcR crosslinking in regard to the induction of different Th cell cytokines.

Since cytokine production by T cells is regulated by key lineage-specific transcription factors, T-bet, Gata-3 and ROR γ t, that program the Th subset

phenotypes Th1, Th2 and Th17, respectively, to produce their signature cytokines (141), I examined whether anti-Thy-1 mAb-induced signaling differed in its ability to regulate the expression of these transcription factors. The fact that anti-Thy-1 mAb-stimulated T cells expressed lower levels of the Th1-defining transcription factor T-bet in comparison to anti-TcR β mAb-stimulated T cells at an early time point after their initial activation could explain the difference seen in IFN γ production. Moreover, the finding that a small population of anti-Thy-1 mAb-stimulated T cells expressed higher levels of the Th2-defining transcription factor Gata-3 in comparison to anti-TcR β mAb-stimulated T cells may explain preferential IL-4 production. The increase in Gata-3 expression; however, was very small and not statistically significant and therefore may not completely explain the statistically significant 4-fold difference in IL-4 production between anti-Thy-1 mAb- and anti-TcR β mAb-stimulated T cells.

T-bet is not expressed in naïve T cells, but is upregulated in response to TcR-mediated T cell activation in the presence of IL-12 (157). Consistently, I also found that T-bet was not expressed by unactivated T cells, but was upregulated in response to anti-TcR β mAb-mediated T cell activation in the presence of LPS-matured BMDCs. T-bet upregulation was not surprising since LPS-matured BMDCs promote Th1 differentiation through the production of IL-12 (155). It is possible that the lower levels of T-bet expression resulting from anti-Thy-1 mAb-mediated T cell activation could be the result of deficient CD40L expression. T cells upregulate CD40L in response to TcR-mediated T cell activation, which subsequently interacts with CD40 on DCs and enhances their production of IL-12 (495). Measurement of IL-12 protein by ELISA following 24 h of activation revealed significantly less IL-12 in supernatants from anti-Thy-1 mAb-activated T cell cultures in comparison to anti-TcR β mAb-activated T cell cultures (Appendix 1.2), suggesting that this may indeed be the case. Alternatively, differences in T-bet expression could be due to the differential activation by the TcR or Thy-1 signal of signaling molecules that directly promote T-bet expression. Naïve T cells activated through the TcR complex in the context of costimulatory signals do not become responsive to IL-12 until early expression of T-bet promotes the

expression of the IL-12R β 2 chain (153). TcR/CD28 signaling in the absence of signals provided by cytokines is sufficient to promote this early expression of T-bet in activated T cells. Placek *et al.* (496) showed that early T-bet expression mediated by the TcR is largely dependent upon the recruitment of NFAT to two DNase-I hypersensitive sites within the *t-bet* gene locus. It is therefore possible that anti-Thy-1 mAb-induced signaling does not activate as much NFAT as anti-TcR β mAb-induced signaling explaining the differences in T-bet expression and IFN γ production that I observed. Consistent with an inability of Thy-1 signaling to activate NFAT to the same level as TcR signaling, Kroczek *et al.* (408) showed that anti-Thy-1 mAb-mediated activation of T cell hybridomas does not induce as strong a rise in cytoplasmic Ca⁺² as anti-TcR mAb-mediated activation.

The strength of the TcR signal can influence the direction of CD4⁺ T cell differentiation. Several studies show that weak TcR signaling favors IL-4 production and Th2 differentiation, whereas strong TcR signaling favor IFN γ production and Th1 differentiation (497-500). On the other hand, other studies have suggested a more biphasic response in which weak and strong TcR signaling favors Th2 differentiation, whereas intermediate strength TcR signaling favors Th1 differentiation (142). It is possible that differences in IL-4 production in anti-Thy-1 mAb- and anti-TcR β mAb-stimulated T cell cultures that I observed could be due to the fact that the Thy-1 signal was weaker than the TcR signal. The mechanism by which weak TcR signaling promotes Th2 differentiation is thought to be due to the early production of IL-4 that is Gata-3-independent, but NFATc-dependent (501). The primary difference in weak and strong TcR signaling in the regulation of early IL-4 and IFN γ production appears to be the extent of ERK activation. Jorritsma *et al.* (502) showed that weak TcR signaling promotes only weak and transient ERK activation compared to strong and sustained ERK activity induced by strong TcR signaling. Weak ERK activation favors the formation of the AP-1 transcription factor complexes composed of Jun-Jun homodimers, whereas strong ERK activation promotes the formation of AP-1 complexes composed by Jun-Fos heterodimers (502). Jun-Jun dimers preferentially associate with NFATc and promote early IL-4 production. In

contrast, strong ERK activation promotes the production of IFN γ (503). The extent of ERK signaling can therefore act as a switch that favors IL-4 production and Th2 differentiation by regulating the composition of the AP-1 transcription factor. As seen in chapter 3, anti-Thy-1 mAb-induces ERK1/2 activation with delayed kinetics compared to anti-TcR β mAb-induced ERK1/2 activation. It is therefore likely that Thy-1 signaling favored the activation of AP-1 transcription factors composed of Jun-Jun dimers early after activation, resulting in the higher levels of IL-4 production I observed in anti-Thy-1 mAb-activated T cell cultures in comparison to anti-TcR β mAb-activated cultures. Moreover, when resting T cells were stimulated with anti-TcR β mAb and anti-Thy-1 mAb together, they produced low levels of early IL-4 production and high levels of IFN γ similar to T cells activated with anti-TcR β mAb alone. This is likely due to the strong induction of ERK1/2 activated by the anti-TcR β mAb.

I demonstrate that anti-Thy-1 mAb-induced signaling in the presence of costimulation provided by LPS-matured BMDCs strikingly upregulates the expression of the Th17-specific transcription factor ROR γ t as early as 12 h following initial T cell activation. In contrast, anti-TcR β mAb-induced signaling did not result in the expression of ROR γ t, even as late as 72 h following initial T cell activation. These findings suggest that the signal generated by crosslinking Thy-1 with mAb somehow differs from the signal that is generated by TcR ligation with regard to the induction of ROR γ t. Since ROR γ t regulates the transcription of IL-17 (294), these findings could also explain the observed higher levels of IL-17 in anti-Thy-1 mAb-activated T cell cultures in comparison to anti-TcR β -activated T cell cultures.

The role that the strength of the TcR signal plays in IL-17 production and Th17 differentiation is less clear than that for Th1 and Th2 differentiation. Two recent studies suggest that strong TcR signaling in mouse T cells is required for IL-17 production and Th17 differentiation (504, 505). In contrast, a study by Purvis *et al.* (506) suggests that weak TcR signaling promotes Th17 differentiation in human T cells. It is therefore possible that the effect of TcR signaling strength on Th17 cell differentiation is species specific. Consistent with

strong TcR signals favoring Th17 differentiation in murine T cells, high levels of CD40L expression on activated T cells is also required for optimal Th17 cell differentiation (507). Since anti-Thy-1 mAb induced a weaker signal than anti-TcR β mAb, the high levels of IL-17 production and ROR γ t expression in the Thy-1 activated cells were likely not merely due to the fact that anti-Thy-1 mAb induces a weaker TcR-like signal. Instead, there must be some inherent difference in the Thy-1 and TcR signaling pathways that regulate of ROR γ t expression and IL-17 production. Consistent with this prediction, T cells that were activated with anti-Thy-1 mAb and anti-TcR β mAb produced similar high levels of IL-17 in comparison to T cells activated with anti-Thy-1 mAb alone. This finding suggests that in the presence of a strong TcR signal, Thy-1 signaling can activate a unique signaling pathway that leads to the upregulation of ROR γ t and IL-17 production.

An essential function of Th effector cells is the production of high levels of cytokines after restimulation through TcR in response to specific Ag (508). This important control mechanism ensures that CD4⁺ T cells that have received priming signals to differentiate into Th effector cells within the LN only produce high levels of cytokines at the site of infection. Having observed that anti-Thy-1 mAb-mediated signaling preferentially promoted early IL-17 and IL-4 production rather than IFN γ production by resting T cells activated in the presence of costimulation provided by LPS-matured BMDCs, I examined whether Thy-1-induced CD4⁺ T cells also preferentially produced high levels of IL-4 and IL-17 upon restimulation. I demonstrated that Thy-1-induced CD4⁺ T cells produced high levels of IL-4 and IL-17 and negligible levels of IFN γ when restimulated with either anti-TcR β mAb or anti-Thy-1 mAb in the presence of costimulatory signals provided by LPS-mature BMDCs. In contrast, TcR-induced T cells preferentially produced high levels of IFN γ in response to both stimuli. These findings suggest that not only does the Thy-1 signal preferentially promote early IL-4 and IL-17 production over IFN γ production, but also preferentially favors Th2 and Th17 cell differentiation. These findings are also consistent with anti-Thy-1 mAb inducing a weak TcR-like signal that favors Th2 differentiation, as well as inducing an unique

signaling pathway that upregulates ROR γ t expression and IL-17 production leading to Th17 differentiation. Moreover, my observation that either soluble anti-Thy-1 mAb alone or anti-Thy-1 mAb in the presence of costimulatory signals induced high levels of cytokine production by TcR-induced and Thy-1 induced Th cells suggests that the Thy-1 signal is sufficient to activate Th effector cells. This finding is particularly significant since it suggests that in a physiological situation, Th effector cells could be activated nonspecifically upon receiving Thy-1-mediated signals generated through the interaction of Thy-1 and its ligand in the lymphoid compartment. If this is the case, then it is likely that the expression of the Thy-1 ligand is stringently regulated so that Th cells are not inappropriately activated.

The cytokine environment in which a T cell receives activating signals can play a large role in the subsequent differentiation of distinct Th cell subsets. As stated earlier, LPS-matured BMDCs are known to favor Th1 differentiation through the production of IL-12 (155). I demonstrated that Thy-1 signaling also promotes the differentiation of resting T cells into Th1, Th2 and Th17 cells under the appropriate polarizing conditions when costimulatory signals are provided by LPS-matured BMDCs. This finding further supports the conclusion that Thy-1 provides a TcR-like signal and indicates that Thy-1 signaling could participate in the priming of CD4⁺ T cells in the physiological situation. Furthermore, consistent with an ability of the Thy-1 signal to preferentially promote IL-4 and IL-17 production, Thy-1-induced Th2 and Th17 cells produce strikingly higher levels of IL-4 and IL-17 than TcR-induced Th2 and Th17 cells, respectively. Increased ROR γ t expression mediated by a pathway that is unique to Thy-1 signaling could explain the higher amount of IL-17 produced by Thy-1-induced Th17 cells. Conversely, the high levels of IL-4 produced by Thy-1-induced Th2 cells in comparison to TcR-induced Th2 cells could be due to an enhancing effect on IL-4 production resulting from a weak TcR-like signal combined with Th2 polarizing conditions. If this is the case, one would expect that there would be higher levels of Gata-3 expression in Thy-1-induced Th2 cells in comparison to TcR-induced Th2 cells. My finding that the opposite is true, i.e., Thy-1-induced Th2 cell

expressed lower levels of Gata-3 than TcR-induced Th2 cells, suggests that Thy-1 signaling may differ from TcR signaling in its ability to regulate some other signaling molecule(s) involved in IL-4 production. My observation that T cells activated with anti-TcR β mAb in the presence of costimulation provided by LPS-matured BMDCs produced significantly higher levels of IL-4 upon restimulation with anti-Thy-1 mAb than when restimulated with anti-TcR β mAb also supports the idea that Thy-1 signaling may regulate another signaling molecule involved in IL-4 production. Several other transcription factors have been implicated in regulating IL-4 production by Th2 cells. For example, the transcription factors c-Maf and JunB play a critical role in promoting *IL-4* gene transcription in Th2 cells (216, 217). It is therefore possible that Thy-1 signaling may preferentially upregulate the expression of one of these transcription factors.

I found that anti-Thy-1 mAb- and anti-TcR β -induced Th cells that were restimulated with anti-TcR β mAb immobilized on microbeads produced similar levels of IFN γ . Moreover, T cells that were activated with anti-Thy-1 mAb under Th1-polarizing conditions produced slightly higher levels of IFN γ than anti-TcR β mAb-activated T cells when restimulated with PMA and ionomycin. These results conflict with my other findings that Thy-1-induced Th cells do not produce significant levels of IFN γ in response to restimulation with either anti-Thy-1 mAb or anti-TcR β mAb, alone or in the presence of costimulation provided by LPS-matured BMDCs. It is likely that these differences are due to the nature and strength of signals used to restimulate the T cells. Immobilized mAbs that cross-link the TcR complex can provide a very strong signal that can activate naïve T cells in the absence of costimulatory signals (460). This is not physiological, however, since naïve T cells clearly require costimulatory signals *in vivo* to become activated. The T cell activating signals provided by immobilized anti-TcR mAb, therefore, were likely not equivalent to those provided by soluble anti-TcR mAb in the presence of costimulation provided by BMDCs. Instead, immobilized anti-TcR mAb may activate certain TcR-mediated signaling pathways more strongly than what would be activated by physiological levels of TcR signaling. Similarly, activation of T cells with PMA and ionomycin is not equivalent to

activation through the TcR in the presence of costimulatory signals. PMA and ionomycin act together to promote T cell proliferation and cytokine production by activating signaling pathways downstream of the membrane proximal events that are initiated by TcR/CD3 complex signal transduction. Ionomycin activates Ca⁺²-dependent signaling pathways, whereas PMA activates DAG-associated molecules such as PKC (462). Since PMA and ionomycin act downstream of the adaptor protein LAT, it is likely that there are signaling pathways activated by TcR signaling that are not activated by PMA and ionomycin. As well, the relative strength of the pathways activated by both TcR signaling and PMA/ionomycin likely differ. The ability of immobilized anti-TcR mAb or PMA/ionomycin to promote IFN γ synthesis by Thy-1-induced Th1 cells was not due to an ability to activate similar pathways, since raising the number of BMDCs and thus the level of costimulation did not permit Thy-1-induced Th cells to produce IFN γ in response to restimulation with either anti-TcR or anti-Thy-1 mAbs. Nevertheless, these results suggest that Thy-1-induced Th cells polarized under Th1 conditions do have the capacity to make more IFN γ than TcR-induced Th1 cells in response to certain T cell activating stimuli. The difference in IFN γ production by TcR- and Thy-1-induced Th1 cells, however, was not as extreme as the difference in IL-17 and IL-4 production seen in Thy-1- and TcR-induced Th17 and Th2 cells, respectively and is consistent with my previous observations that Thy-1 preferentially favors IL-17 and IL-4 production.

It is interesting that under Th1- and Th2-polarizing conditions, the potential of Thy-1-induced Th cells to produce more of the signature cytokines IFN γ and IL-4 than TcR-induced Th cells did not correlate with the expression of higher amounts of the lineage-defining transcription factors, T-bet and Gata-3. Instead, Thy-1-induced Th1 and Th2 cells expressed strikingly less T-bet and Gata-3, respectively. It is important to note that greater expression of the key-lineage transcription factors does not always correspond to greater Th cell functionality. For example, low levels of Gata-3 expression are sufficient to induce a maximal Th2 response, and forced expression of Gata-3 does not further enhance Th2 cytokine production (209). Moreover, Gata-3 expression is dispensable for IL-4

production once Th2 cells are differentiated. A major consequence of Th cell differentiation is the epigenetic modification of cytokine gene loci. All of the key-lineage transcription factors promote remodeling at Th cell cytokine gene loci, resulting in the increased accessibility of transcription factors to the respective signature Th cell cytokine gene loci, while silencing transcription at all other Th cell cytokine gene loci (159, 160, 209, 215). For example, Gata-3 promotes chromatin remodeling at the *IL-4* gene locus, thereby increasing accessibility to transcription and chromatin remodeling at the *IFN γ* gene locus that results in the repression of transcription. It would therefore be interesting to determine whether the greater ability of Thy-1-induced Th1, Th2 and Th17 cells to produce the respective signature cytokine in comparison to TcR-induced Th1, Th2 and Th17 in response to restimulation with PMA and ionomycin is because Thy-1 signaling somehow enhances chromatin remodeling.

Unlike Thy-1, I found that the crosslinking two other GPI-APs, CD48 and Ly6A/E, on the surface of murine T cells did not promote T cell activation or cytokine production in the presence of strong costimulation provided by LPS-matured BMDCs. These findings suggest that the ability of Thy-1 to provide a nonspecific TcR-like signal is not a common feature to all GPI-APs. In fact, it appears that CD48 provides more of a costimulatory-like signal during T cell activation, since CD48 signaling enhances TcR-driven T cell activation (464). A hallmark of T cell costimulatory signals is the regulation of cytokine production through the promotion of increased mRNA stability. Many cytokine mRNAs contain AU-rich elements (AREs) in their 3'-untranslated region that regulate the stability of the mRNA by binding to trans-factors that promote either mRNA decay or stability (509, 510). CD28 signaling promotes increased IL-2 mRNA stability, in part by the Akt-dependent activation of the ARE-binding protein, NF90, which prevents decay of IL-2 mRNA (511). CD48 signaling also enhances TcR-mediated T cell activation by promoting the stabilization of IL-2 and IFN γ mRNA (463), consistent with its role in providing costimulatory signals during T cell activation. In contrast, Ly6A/E signaling prevents TcR-mediated IL-2 production (467), suggesting that Ly6A/E negatively regulates T cell responses. Taken

together these observations suggest that different GPI-APs can have distinct roles in T cell activation.

Ly6A/E signaling can induce T cell hybridoma cells to proliferate and produce IL-2 in the presence of splenic accessory cells when PMA is also present. I demonstrated that Ly6A/E signaling in the presence of costimulation provided by LPS-matured BMDCs can also induce highly purified T cells to proliferate in the presence of PMA. These findings suggest that, compared to TcR, Ly6A/E signaling is deficient in its ability to activate the necessary DAG-mediated pathways that are required for T cell proliferation. Additionally, Thy-1-mediated T cell proliferation and IL-2 production is also enhanced by PMA (468) and Thy-1-mediated T cell activation in the presence of costimulation provided by splenic accessory cells is more sensitive to PKC inhibition than TcR-mediated T cell activation (406), suggesting that Thy-1 does not induce as strong PKC activation as the TcR. Consistent with this hypothesis, I showed that PMA enhanced Thy-1-induced IL-2 production to a greater extent than TcR-induced IL-2 production in the presence of costimulation provided by LPS-matured BMDCs. Interestingly, Zhu et al. (512) recently showed that PMA enhances IL-2 mRNA stability through the activation of PKC β 1. PKC β 1 is a PKC isoform that mediates increased IL-2 mRNA stability through the activation NF90. TcR-mediated IL-2 production is also dependent upon the activation of PKC β 1 (513). Therefore, it would be interesting to determine whether Thy-1 signaling activates PKC β 1 to a lesser extent than TcR signaling.

My observation that Ly6A/E signaling in the presence of costimulatory signals provided LPS-matured BMDCs with or without PMA did not induce appreciable levels of IL-2, IFN γ , IL-4 or IL-17, suggests that the ability of Thy-1 signaling to provide a TcR-like signal that can modulate T cell cytokine production is not a common ability of all GPI-APs. This does not mean, however, that other GPI-APs may not play a role in the differentiation of distinct Th cell phenotypes. For example, it would be interesting to determine whether CD48-mediated costimulation preferentially skews the T cell response towards a particular Th cell subset. In this regard, Ingram *et al.* (514) recently showed that mice deficient in

the cellular prion protein, which is also a GPI-AP implicated in T cell activation, produce lower levels of IL-17, IL-4 and IFN γ in response to T cell activation and develop decreased EAE as well as weaker responses to bacterial challenge. These findings suggest that cellular prion protein signaling is required for optimal cytokine production.

Collectively, my findings strongly support a model whereby Thy-1 signaling in the presence of costimulatory signals provides a weak TcR-like stimulus that favors IL-4 production and Th2 differentiation over IFN γ and Th1 development (Figure 5.1). Moreover, my findings reveal a novel role for Thy-1 signaling in promoting IL-17A production, ROR γ t expression and enhancing Th17 differentiation. Furthermore, I show for the first time that, similar to the TcR, the Thy-1 signal is not only sufficient to promote the differentiation of CD4⁺ T cells into cytokine-producing Th effector cells, but can also provide the signals that are necessary to reactivate Th effector cells to produce high levels of particular cytokines in the absence of costimulation.

5.3 Limitations of This Study

A major limitation with this study is that the ligand for T cell-associated Thy-1 is not yet known. Like numerous other Thy-1 researchers, I have utilized a mitogenic Thy-1 mAb in order to mimic the endogenous Thy-1 ligand. It is worth noting that there are mAbs that recognize several different domains on Thy-1 and therefore differ in terms of their mitogenicity. For example, the 30-H12 mAb clone is not mitogenic and recognizes an epitope specific to Thy-1.2 (376, 515). In contrast, the mitogenic G7 mAb clone used in this study binds a specific non-polymorphic epitope on Thy-1 (400, 515). It is therefore possible that the putative Thy-1 ligand binds to a different domain on Thy-1 than G7 and could trigger different signaling pathways with distinct strength and kinetics compared to what was seen in this study. It is worth noting, however, that of all the anti-Thy-1 mAb clones tested to date, cross-linking of Thy-1 on T cells by G7 results in the strongest mitogenic response (400, 401), and could therefore be analogous to strong signaling mediated by ligand binding. Moreover, Thy-1 possesses an RLD integrin binding motif within the nonpolymorphic region that mediates the

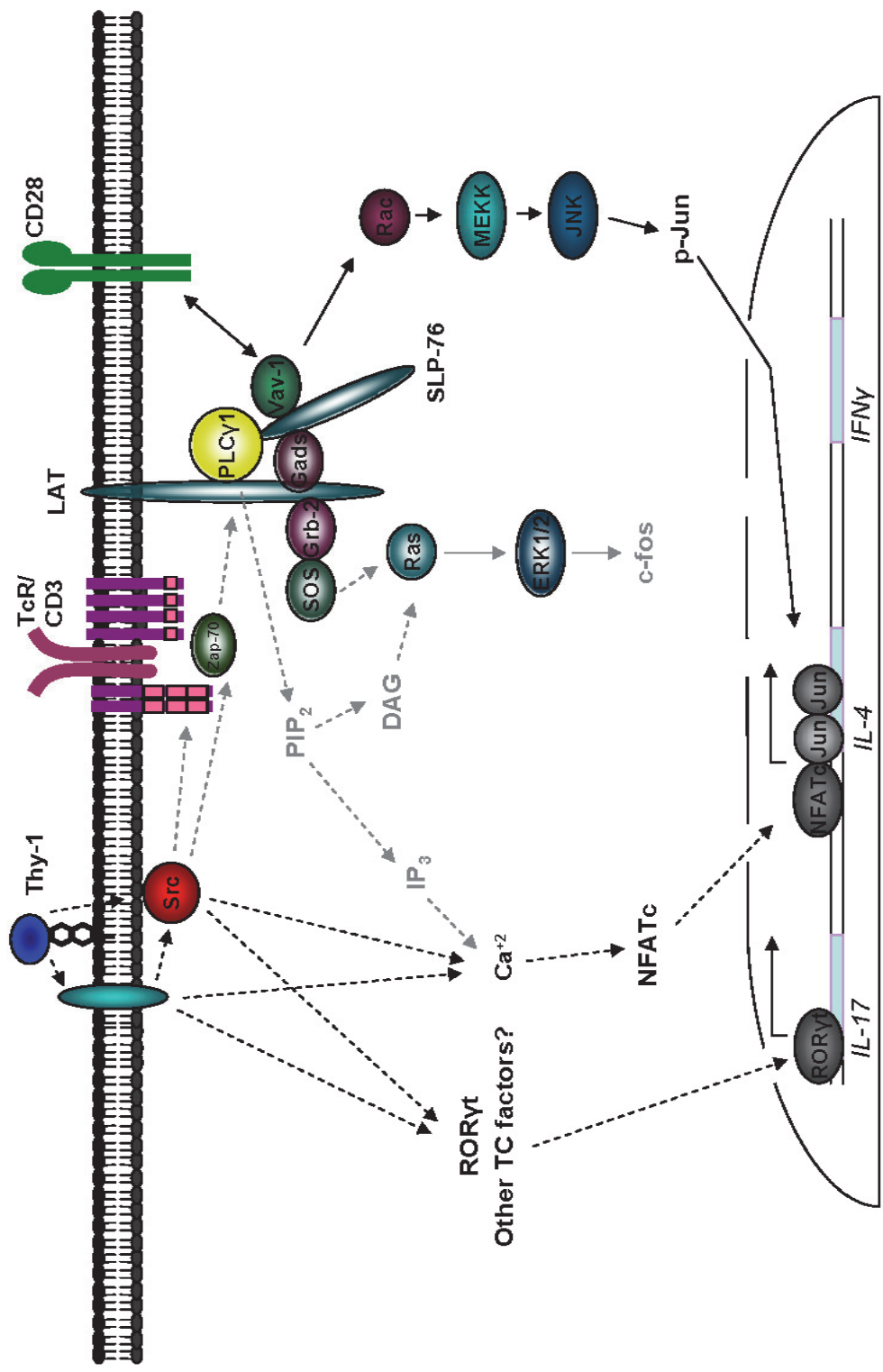


Figure 5.1. Proposed Model for Thy-1 Signal Transduction Leading to Preferential Production of IL-17 and IL-4. Dashed lines indicate proposed signaling pathways involved in Thy-1-mediated signal transduction based on my study as well as previous other studies (see text). Grey lines indicate pathways that may be activated with delayed signaling kinetics by Thy-1-mediated signaling in comparison to TcR-mediated signaling.

interaction of Thy-1 with several integrins within the neurological compartment (492). Since G7 also binds to a nonpolymorphic portion of Thy-1 (400), it is possible that G7 engages this potential ligand binding domain. Another limitation of this study is that since the Thy-1 ligand is not known, we do not know where and under what circumstances it is expressed. It is therefore unclear whether Thy-1 signaling would even occur in the presence of the costimulatory signals that are provided by mature BMDCs. Consequently, until the ligand is identified for T cell-associated Thy-1 the physiological relevance of my findings, as well as that of many other studies examining the role of Thy-1 signaling in T cell function will remain uncertain.

Another limitation with this study is that anti-TcR β mAb was used to simulate the interaction of the TcR with its physiological ligand, pMHC. Although this is a commonly used method for studying TcR-mediated T cell activation, the physiological relevance can be questioned. Importantly, there is variability in the affinity of different TcRs for their cognate pMHC that, as discussed above, plays a major role in determining the strength and kinetics of TcR-mediated signal transduction and consequently the nature of T cell responses. In contrast, anti-TcR β mAb likely has the same affinity for all TcRs. In terms of strength, it is unclear if concentrations of anti-TcR β mAb that induce a maximal mitogenic response in the presence of BMDCs activate TcR signal transduction pathways within the physiological range generated by TcRs engaged by cognate pMHC. Another potential difference is the involvement of CD4 and CD8 coreceptors. Engagement of the TcR complex by pMHC results in the recruitment of CD4 or CD8 through interaction with the invariant region on the MHC molecules (13). This brings Ick, which is associated with CD4 and CD8, in close proximity to the ITAMs of the TcR ζ chains that it subsequently phosphorylates. In contrast, anti-TcR β mAb promotes TcR signaling by crosslinking TcR complexes by binding to specific epitopes on the TcR β chains and likely acts independently of CD4 and CD8. It will therefore be necessary to compare Thy-1 signaling to pMHC-mediated TcR signaling to determine if differences observed in this study have physiological relevance.

5.4 Future Directions and Concluding Remarks

My research provides several novel insights into the possible immunological relevance of T cell-associated Thy-1, although many unanswered questions remain. I show clear evidence that the signal generated by Thy-1 in response to crosslinking with anti-Thy-1 mAb is weaker and has delayed kinetics in comparison to TcR signaling. Consequently, some of the outcomes of Thy-1 signaling in the presence of costimulation provided by LPS-matured BMDCs in T cells appear to emulate weak TcR signaling generated by low affinity Ag seen in other studies (502), suggesting that Thy-1 signaling may merely be providing a weaker TcR signal. Some low affinity Ags can induce a TcR signal that activates signaling molecules with similar kinetics to strong affinity Ag but to an overall less extent (502). Other low affinity Ags have been shown to activate signaling molecules, such as ERK, to a similar extent but with delayed kinetics compared to high affinity Ag (430). The difference between these Ags may be due to the mechanics of signaling rather than the affinity of the Ag for the TcR. For example, Yachi *et al.* (516) showed that one Ag activates ERK with delayed kinetics because its ability to recruit CD8 to the TcR complex was delayed compared to other Ag. Therefore, the finding that the kinetics of Thy-1 signaling are delayed compared to TcR signaling suggests that the mechanics of Thy-1 signaling mediated by mAb crosslinking may also be delayed compared to TcR signaling. Once the Thy-1 ligand is identified, it will be worthwhile to investigate whether the signaling kinetics initiated by Thy-1 binding to its ligand are similarly delayed.

I show clear evidence that the Thy-1 signal-generated can not only substitute for TcR signaling in CTL differentiation, but can also trigger CTLs to destroy target cells, albeit with delayed kinetics. Several methods could be used to determine the mechanism(s) by which anti-Thy-1 mAb-induced CTLs destroy target cells. The ability of anti-Thy-1 mAb-induced CTLs to polarize their lytic granules towards target cells could be visualized by fluorescence microscopy using specific labeling of the lytic granules with fluorochrome-conjugated Ab, such as anti-cathepsin D or anti-granzyme B mAbs. Alternatively, the release of the contents of lytic granules could also be measured by surface labeling of the

lysosomal membrane protein, LAMP-1 (CD107a), which appears on the surface of CTLs upon fusion of lytic granules with the plasma membrane (517). Moreover, since the cytotoxic activity of perforin is dependent upon extracellular Ca^{+2} , a chelator such as EGTA that targets only extracellular Ca^{+2} should inhibit anti-Thy-1 mAb-induced CTL-mediated cytotoxicity if it is due to granule-mediated cytotoxicity but not if it is due to the Fas-mediated pathway. It would also be interesting to try different target cells to determine if Thy-1-induced CTLs can destroy other cells.

It is also possible that the differential production of cytokines by TcR- and Thy-1-activated T cells could play a part in the differences seen in cytotoxic effector function by TcR- and Thy-1-induced CTLs. Interestingly, it has been shown that IL-4 downregulates CTL responses (518), whereas IL-17 can promote CTL development (519). It will therefore be necessary to block IL-4 signaling to determine if the higher levels of IL-4 production by Thy-1-induced CTLs could explain the delayed kinetics of CTL function.

I also found that Thy-1 signaling in the presence of costimulatory signals provided by BMDCs also promotes the differentiation of CD4^{+} T cells into cytokine producing Th cells when in the presence of appropriate polarizing conditions. Effector T cells have less stringent activating requirements than naïve T cells as naïve T cells require CD28-mediated costimulation but effector T cells generally do not. I demonstrated that Thy-1 signaling in the absence of costimulation is also sufficient to activate both TcR- and Thy-1-induced Th effector cells to produce high levels of cytokines. These findings provide further evidence that Thy-1 can provide a nonspecific TcR-like signal and have major implications for the possible *in vivo* role of Thy-1. Since the Thy-1 signal in the presence of costimulatory signals provided by DCs results in the differentiation of CD8^{+} and CD4^{+} T cells into fully-armed CTLs and cytokine-producing Th cells, if DCs do indeed express the Thy-1 ligand, the timing of its expression would need to be tightly regulated to ensure that T cells are not inappropriately activated. Similarly, since Thy-1 signaling in the absence of costimulatory signals is sufficient to activate effector T cells to produce cytokines and destroy target cells, if the Thy-1

ligand is expressed on any other cell-type that may interact with T effector cells, its expression would also have to be tightly regulated to prevent the erroneous activation of T cell effector responses. It is therefore possible that the Thy-1 ligand is only expressed under certain inflammatory conditions. My observation that Thy-1 signaling has delayed kinetics compared to TcR signaling may also contribute to preventing the activation of robust nonspecific T cell responses initiated by Thy-1 signaling. It is possible that the Thy-1 ligand may only be expressed for a short time period, which could allow for only enough Thy-1 signaling to induce small amounts of proliferation and survival signals but not the acquisition of effector molecules. Alternatively, interaction of Thy-1 with its ligand may not induce as robust a response as cross-linking Thy-1 with mAb and may only promote low levels of T cell proliferation and cytokine production. Intriguingly, I consistently observed a small amount of proliferation in T cells that were cultured alone with LPS-matured BMDCs. Since the T cells and BMDCs were cultured in fetal calf serum, this response may simply be the result of the presentation by the BMDCs of foreign Ag from the serum to the murine T cells. However, human DCs will promote autologous CD4⁺ T cells to proliferate at low levels in the absence of serum and thus xenogenic Ag (520). This response appears to be dependent upon MHC II and CD28-mediated costimulation, which has led to the suggestion that presentation of endogenous Ag to T cells may be important in T cell homeostasis. Interestingly, I found that blocking Thy-1 signaling with the non-mitogenic anti-Thy-1 mAb 30-H12 clone prevented the low levels of proliferation (Appendix 1.3). This finding is consistent with LPS-matured BMDCs expressing the Thy-1 ligand. Another possible explanation is that the background proliferation observed in cultures containing T cells and BMDCs may be due to the proliferation of BMDCs. Indeed, when T cells were labeled with CFSE and cultures with BMDCs, no evidence of cellular division was observed (Figure 3.4A). It is therefore possible that the anti-Thy-1 mAb 30-H12 clone prevented low levels of observed proliferation in T cell and BMDCs cultures by blocking BMDC proliferation, possibly by binding to FcγRs. It would thus be

worthwhile to determine if the anti-Thy-1 mAb 30-H12 clone can still prevent proliferation in T cell and BMDC cultures when FcγRs are blocked.

The observation that Thy-1 signaling preferentially skews Th cell cytokine production towards IL-4 and IL-17 synthesis provides evidence that Thy-1 signaling may preferentially promote Th2 and Th17 responses over Th1 responses. These results suggest that Thy-1 signaling may be important in immune responses against Th2- and Th17-type pathogens, as well as pathogenic T cell immune responses involved in allergy and EAE. It will therefore be interesting to determine whether Thy-1 signaling *in vivo* also favors Th2 and Th17 responses and is required for optimal host defense against helminthes and extracellular bacteria, respectively. Although not readily available, Thy-1^{-/-} mice have been generated. However, very few studies have examined T cell responses within these animals and none have examined whether different types Th cell responses are compromised (402). It will also be important to determine whether these animals have decreased susceptibility to allergy and EAE.

It is currently unclear whether Thy-1-mediated signaling induces T cells to produce both IL-17 and IL-4, or distinct subsets of T cells that produce only IL-17 or IL-4. Interestingly, Th cell subsets that are Gata-3⁺RORγt⁺ and produce both IL-4 and IL-17 have been identified in both patients and mice with allergic asthma (521). In this study, IL-4/IL-17 producing Th cells were found in the lungs of mice at both the early and chronic stages of allergic inflammation. These cells were found to be more pathogenic than conventional Th2 or Th17 cells since adoptive transfer of IL-4/IL-17 producing Th cells resulted in more robust recruitment of neutrophils, eosinophils, macrophages and lymphocytes (521). It is possible that IL-4/IL-17 producing Th cells either develop directly from naïve CD4⁺ T cells or from conventional Th2 and Th17 cells. The latter hypothesis is supported by increasing evidence that different Th cell lineages have a considerable amount of plasticity, which is greatly influenced by the inflammatory environment. Importantly, Cosmi *et al.* (522) showed that Th17 cells start to produce IL-4 in environments rich in IL-4, suggesting that IL-4/IL-17 producing Th cells could develop from Th17 cells within the right cytokine environment. Moreover, Wang

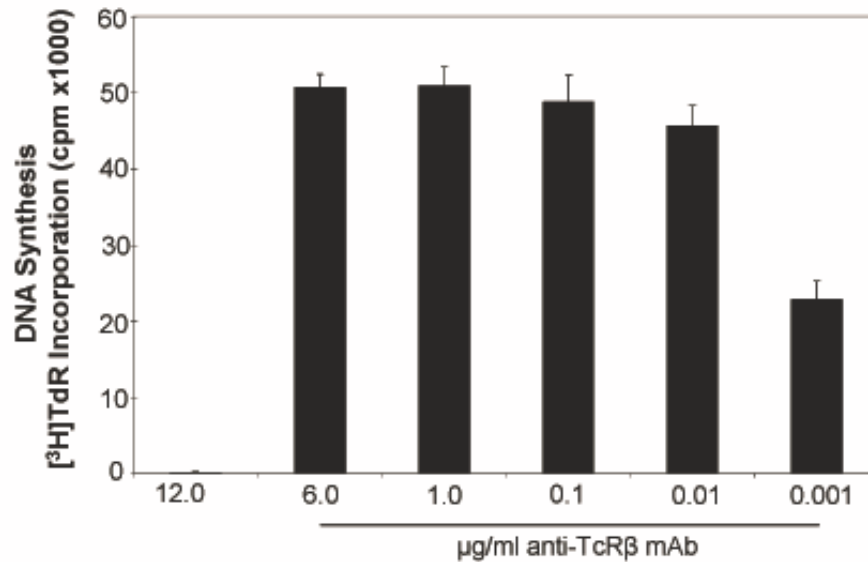
et al. (521) showed that Th2 cells will upregulate ROR γ t expression and produce IL-17 when in the presence of the proinflammatory cytokines IL-1 β , IL-6 and IL-21. Whether Thy-1 signaling promotes the differentiation of IL-4/IL-17 producing Th cells should be determined by two-color intracellular cytokine staining. If this hypothesis is confirmed, it will indicate that Thy-1 signaling also may be important in the development of IL-4/IL-17 responses and will further support a possible role for Thy-1 signaling in allergic asthma.

Nevertheless, until the physiological ligand of T cell-associated Thy-1 is discovered, the true immunological function of Thy-1 will not be fully understood. The characterization of an RLD integrin-binding domain within the Thy-1 protein has led to the identification of several physiologically significant integrins that can bind to Thy-1. Astrocyte β_3 integrin and leukocyte $\alpha_m\beta_2$ integrin interact with Thy-1 and promote astrocyte adhesion and human leukocyte adhesion to Thy-1 expressing endothelium, respectively (523, 524). It is therefore possible that the ligand for T cell-associated Thy-1 might be one of these integrins. Alternatively, the true ligand might bind to another domain on Thy-1. For example, a soluble sugar binding protein, galectin-1, has been shown to bind to Thy-1 in a carbohydrate-dependent manner (525). Interestingly, galectin-1 has been shown to favor Th2 differentiation over Th1 differentiation (526). Future investigations focusing on these possible Thy-1 ligands are certainly warranted.

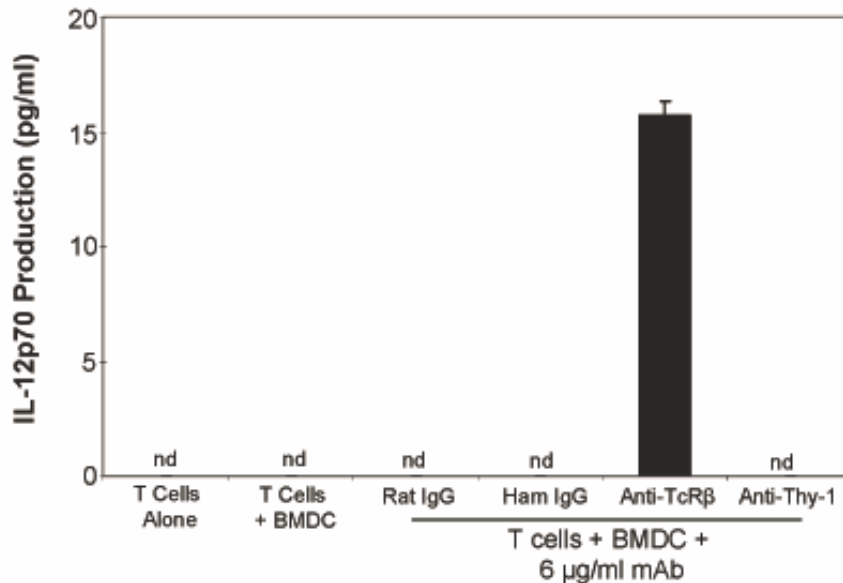
In summary, my results strongly support the growing body of evidence suggesting that Thy-1 signaling is analogous to TcR signaling, albeit with delayed kinetics and certain differences in outcomes. I show that like the TcR, Thy-1 provides a signal that not only promotes the differentiation of T cells into CTL and Th effector cells, but also is sufficient to trigger CTL and Th effector function. I confirm that PTKs and ERK are activated by both Thy-1 and TcR signaling, and I show for the first time that a central player in TcR signaling, Zap-70 is also phosphorylated as a result of Thy-1 signaling. Moreover, my research also strongly supports a model whereby Thy-1 additionally signaling pathways that are distinct from those activated by TcR signaling. Importantly, Thy-1 signaling uniquely promoted ROR γ t and IL-17 production either alone or in the presence of

TcR-signaling, suggesting a possible physiological role for Thy-1 in Th17 cell function.

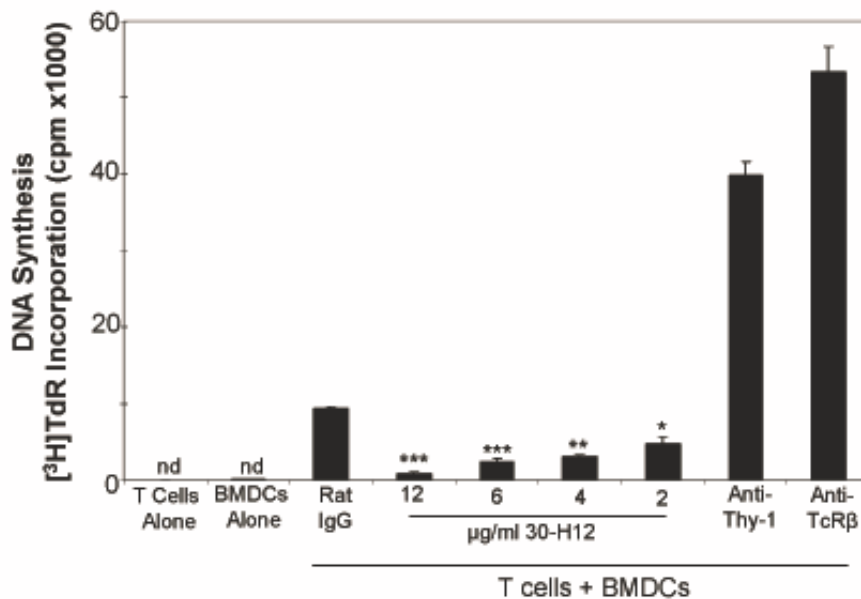
Appendix – Supplementary Figures



Appendix 1.1. Dose response of anti-TcRβ mAb-induced T cell proliferation in the presence of costimulation provided by LPS-matured BMDCs. Highly purified CD3⁺ T cells with or without LPS-matured BMDCs were seeded in triplicate into 96-well round-bottom plates. T cells were then cultured in the presence or absence of the indicated concentrations of anti-TcRβ mAb for 72 h. Wells were pulsed with [3H]TdR 6 h before the endpoint at which time the cells were harvested and DNA synthesis was determined based on [3H]TdR incorporation. Data are representative of 2 independent experiments ± SD.



Appendix 1.2. Anti-Thy-1 mAb-Activated T Cell Cultures Produce Less IL-12p70 than Anti-TcR β mAb-Activated T Cell Cultures. CD3⁺ T cells with or without LPS-matured BMDCs were seeded in quadruplicate into 96-well round-bottom plates. T cells were then cultured in the presence of 6 μ g/ml anti-Thy-1 mAb (clone G7), anti-TcR β mAb or the appropriate isotype control for the 24 h. (A) Supernatants were isolated and analyzed for the production of IL-12p70 by ELISA. Data shown are representative two separate experiments +/- SD; nd denotes not detected.



Appendix 1.3. Non-mitogenic Anti-Thy-1 mAb Clone 30-H12 Blocks Low Levels of T Cell Proliferation Induced by LPS-Matured BMDCs. CD3⁺ T cells with or without LPS-matured BMDCs were seeded in quadruplicate into 96-well round-bottom plates. T cells were then cultured in the presence or absence of 6 µg/ml anti-Thy-1 mAb (clone G7), anti-TcRβ mAb, the indicated concentrations of anti-Thy-1 mAb (clone 30-H12) or the appropriate isotype control for 72 h. Wells were pulsed with [³H]TdR 6 h before the endpoint at which time the cells were harvested and DNA synthesis was determined based on [³H]TdR incorporation. Data are representative of 2 independent experiments ± SD nd denotes not detected. * denotes p < 0.05, ** denotes p < 0.01 and *** denotes p < 0.001 as determined by the Bonferroni multiple comparisons test.

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