THY-1 SIGNALLING IN T CELLS IS REGULATED BY MITOGEN-ACTIVATED PROTEIN KINASES AND INDUCES A REGULATORY PHENOTYPE

by

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To my beloved wife

The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny ...'

-Isaac Asimov (1920 - 1992)

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Abstract

Antibody-mediated Thy-1 stimulation was first shown to activate T cells more than two decades ago. Since then, our knowledge of the mechanism of Thy-1 signal transduction has remained limited, in large part due to the absence of a known stimulatory Thy-1 ligand in the lymphoid compartment. We therefore also know little of the function of this glycophosphatidylinositol-anchored protein, beyond T cell mitogenesis. The purpose of this work was to enhance our understanding of the nature, and role, of Thy-1 signaling in T cells. In the context of Thy-1- and CD28-mediated T cell activation induced by monoclonal antibody (mAb)-coated microbeads, I found that extracellular signal-regulated kinase (ERK)1/2, p38 mitogen-activated protein kinase (MAPK), and c-Jun N-terminal kinase (JNK) each carry out different, but important, roles. The major function of ERK1/2 is to mediate interleukin (IL)-2 expression, while JNK is involved in the production of IL-2 and surface expression of the IL-2 receptor (IL-2R) α chain (CD25). Interestingly, in addition to mediating IL-2R signal transduction, p38 MAPK also negatively regulates IL-2 gene transcription. The observation that a Thy-1 signal *per se* induces CD25 expression in T cells allowed me to use a panel of selective pharmacological inhibitors to identify components of the Thy-1 signaling pathway. Thy-1-induced CD25 expression is dependent on ERK1/2, JNK, phospholipase C, protein kinase C, calmodulin-dependent kinase II, calcineurin, phosphatidylinositol-3 kinase, and protein kinase A, many of which are also associated with T cell receptor signaling. Unexpectedly, I found that p38 MAPK is not involved in Thy-1-induced CD25 expression. Lastly, I discovered that Thy-1 signaling induces an atypical regulatory phenotype in normal T cells. mAb-mediated stimulation of Thy-1 on $CD4^+CD25^-$ forkhead box protein P3 $(FoxP3)^-$ T cells generates $CD4^+CD25^+FoxP3^-$ T cells that do not proliferate in response to CD3/CD28 stimulation despite producing substantial amounts of IL-2, and are able to suppress normal T cell responses. Taken together, my findings clarify the role of MAPKs in Thy-1-induced T cell activation, reveal elements of the Thy-1 signal transduction pathway in T cells, and suggest a novel function for Thy-1 in the induction of atypical regulatory T cells.

List Of Abbreviations And Symbols Used

A	amperes
AIDS	acquired immunodeficiency syndrome
ALX	adaptor in lymphocytes of unknown function, X
ANOVA	analysis of variance
AP-1	activator protein-1
APC	antigen-presenting cell
APS	ammonium persulfate
ASK	activator of S-phase kinase
ATF	activating transcription factor
AU	adenosine-uridine
Bcl-X_L	long isoform of B cell lymphoma-X
BfA	brefeldin A
bp	base pair
BSA	bovine serum albumin
°C	degrees Celcius
Ca^{2+}	calcium ion
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CD	cluster of differentiation
CD25	IL-2R α chain
cDNA	complementary DNA
CFSE	carboxyfluoroscein succinimidyl ester
Ci	Curie

CO_2	carbon dioxide
ConA	concanavalin A
cpm	counts per minute
CREB	cyclic adenosine monophosphate response element-binding protein
cRPMI	complete RPMI medium
CsA	cyclosporine A
CTLL-2	cytotoxic lymphoid line-2
DAG	diacylgylcerol
DC	dendritic cell
DMSO	dimethyl sulfoxide
DN	double-negative
DNA	deoxyribonucleic acid
DP	double-positive
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
$F(ab')_2$	antibody-binding fragment
FADD	Fas-associated death domain
Fas	FS-7 cell-associated cell surface
FasL	Fas ligand

Fc	fragment, crystalizable
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FLICE	FADD-like IL-1β-convertine enzyme (caspase 8)
FLIP	FLICE-inhibitory protein
FoxP3	forkhead box protein P3
g	gram(s)
g	force of gravity
Gads	Grb2-related adaptor downstream of Shc
GPI	glycophosphatidylinositol
Grb2	growth factor receptor-bound protein 2
GTP	guanosine triphosphate
[^{3}H]TdR	methyl ^{3}H-thymidine deoxyribonucleotide
h	hour(s)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
ICOS	inducible costimulatory molecule
IF	immunofluorescence
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL-2R	IL-2 receptor
IP_3	inositol 1,4,5-trisphosphate

IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
ITAM	immunoreceptor tyrosine-based activation motif
itk	IL-2-inducible T cell kinase
JAK	Janus tyrosine kinase
JNK	c-Jun NH_2-terminal kinase
κ	kappa
kDa	kilodalton
l	litre
LAT	linker of activated T cells
lck	leukocyte-specific protein tyrosine kinase
LFA-1	lymphocyte function-associated antigen 1
LPS	lipopolysaccharide
m	metre
M	molar
mAb	monoclonal antibody
MACS	magnetic cell sorting
MAPK	mitogen-activated protein kinase
MAPKAPK	MAPK-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MEK	MAPK/ERK kinase
MEKK	MEK kinase
MKK	MAPK kinase

MHC	major histocompatibility complex
min	minute(s)
MLK	mixed lineage protein kinase
mRNA	messenger ribonucleic acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	molecular weight
NFAT	nuclear factor of activated T cells
NFκB	nuclear factor kappa B
NK	natural killer
p	probability
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain-reaction
PD98059	a MEK1 inhibitor
PE	phycoerythrin
PFA	paraformaldehyde
pH	potential of hydrogen
PI3K	phoshpatidylinositol-3 kinase
PIG-A	phosphatidylinositol glycan class A
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C

PNH	paroxysmal nocturnal haemoglobinuria
PTEN	phosphatase and tensin homolog deleted on chromosome 10
PTK	protein tyrosine kinase
RAG	recombination-activating gene
RLD	arginine-leucine-aspartic acid
RNA	ribonucleic acid
RNA Pol	RNA polymerase
RPMI	Roswell Park Memorial Institute
s	second(s)
SAPK	stress-activated protein kinase
SB203580	a p38 MAPK inhibitor
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SH	src homology
Shc	SH2 domain-containing
siRNA	small interfering RNA
SLP-76	SH2 domain-containing leukocyte-specific phosphoprotein of 76 kDa
SOS	son of sevenless
SP	single-positive
SP600125	a JNK inhibitor
STAT	signal transducer and activator of transcription
T	threonine

TAE	tris-acetic acid-EDTA
TBS	tris-buffered saline
TBST	TBS with 0.05% (v/v) Tween-20
Tc	cytotoxic T
Tc1	type-1 Tc
Tc2	type-2 Tc
TCR	T cell receptor
TEMED	tetramethylethylenediamine
TGF	tumour growth factor
Th	T helper
Th1	type-1 Th
Th17	interleukin-17-producing Th
Th2	type-2 Th
Th3	type-3 Th
Thy	thymus cell antigen
Thy-1-Fc	a chimeric protein composed of the binding region of Thy-1 and the Fc portion of human IgG1
TNF	tumour necrosis factor
Tr1	type-1 regulatory T
Treg	regulatory T
U	units
UV	ultraviolet
V	Volts
VASP	vasodilator-stimulated phosphoprotein

WT	wild-type
Y	tyrosine
ζ	zeta
ZAP-70	zeta chain-associated protein 70 kDa

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First and foremost I must thank my supervisor, Dr. David Hoskin, for welcoming me into his lab as a volunteer in 2001, and for keeping me around ever since as a summer student, an honours student, and, most recently as a graduate student. I owe you a great deal of gratitude for your encouragement, patience, mentorship, and infectious enthusiasm for research. Even when things were not working in the lab and I wanted to be anywhere but the Tupper Building, you always knew just how to motivate/scare me back to the bench. You have made me a better thinker, a better writer, and a better researcher. For all of this I am forever grateful.

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Chapter 1. Introduction

1.1. T Cells

The immune system is responsible for the never-ending task of monitoring for, and eliminating, pathogens and cancer. However, the immune system itself can also cause detrimental outcomes for the host, should aberrant responses be triggered, or permitted to continue, in the absence of appropriate regulation. A delicate balance therefore must be maintained among the many cells involved in normal and effective immunity in order to promote proper health and protect against disease. Immunity is the outcome of the collective efforts of the cells that comprise innate and adaptive branches of the immune system. The innate arm of immunity is charged with the rapid response to pathogens (Akira et al., 2006), while the adaptive arm of the immune system mediates specific and long-term protection against infections and cancer (Pancer and Cooper, 2006). B cells and T cells are the major components of the adaptive immune system. Antibodies are produced by B cells to protect against pathogens, while T cells respond to foreign antigen and altered self-cells.

T cells are antigen receptor-bearing cells of the lymphoid lineage. They develop from progenitor bone marrow cells and undergo thymic education before exiting to the periphery (Rothenberg and Taghon, 2005). Among the immunological functions of T cells are the destruction of target cells, the production of cytokines that support ongoing immune responses, and the negative regulation of normal immunity. The importance of T cells to our health is exemplified by patients with acquired immunondeficiency syndrome (AIDS), the

diagnosis of which is based on critically low numbers of peripheral T cells. AIDS patients suffer from an elevated incidence of opportunistic infections and certain types of cancer (Cheung, 2004a; Cheung, 2004b; Martinez and Temesgen, 2006). At the other end of the spectrum, errant T cell responses directed against self tissues can result in autoimmune disorders, such as multiple sclerosis. Even the predominant activation of one type of T cell over another can contribute to the pathology of infectious diseases (Hernandez-Pando et al., 1996). T cell biology is a field of intensive biomedical research, and our understanding of T cell function, as well as our ability to manipulate the activity of these important cells, is ever-expanding.

1.1.1. T Cell Development

T cells develop from haematopoietic stem cells in the bone marrow that transit to the thymus. In the thymus, these cells, called thymocytes, develop through stages that are identifiable by surface expression patterns of CD4, CD8, CD44, and the interleukin (IL)-2 receptor (IL-2R) α chain (CD25), as well as by physical location within the organ itself. Thymocytes begin this developmental process as $CD4^-CD8^-$ double-negative (DN) cells. DN cells enter the thymus at the cortico-medullary junction and transition from a $CD44^+CD25^-$ phenotype to a $CD44^+CD25^+$ phenotype as they move through the cortex (Lind et al., 2001). Thymocytes are $CD44^-CD25^+$ once they reach the subcapsular zone of the thymus, at which point T cell receptor (TCR) β chain gene rearrangement can already be detected. In fact, thymocytes that do not undergo gene rearrangement at this point fail to develop further into $CD44^-CD25^-$ DN cells (Mombaerts et al.,

1992; Shinkai et al., 1992). Thymocytes that have successfully rearranged their TCR β chain, which is expressed in association with a pre-Tα chain and the CD3 signalling complex (collectively refered to as the pre-TCR), become $CD4^+CD8^+$ double-positive (DP) cells. After successfully rearranging the TCR α chain, the DP cells that survive thymic selection exit to the periphery as $CD4^+$ or $CD8^+$ single-positive (SP) T cells.

To leave the thymus as fully mature T cells, thymocytes must first undergo two selection processes. The first process, or positive selection, takes place in the thymic cortex (Minter and Osborne, 2003). Here, immature thymocytes interact with thymic epithelial cells that express major histocompatibility complex (MHC) molecules. Interaction between the TCR and MHC molecules elicits a signal that promotes the survival of cells bearing TCRs capable of engaging self MHC. The surviving MHC-restricted DP thymocytes then travel to the medulla of the thymus, where MHC-bearing stromal cells induce thymocyte death if TCR-MHC interactions are too strong. The negative selection process involves the interaction of thymocytes and mesenchymal fibroblasts via their TCR and MHC molecules, respectively. Cells bearing TCR molecules with high affinity for self MHC complexed with self peptides are eliminated by apoptosis, allowing only self-tolerant T cells to mature further. The end result of these thymic selection processes is the development of self MHC-recognising, self-tolerant T cells.

1.1.2. T Cell Subsets

The processes described above pertain to the development of αβ T cells. While γδ T cells also populate the body, albeit in far smaller numbers, their

developmental processes remain unclear. Beyond differences in TCR chain expression, T cells differ by function. Presently three different functional T cell subsets are recognised, namely T helper (Th) cells, cytotoxic T (Tc) cells, and regulatory T (Teg) cells.

1.1.2.1. Th Cells

Th cells are CD4 coreceptor-expressing T cells. The mechanism by which DP thymocytes become either $CD4^+$ or $CD8^+$ SP cells is not known. The two models that describe how this process might occur are the instructive and stochastic models (Robey, 1994). According to the instructive model, expression of CD4 or CD8 is dictated by MHC class II or class I binding, respectively, during thymic selection. Commitment to CD4 or CD8 expression is completely random according to the stochastic model. Th cells are stimulated by antigen presented in the context of class II MHC on the surface of antigen-presenting cells (APCs). Exogenous antigens are phagocytosed or endocytosed and loaded into class II MHC after endosomal processing. The major function of Th cells is to express cytokines that support immune responses, such as B cell or Tc cell activation. Th cells are further classified as type-1 Th (Th1) or type-2 Th (Th2) cells, based on their cytokine expression profiles (Mosmann and Sad, 1996). Th1 cells express so-called type-1 cytokines, such as IL-2, interferon (IFN)-γ, and tumour necrosis factor (TNF)-α, which promote inflammation and Tc cell activation. Type-2 cytokines, such as IL-4, IL-5, and IL-10 are produced by Th2 cells, which support allergy and antibody responses. $CD4^+$ type-3 Th (Th3) cells, which express the cytokine tumour growth factor (TGF)-β, have also been described, but are now

recognised as a type of Treg cell, and will be discussed as such below. Likewise, IL-17-producing Th (Th17) cells are $CD4^+$ T cells whose function is sufficiently different those of other $CD4^+$ cells to warrant their classification as a new functional subset of Th cells.

The discovery of Th17 cells stemmed from the observation that IL-23 plays a critical role in autoimmunity (Cua et al., 2003; Murphy et al., 2003). This finding, coupled with the ability of IL-23 to induce the production of IL-17 by $CD4^+$ T cells (Aggarwal et al., 2003) suggested that IL-17-producing $CD4^+$ T cells might represent a distinct subset of pathogenic Th cells. Later studies showed that passive transfer of IL-23-treated, IL-17-producing $CD4^+$ T cells results in severe experimental autoimmune encephalopathy (Langrish et al., 2005). Here, Th17 (or Th_{IL-17}) cells were first described as a unique effector $CD4^+$ T cell population.

Th17 cells were originally thought to differentiate in parallel with Th1 cells from a common precursor cell. However, recent evidence suggests that Th1 and Th2 cell development antagonises Th17 cell development, and it is now believed that Th17 cells differentiate along a completely separate lineage from Th1/Th2 cells (Weaver et al., 2006). It is interesting to note that TGF-β is a critical cytokine in the development of the Th17 lineage. Because this cytokine is also known to induce forkhead box protein P3 $(FoxP3)^+$ Treg cells (Chen et al., 2003), there has been much interest in identifying other factors involved in promoting the development of the unique Th17 cell type. IL-6 was identified as a master regulator of TGF-β-induced Th17 cell differentiation; in the presence of IL-6, TGF-β induces Th17 cells instead of Treg cells (Bettelli et al., 2006). The

implications of the existence of a third Th cell subset in health and disease remain to be fully explored in the context of human immunity.

1.1.2.2. Tc Cells

Tc cells bear the CD8 coreceptor, which allows them to interact with class I MHC. Class I MHC molecules present endogenous antigens, including self-peptides, viral peptides, and peptides from intracellular bacteria, on the surface of virtually all nucleated cells. The major function of Tc cells in immunity is to mount cellular immune responses. Activation of a Tc cell by antigenic stimulation results in the expression of cytotoxic effector molecules, such as the FS-7 cell-associated cell surface (Fas) antigen ligand (FasL), perforin, and granzymes (Schachter et al., 1998). Activated Tc cells form conjugates with target cells, in which apoptosis is subsequently induced. Ligation of death ligands with the death receptors on targets cells induces the extrinsic apoptotic program, which culminates in deoxyribonucleic acid (DNA) fragmentation and deconstruction of the target cell. Tc cells can also employ perforin and granzymes to eliminate infected or foreign cells. These cytotoxic molecules are released into the synapse between the Tc cell and the target cell. Perforin and granzymes create transmembrane pores in the target cell and induce DNA fragmentation, respectively. Like Th cells, Tc cells can be described as either type-1 Tc (Tc1) or type-2 (Tc2) cells, where Tc1 refers to IFNγ-secreting cells and Tc2 cells produce IL-4 and IL-5 (Mosmann et al., 1997).

1.1.2.3. Treg Cells

Treg cells are T cells that suppress or regulate normal immune responses. Most commonly described as $CD4^+CD25^+FoxP3^+$ T cells, the term Treg cell is applied to any cell that shares this characteristic suppressor function. Indeed, there is no known cell marker that identifies all Treg cells. Treg cells can be $CD4^+$ or $CD8^+$, and mediate suppression through contact-dependent or contact-independent mechanisms. Relatively little is known about $CD8^+$ Treg cells, thus $CD4^+$ Treg cells will be the focus of this section.

Some of the earliest work that suggested certain subsets of T cells might possess a suppressive phenotype was carried out by Gershon and colleagues. They showed that T cells are required to tolerise B cell precursors against sheep red blood cells (Gershon and Kondo, 1970; Gershon and Kondo, 1971). In fact, Gershon et al. subsequently coined the term "suppressor cell" to describe these unique T cells (Gershon et al., 1972). Unfortunately, owing to the inability to clearly characterise these cells or the factors utilised to mediate suppression, the notion of the existence of T cells with suppressor function soon fell out of favour. The theory of suppressor cells was not fully revived until the mid-1990s, when $CD4^+CD25^+$ T cells were shown *in vivo* to mediate self-tolerance (Sakaguchi et al., 1995).

$CD4^+CD25^+$ T cells are considered to be "naturally occurring" Treg cells. They constitute 5-10% of the $CD4^+$ T cell population in mice, and develop in the thymus (Sakaguchi et al., 1995). In addition to CD25, FoxP3 is also expressed by $CD4^+CD25^+$ Treg cells. FoxP3 is a member of the forkhead-winged-helix family

of transcription factors, and was first identified in scurfy mice (Brunkow et al., 2001). The scurfy phenotype is a multi-organ lymphoproliferative disorder that adheres to an X-linked recessive inheritance pattern. The equivalent condition in humans, called immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), is also causally linked to mutations in the FoxP3 gene (Bennett et al., 2001b; Wildin et al., 2001). FoxP3 acts as a transcriptional repressor, attenuating cytokine expression and proliferation by T cells (Schubert et al., 2001). Interestingly, IL-2 is a cytokine whose expression is repressed in $CD4^+CD25^+$ Treg cells (Su et al., 2004), but has also been shown to regulate Treg cell function (Thornton et al., 2004). Here, the authors used neutralising anti-IL-2 antibodies to reverse Treg cell-mediated suppression of IL-2 expression by normal T cells, suggesting that responder T cell-derived IL-2 actually promotes the suppression mediated by $CD4^+CD25^+$ Treg cells. The exact mechanism of $CD4^+CD25^+$ Treg cell-mediated suppression is unknown; however, these cells fail to suppress T cell responses across a semi-permeable membrane, which implicates a contact-dependent mechanism (Takahashi et al., 1998). Membrane-bound TGF-β was among the first proposed mediators of contact-dependent suppression (Nakamura et al., 2001), though the role of this cytokine in Treg cell function remains contentious, as Treg cell-mediated suppression is intact in the absence of TGF-β (Piccirillo et al., 2002). It has been suggested that Fas/FasL killing is carried out by Treg cells to suppress responder cells; however, Fas-deficient B cells are suppressed by $CD4^+CD25^+$ T cells in a manner dependent on upregulation of perforin and granzyme B (Zhao et al.,

2006). In fact, $CD4^+CD25^+$ Treg cells from granzyme B-deficient mice have an impaired suppressive capacity compared to wild-type (WT) $CD4^+CD25^+$ Treg cells (Gondek et al., 2005). Although these reports strongly support a major role for perforin/granzyme in Treg cell-mediated immune suppression, this topic remains an area of active research, as a widely accepted mechanism of Treg cell suppression has yet to be described.

Most, if not all, naturally-arising Treg cells develop in the thymus (Sakaguchi et al., 1995), though the exact processes that dictate development along the Treg cell lineage are not well known. Type-1 regulatory T (Tr1) cells and Th3 cells are known as "adaptive" Treg cells, because they are believed to arise in the periphery from antigen-activated $CD4^+$ T cells. Adaptive Treg cells also differ from naturally-arising Treg cells by virtue of the contact-independent and cytokine-dependent immune suppression that they exhibit. Cell-free supernatants from anti-CD3/anti-CD28 antibody-activated Tr1 cells inhibit dendritic cell (DC)-mediated T cell proliferation (Lecart et al., 2001), and neutralising antibodies against IL-10 and TGF-β reverse the suppressive effects of these cells (Groux et al., 1997). Similarly, Th3 cell-mediated suppression is dependent on TGF-β and can be abrogated with anti-TGF-β antibodies (Chen et al., 1994). A number of theories exist on how normal T cells are transformed into Treg cells in the periphery. Chen et al. showed that TCR signalling in the context of TGF-β costimulation induces expression of FoxP3 in $CD4^+CD25^+$ T cells, which is associated with hyporesponsiveness to subsequent TCR stimulation, the production of TGF-β, and the ability to suppress T cell proliferation (Chen et al.,

2003). The combination of IFN-α and IL-10 induces a Tr1 cell phenotype in cord blood T cells, which exhibit impaired responsiveness to CD3/CD28 stimulation, and the ability to suppress T cell responses (Levings et al., 2001). Concomitant CD3 and CD46 stimulation (Kemper et al., 2003), as well as blockade of CD40/CD40L interactions (Taylor et al., 2002), have also been shown to induce a regulatory phenotype in T cells. As mysterious as naturally-arising Treg cells remain, far less is known about their adaptive counterparts. However, the ability to induce a Treg cell phenotype could potentially be used to treat lymphoproliferative or autoimmune disorders. Hopefully, the biology of these cells will become clearer in the coming years.

1.2. T Cell Activation

T cells possess the potential to eliminate infection and cancer, but also to cause serious harm. One level of regulation governing the function of T cells is the process of T cell activation. In order for a T cell to become fully equipped with the gene products necessary to kill a target cell, in the case of Tc cells, or to produce cytokines, in the case of Th cells, the T cell must first receive very disctinct signals to enable transcription of effector genes, stabilise cytokine messenger ribonucleic acid (mRNA) molecules, and drive cell division. Presently, this process is believed to involve at least three separate signals.

1.2.1. The 3-Signal Model Of T Cell Activation

The 2-signal model has been used to describe T cell activation for many years (Bretscher and Cohn, 1970). As the name suggests, it was thought that T cells required two signals in order to undergo T cell activation: signal 1,

generated as a result of antigenic stimulation of the TCR; and signal 2, triggered by costimulatory molecule interactions. However, in recent years, it has become apparent that these two signals alone are not sufficient to drive T cell activation. A third signal, provided by cytokines, is now considered to be an integral part of the T cell activation process.

1.2.1.1. Signal 1

The TCR is stimulated by antigen presented in the context of self-MHC, which triggers signal 1 for T cell activation. Because the α and β chains of the TCR possess no enzymatic activity, TCR signal transduction depends upon the participation of many adaptor proteins and associated signalling molecules (Figure 1.1). The TCR is associated with ζ chain homodimers, and CD3 ε/δ or ε/γ chain heterodimers. The CD3 complex and ζ chains possess immunoreceptor tyrosine-based activation motifs (ITAMs), which are phosphorylated during TCR signalling, creating docking sites for signalling molecules involved in TCR signalling. Phosphorylation of ITAMs in CD3 and ζ chains may simply promote T cell activation or reduce costimulation requirements; however, each chain is also associated with other functions (Pitcher and van Oers, 2003). CD3 δ chains are associated with activation of extracellular signal-regulated kinase (ERK)(Delgado et al., 2000), CD3 ε chains interact with the adaptor protein Nck (Gil et al., 2002), and CD3 γ chains are associated with cell survival (Torres et al., 2002). TCR ζ chains serve as docking sites for ζ (zeta) chain-associated protein 70 kDa (ZAP-70), which is a critical signalling molecule in the TCR pathway.

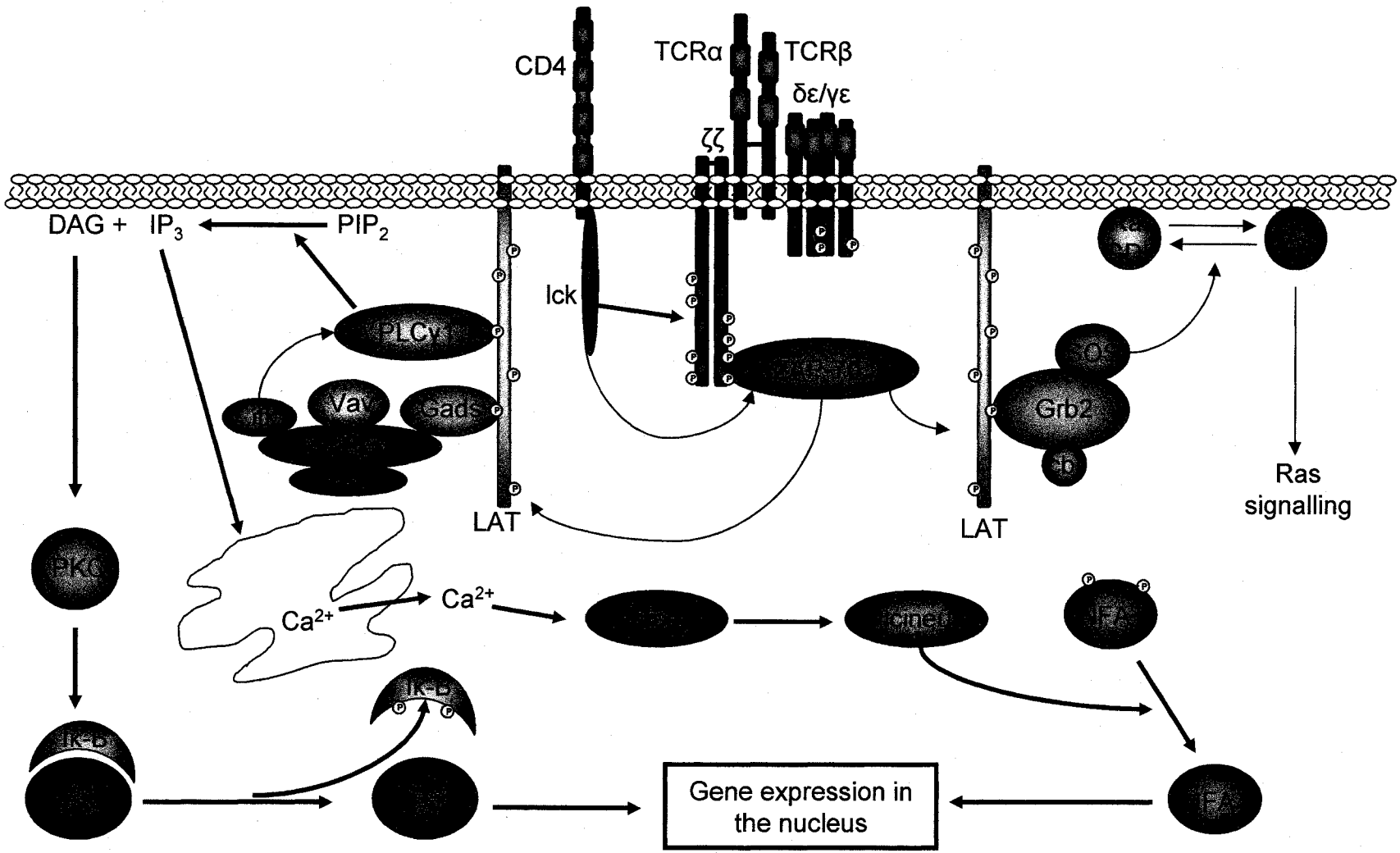


Figure 1.1. Signal 1 In T Cell Activation. Stimulation of the TCR activates multiple signalling molecules and associated pathways in the T cell, ultimately culminating in transcription factor activation and gene expression.

Activation of the Src tyrosine kinase leukocyte-specific protein tyrosine kinase (lck) is one of the earliest events in TCR stimulation (Qian and Weiss, 1997). Lck is associated with the coreceptors CD4 and CD8 (Veillette et al., 1988), which cluster with the TCR and bind MHC molecules on APCs to stabilise TCR-antigen/MHC interactions (Owens et al., 1987; Wooldridge et al., 2005). Lck phosphorylates the TCR ζ chains, at sites where ZAP-70 binds and is activated, enabling ZAP-70 to phosphorylate linker of activated T cells (LAT) (Paz et al., 2001). LAT is an integral membrane adaptor protein that associates with growth factor receptor-bound protein 2 (Grb2), Grb2-related adaptor downstream of Shc (Gads), son of sevenless (SOS), phospholipase (PLC)γ1, Vav, src homology (SH)2 domain-containing leukocyte-specific phosphoprotein of 76 kDa (SLP-76), Cbl, and phosphatidylinositol 3-kinase (PI3K), and may also be activated by lck (Jiang and Cheng, 2007). The complex of LAT, SOS, and Grb2 mediates activation of the Ras pathway (Chardin et al., 1993). SLP-76 is activated by ZAP-70 (Wardenburg et al., 1996) and, together with Gads, recruits IL-2-inducible T cell kinase (itk), which, along with ZAP-70, activates PLCγ1 (Su et al., 1999; Zhang et al., 1998). Cbl can also mediate PLCγ1 activation independently of LAT and SLP-76 (Graham et al., 2003). Vav is a Rho GTPase-specific guanine nucleotide exchange factor associated with the regulation of TCR-mediated Ca^{2+} flux and reorganisation of the cytoskeleton (Fischer et al., 1998).

PLCγ1, which can also be activated independently of protein tyrosine kinases (PTKs) (Cockcroft and Thomas, 1992), hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) to yield diacylglycerol (DAG) and inositol 1,4,5-

trisphosphate (IP_3), which activate protein kinase C (PKC) and induce the release of Ca^{2+} from intracellular stores, respectively (Bell, 1986; Berridge, 1987). PKC mediates activation of nuclear factor kappa B (NFκB), and a Ca^{2+}-dependent pathway involving calmodulin and calcineurin, which mediates activation of nuclear factor of activated T cells (NFAT) (Crabtree, 1999; Lin et al., 2000). NFκB and NFAT are transcription factors that mediate the expression of genes involved in T cell activation, including genes coding for IL-2 and CD25 (Schuh et al., 1998).

In addition to classical antigen-mediated stimulation of the TCR, the lectins concanavalin A (ConA) and phytohemagglutinin, superantigens, and mitogenic TCR or CD3 antibodies can induce signalling via the TCR to drive T cell activation (Chong et al., 1992; Coutinho et al., 1979; Majumdar et al., 1990; O'Flynn et al., 1986). Phorbol esters in combination with ionophores are also commonly used to bypass TCR stimulation and induce T cell activation at the level of PKC activation and Ca^{2+} mobilisation (Truneh et al., 1985). But perhaps the most peculiar means of T cell activation involves glycophosphatidylinositol (GPI)-anchored proteins. These proteins, which do not even span the plasma membrane, are believed to possess the ability to transduce signal 1 in order to cause T cell activation (Loertscher and Lavery, 2002).

1.2.1.2. Signal 2

Signal 2, or costimulation, is generally characterised as a signal that reduces the requirements for signal 1 to induce T cell activation. CD28 is the most extensively studied costimulatory molecule, and delivers the most robust

costimulatory signal (Acuto and Michel, 2003; Lenschow et al., 1996). CD28-mediated costimulation of T cells was first described in the early 1990s (Harding et al., 1992; Jenkins et al., 1991). However, no discreet signalling pathway has yet been ascribed to CD28, although many, if not all, of signalling molecules identified as components of the CD28 signalling machinery are involved in TCR signalling (Acuto and Michel, 2003; Diehn et al., 2002). CD28 signalling activates many factors involved in cell survival and differentiation, including the transcription factors NFκB, NFAT, and activator protein-1 (AP-1) (Diehn et al., 2002; Kane et al., 2002; Michel et al., 2000; Rincon and Flavell, 1994), and drives proliferation by inducing expression of D cyclins (Boonen et al., 1999; Kovalev et al., 2001), which are important in the progression of the cell cycle. During T cell activation, CD28 associates with and activates PI3K (Prasad et al., 1994). The src kinases lck and fyn phosphorylate CD28, creating docking sites for PI3K (Raab et al., 1995). Activation of PI3K is required for optimal IL-2 production and expression of the anti-apoptotic protein, Bcl-X_L (Burr et al., 2001; Okkenhaug et al., 2001). CD28 signalling also enhances transcription of the IL-2 gene (Fraser et al., 1991) and promotes cytokine mRNA stability (Lindstein et al., 1989), the latter of which likely involves a p38 mitogen-activated protein kinase (MAPK)- or c-Jun NH_2-terminal kinase (JNK)-dependent pathway (Chen et al., 1998; Dean et al., 2004; Ming et al., 1998). Cytokine mRNA stability and transcriptional regulation are governed by different signalling pathways (Sanchez-Lockhart et al., 2004).

While CD28 is simply one of several costimulatory molecules involved in T cell activation, it is considered to be one of the most important. Indeed, a decline in CD28 expression may be responsible for age-related defects in T cell function (Vallejo et al., 1998). A major role of CD28 signalling in T cell activation is also thought to be the induction of other costimulatory molecules on T cells and their corresponding ligands on APCs, in order to promote subsequent costimulation and ensure effective T cell activation (Acuto and Michel, 2003). OX40, 4-1BB, CD40 ligand, inducible costimulatory molecule (ICOS), CD2, and lymphocyte function-associated antigen 1 (LFA-1) are simply a few of the known costimulatory molecules (Bierer and Hahn, 1993; Dustin, 2001; Richter and Burdach, 2004; Watts, 2005). CD2 and LFA-1 also function as adhesion molecules, while 4-1BB and OX40 are not expressed until late (~24 h) in the T cell activation process. The costimulatory molecules are a heterogenous group, and can serve multiple functions in addition to costimulation. Interestingly, some GPI-anchored proteins, such as thymus cell antigen (Thy)-1, CD48, and CD73 are also believed to provide costimulation to T cells (Haeryfar and Hoskin, 2004; Moran and Miceli, 1998; Resta and Thompson, 1997; Robinson et al., 1989; Stillwell and Bierer, 2001).

1.2.1.3. Signal 3

Before the importance of costimulatory molecules was appreciated in the context of T cell activation, cytokines, such as IL-1 and IL-6 were thought costimulate T cells (Ceuppens et al., 1988; Davis and Lipsky, 1986; Houssiau et al., 1989). Since this time, however, costimulation has become synonymous with

signal 2, and the contribution of cytokines to T cell activation has been more specifically associated with promoting the acquisition of effector function. Many of the recent studies on signal 3 have focused on the requirement for IL-12 in the activation of $CD8^+$ T cells. Mescher's group has been very active in deciphering the role of IL-12 as a third signal in $CD8^+$ T cell activation. They have shown that IL-12 is required for T cells to respond to MHC/antigen-coated microspheres plus IL-2 (Curtsinger et al., 1999), likely because IL-12 enhances the intensity and duration of CD25 expression (Valenzuela et al., 2002). Brief stimulation of the TCR by antigen (signal 1) induces expression of the genes required for cell division and effector function, while costimulation (signal 2) and IL-12 (signal 3) are required to sustain expression of these genes (Curtsinger et al., 2003). Signal 3 is also thought to be the determining factor in the induction of tolerance versus the development of effector function, because IL-12 appears to promote expression of granzyme B. Although a $CD8^+$ T cell activated in the absence of signal 3 can degranulate at the T cell – target cell interface, the target cell is unaffected due to the absence of granzyme B (Curtsinger et al., 2005).

Little is known about the requirements for signal 3 in $CD4^+$ T cell activation. IL-1 has been shown to promote the proliferation of $CD4^+$ T cells (Curtsinger et al., 1999), and even melatonin has been suggested to provide signal 3 to these cells. (Raghavendra et al., 2001). However, the same study also indicates that melatonin depresses the expression of B7 molecules on APCs, so the role of melatonin in T cell activation remains unclear. Many other cytokines may serve to generate signal 3. Blockade of Janus tyrosine kinase (JAK)3 is

additively immunosuppressive in combination with rapamycin treatment (Stepkowski et al., 2002). Rapamycin blocks IL-2 responsiveness, and is considered a signal 3 inhibitor. By this reasoning, all cytokines that signal through a JAK3-dependent mechanism may contribute to signal 3 in T cell activation.

Another theory on the mechanism of T cell activation is the danger theory. Here, T cells are not thought to discriminate between self and non-self, rather the requirements for T cell activation are more generally described as "danger", in that the T cell must sense a reason to become activated, such as the presence of an infection (Matzinger, 1994). It has been suggested that costimulation and inflammatory cytokines may in fact serve to indicate danger to a T cell (Janeway et al., 1996). To this end, the 3-signal model and danger theory might be one and the same, thus unifying the latter with more conventional notions of T cell activation.

1.2.2. Induction Of Anergy

T cell anergy was first described two decades ago by Jenkins and Schwartz (Jenkins and Schwartz, 1987), who showed that chemically-fixed APCs not only fail to activate T cells, but also induced a state of non-responsiveness that lasts for days. Today, antigenic stimulation of a T cell, or the provision of signal 1 alone, is known to induce anergy, a hyporesponsive state also seen in Treg cells. The true physiological function of anergy remains unclear, despite a great deal of interest into the mechanisms that govern this phenomenon. Clearly, in the absence of costimulation, TCR stimulation is less likely to induce sufficient cytokine expression due to the short half-life of cytokine mRNA. However, recent

evidence suggests that T cell anergy is an active process, and not simply a failure to adequately stimulate a T cell. For example, calcineurin-dependent ubiquitination and proteasomal degradation of PKCθ and PLCγ are associated with T cell anergy (Heissmeyer et al., 2004). Moreover, costimulation is required to overcome negative regulation of TCR signal transduction imposed by phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (Buckler et al., 2006). Recently, defects in TCR signalling were localised to LAT in anergic cells. Phosphorylation of TCR ζ chains and ZAP-70 after CD3/CD28 stimulation were normal in anergic $CD4^+$ T cells; however, phosphorylation of LAT and PLCγ were impaired (Hundt et al., 2006).

1.3. MAPKs

MAPKs are proline-directed serine/threonine protein kinases (English et al., 1999), aptly named for their activation following mitogenic stimulation of cells (Rossomando et al., 1989). MAPKs are evolutionarily conserved, and participate in myriad biological processes in organisms ranging from the budding yeast *Saccharomyces cerevisiae* to complex eukaryotic cells in humans. These important signalling molecules are grouped into three subfamilies, namely the ERK, p38 MAPK, and JNK subfamilies. The crystal structures have been solved for several MAPKs (Wilson et al., 1996; Xie et al., 1998; Zhang et al., 1994). All three subfamilies share a conserved catalytic core, and are activated in a cascade fashion (depicted in Figure 1.2) by phosphorylation of threonine (T) and tyrosine (Y) residues in a T-X-Y motif, where X is glutamic acid for ERKs, glycine for p38 MAPKs, and proline for JNKs (Cobb and Goldsmith, 1995). The ERK

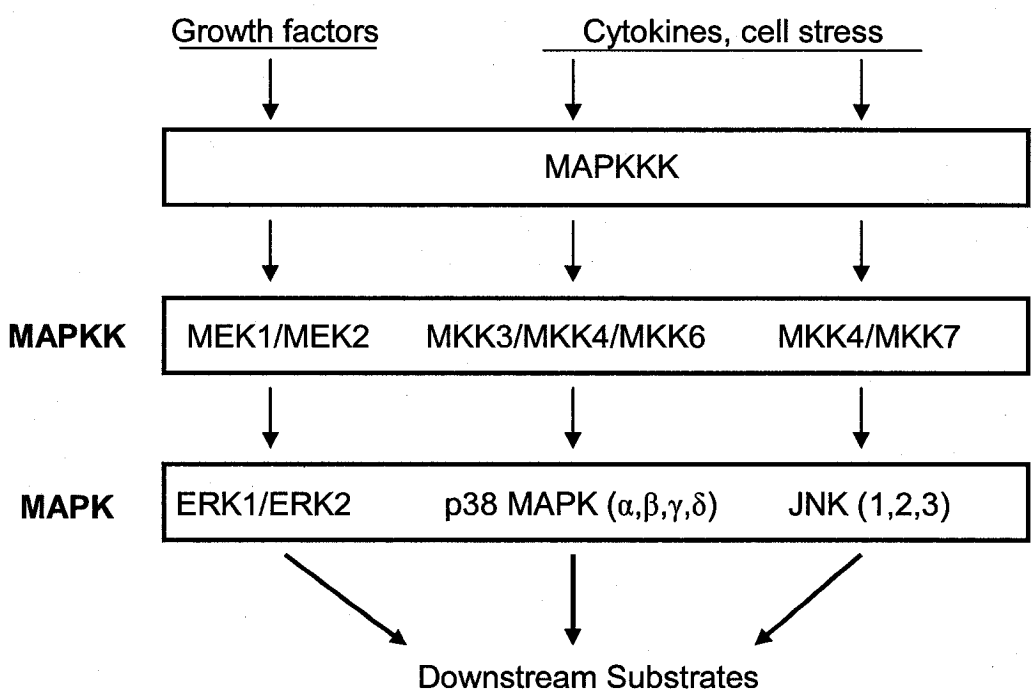


Figure 1.2. The Hierarchical Activation Of MAPKs. The MAPKs ERK1/2, p38 MAPK, and JNK are activated in a cascade fashion. Growth factors, cytokines, or cell stress activate MAPK kinase (MAPKK or MKK) kinases (MAPKKKs) to initiate signalling. MAPKKKs phosphorylate and activate MAPKKs, which activate the MAPKs. ERK1/2 are substrates of MAPK/ERK kinase (MEK)1/2, p38 MAPK is the substrate of MKK3/MKK4/MKK6, and JNK is the substrate of MKK4/MKK7. Stimulation of the MAPK signalling modules culminates in the activation of downstream substrates, such as transcription factors.

subfamily is associated with cell growth and survival, while p38 and JNK MAPKs are more commonly thought to participate in cell death and responses to stress (Pearson et al., 2001).

1.3.1. The ERK Subfamily

The ERK signalling cascade was the first signalling network discovered to be associated with receptor tyrosine kinase activation (Seger and Krebs, 1995). To date, eight ERKs have been described. ERK1 was found to phosphorylate microtubule-associated protein 2 in adipocytes in response to insulin stimulation (Ray and Sturgill, 1987). ERK2 and ERK3 were subsequently identified by screening a rat brain complementary DNA (cDNA) library with the same probe that was used to identify ERK1 (Boulton et al., 1991). ERK1 and ERK2 share 90% sequence identity, and are the members of the ERK subfamily most commonly studied in the context of MAPK signalling cascades. Like ERK1 and ERK2, ERK5 is also activated by a MAPK kinase (MAPKK) and is therefore considered to be a "conventional" MAPK. In fact, ERK5 is thought to constitute a distinct fourth MAPK subfamily of its own (Coulombe and Meloche, 2006) and is arguably the third class of stress-activated protein kinases (SAPKs). ERK3, ERK4, and ERK7 are "atypical" MAPKs, and much less is known about their biological functions. Because of its sequence homology with the p38 MAPK subfamily (Lechner et al., 1996), ERK6 is also known as p38γ, and will be discussed with the other p38 MAPK molecules below. ERK8, the most recently discovered ERK molecule, was identified in 2002 (Abe et al., 2002). Because a proto-oncogene activates ERK8 (Iavarone et al., 2006), this MAPK should also

be considered "atypical", and like the other atypical MAPKs, little is known about its biological function(s).

ERK1 and ERK2 are activated by the MAPKKs, MEK1 and 2. MEK1 and MEK2 can be activated by autophosphorylation (Deak and Templeton, 1997), downstream MAPKs (Matsuda et al., 1993), MEK Kinase (Lange-Carter et al., 1993), and the kinase Raf-1 (Kyriakis et al., 1992). The ERK1/2 signalling cascade is associated with cellular proliferation, differentiation, and survival, and is triggered by growth factors that activate receptor tyrosine kinases. TCR signalling can also activate the ERK1/2 cascade, through activation of Ras (Marais and Marshall, 1996), which mediates phosphorylation of Raf-1 (Marais et al., 1995). Stimulation of the ERK1/2 MAPK module culminates in the activation of many downstream targets. To date, roughly 160 substrates have been identified for the ERK signalling pathway (Yoon and Seger, 2006), including transcription factors, cytokine receptor-associated signalling molecules, and other MAPKs.

1.3.2. The p38 MAPK Subfamily

p38 MAPK was first identified in murine pre-B cells engineered to express human CD14. Lipopolysaccharide (LPS) stimulation of these cells induced the rapid phosphorylation of a 38 kDa molecule (Han et al., 1993). This molecule, later known to be p38α, is one of four splice variants that constitute the p38 MAPK subfamily, including p38β (Jiang et al., 1996), p38γ (Lechner et al., 1996; Li et al., 1996), and p38δ (Jiang et al., 1997). p38 MAPK activity is associated with cell death, and occurs in response to stress stimuli, such as ultraviolet (UV)

light, heat, osmotic shock, and inflammatory cytokines such as TNF-α and IL-1 (Freshney et al., 1994; Han et al., 1994; Raingeaud et al., 1995; Rouse et al., 1994). For this reason, p38 MAPK are also known as SAPKs. p38α and p38β MAPK are thought to be ubiquitously expressed, while expression of the γ and δ isoforms is tissue-specific and developmentally-regulated (Hale et al., 1999; Hu et al., 1999; Uddin et al., 2004; Zarubin and Han, 2005). However, some studies in disease models suggest that p38 MAPK expression may be even more complicated (Korb et al., 2006).

The two main MAPKKs known to activate p38 MAPK are MAPKK3 and MAPKK6 (or MKK3 and MKK6, respectively)(Enslen et al., 1998; Zarubin and Han, 2005). p38 MAPK is a substrate of MKK4 (Derijard et al., 1995), but this MAPKK also activates JNK (Cuenda, 2000). Upstream MAPKKKs in this cascade can be activated by a sequence of signalling events believed to involve G protein-coupled receptors, small G proteins, and other protein kinases (Yamauchi et al., 2001). The first downstream target of p38 MAPK (α isoform) is MAPK-activated protein kinase (MAPKAPK)2 (Freshney et al., 1994; Rouse et al., 1994). One of the targets of MAPKAPK2 is tristetraprolin (Mahtani et al., 2001). Because tristetraprolin is known to modulate mRNA stability, the discovery that this substrate of p38 MAPK phosphorylates tristetraprolin suggested that the p38 MAPK cascade may be involved in regulating gene expression. Indeed, in addition to activating transcription factors such as activating transcription factor (ATF)-1, -2, and 6, ELK1, and NFAT, to name a few, p38 MAPK regulates mRNA stability via adenosine-uridine (AU)-rich element-binding proteins, and may

promote initiation of translation by activating proteins like MNK1, which phosphorylates eukaryotic initiation factor-4E (Wang et al., 1998).

One of the current controversies surrounding the function of p38 MAPK is its involvement in apoptosis. For years, this kinase has been described as a pro-apoptotic signalling molecule, because triggering apoptosis with such classical stimuli as Fas ligation also induces p38 MAPK phosphorylation (Juo et al., 1997). Evidence has been presented in recent years that suggests p38 MAPK may also carry out an anti-apoptotic role, likely in a cell type- or apoptosis stimulus-specific manner. For example, inhibition of p38 MAPK enhances apoptosis induced by okadaic acid treatment of human T leukaemia cells (Boudreau et al., 2007), possibly because p38 MAPK can phosphorylate and thereby deactivate caspase-3 (Alvarado-Kristensson and Andersson, 2005), which plays a critical role in the execution of apoptosis. Studies on the role of p38 MAPK in myriad biological functions are ongoing, and continue to define the activity of this important signalling molecule.

1.3.3. The JNK Subfamily

JNK was first identified in the livers of rats that had been treated with cycloheximide (Kyriakis and Avruch, 1990) and, like p38 MAPK, is a SAPK. JNK is activated by inflammatory cytokines and environmental stresses (Ip and Davis, 1998) and is involved in several different physiological and biological processes. Three genes encode the JNK1, JNK2, and JNK3 proteins; however, alternative RNA splicing generates ten isoforms of JNK (Gupta et al., 1996). The genes for

JNK1 and JNK2 are found in all tissues, whereas expression of the JNK3 gene is tissue-specific.

MKK4 and MKK7 are upstream activators of JNK in this MAPK cascade. Interestingly, activation of MKK4 is predominantly observed in response to cellular stress, while inflammatory cytokines are the main activating stimulus for MKK7. Although MKK4 is also an activating kinase of p38 MAPK, MKK4 and MKK7 may activate JNK in a cooperative manner, as MKK4 appears to phosphorylate the T residue in the T-X-Y motif, while MKK7 phosphorylates the Y residue (Lawler et al., 1998). It is not clear if there are upstream kinases that act as dedicated activators of MKK4 and MKK7, though members of the activator of S-phase kinase (ASK), mixed-lineage protein kinase (MLK), and MEK kinase (MEKK) groups of MAPKKKs, for which there are many substrates, are believed to be involved in activation of the JNK signalling module (Davis, 2000).

As its name implies, JNK plays an active role in modulating the transcriptional activity of c-Jun (Pulverer et al., 1991; Smeal et al., 1991). Members of the Jun and Fos gene families physically associate to form the dimeric transcription factor complex AP-1 (Curran and Franza, 1988). JNK also activates the AP-1 proteins JunB, JunD, and ATF-2 (Ip and Davis, 1998), and mediates AP-1 activation in response to environmental stress and certain cytokines, although other stimuli can induce AP-1 activity in a JNK-independent fashion (Yang et al., 1997). Like p38 MAPK, JNK is involved in apoptosis (Tournier et al., 2000). However, it seems that only sustained JNK activity mediates apoptosis (Chen and Tan, 2000). Indeed, not all stimuli that activate

JNK also induce apoptosis (Liu et al., 1996). Enhanced cell death is observed in the forebrain of JNK-deficient mice (Sabapathy et al., 1999b), suggesting that, like p38 MAPK, JNK may also play dual roles in the regulation of apoptosis.

1.3.4. MAPKs In Health And Disease

Despite their involvement in critical developmental processes, not all of the MAPKs are essential for life. Knocking out ERK2 in mice results in embryonic lethality (Hatano et al., 2003; Saba-El-Leil et al., 2003), as does MEK1 deficiency (Giroux et al., 1999). Interestingly, MEK2-deficient mice develop normally, suggesting that MEK1 and MEK2 carry out different roles in development, or that the function of MEK2 is redundant (Belanger et al., 2003). Likewise, absence of function of ERK1 is not embryonic lethal (Mazzucchelli et al., 2002), which indicates that ERK1 and ERK2 carry out different functions, or that ERK2 can carry out ERK1-like function in its absence. ERK5 is essential to embryonic development (Yan et al., 2003), but no other ERK subfamily members have been examined in knockout studies. p38α knockouts are not viable (Allen et al., 2000), but null mutations in the β, γ, or δ isoforms of p38 MAPK have no dramatic effect on embryonic development (Beardmore et al., 2005; Sabio et al., 2005). Likewise, mutations of the genes for JNK1, JNK2, or JNK3 are not embryonic lethal in mice (Kuan et al., 1999). To date, there is no clear association between null mutations in the ERK, p38, or JNK MAPKs and human disease. However, owing to the important role that these signalling molecules play in the regulation of cell growth and apoptosis, MAPKs have been implicated in a number of medical conditions.

Parkinson's disease is a neurodegenerative disorder characterised by the loss of dopaminergic neurons (Chen and Le, 2006), and Alzheimer's disease is also caused by neuronal death (LeBlanc, 2005). Although conventionally associated with cell survival, ERK1/2 is believed to play a role in promoting cell death (Zhuang and Schnellmann, 2006), specifically in the context of neuronal apoptosis (Cheung and Slack, 2004; Subramaniam et al., 2004). p38 MAPK is activated in 6-hydroxydopamine-induced neuronal death, which can be attenuated by treatment with a p38 MAPK inhibitor or expression of a dominant negative form of p38 MAPK (Choi et al., 2004). Also, p38 MAPK activation has been observed in the brains of Alzheimer's patients, but is almost absent in normal controls (Hensley et al., 1999). Inhibition of JNK is protective for dopaminergic neurons *in vitro*, as well as in animal models of Parkinson's disease (Wang et al., 2004). Therefore, pharmacological agents that ablate the activity of MAPKs might be useful in preventing the progression of these devastating neurological conditions.

There is a growing literature on the role of MAPKs in cancer and tumour development. In fact, despite the controversy over how MAPKs might influence cell death compared rather than cell growth, a number of small molecule inhibitors that block MAPK activity are in clinical trials for cancer therapy. Studies on MEK1/2 inhibitors have progressed to Phase II clinical trials for the treatment of colorectal, non-small cell lung, breast, and pancreatic cancer (Rinehart et al., 2004). The JNK inhibitor CC-401 has supposedly also been brought to Phase II in clinical trials for the treatment of acute myelogenous leukaemia. MAPKs are

also critically involved in the pathology of inflammatory bowel disease, rheumatoid arthritis, and psoriasis, as p38 MAPK inhibitors have been assessed in the treatment of these conditions in clinical trials.

1.3.5. The Controversial Role Of MAPKs In T Cell Activation

T cell activation is a complex biochemical process that is governed by numerous signalling cascades. The role of MAPKs in T cell activation has been the object of investigation for many researchers since the early 1990s. Indeed, a great deal is known about the role of ERK1/2, p38 MAPK, and JNK in the differentiation and activation of T cells. These signalling molecules are all critical to proper T cell function, and have each been implicated in the integration of signal 1 and signal 2 during T cell activation (Chen et al., 1997; Su et al., 1994; Zhang et al., 1999). For the sake of brevity, and because it was a focus of my research, the roles of MAPKs in T cell activation described in this section will be limited to IL-2 expression, IL-2R expression, IL-2R signalling, and T cell proliferation.

1.3.5.1. The Role Of ERK1/2 In T Cell Activation

The ERK1/2 MAPK module is activated in the TCR signalling pathway (Izquierdo et al., 1993; Su et al., 1994). There is evidence that ERK activation is mediated by the CD3 δ chain of the TCR signalling complex during TCR signal transduction (Delgado et al., 2000). However, because ERK activation, which is absent in CD3 $\delta^{-/-}$ thymocytes, can be restored by the expression of CD3 δ chains that lack a cytoplasmic domain, the CD3 complex is likely not involved in the direct activation of ERK. CD3 δ may promote ERK activation by facilitating

clustering and phosphorylation of TCR ζ chains within lipid rafts, ultimately leading to Ras activation, since LAT phosphorylation is also impaired in the CD3 $\delta^{-/-}$ thymocytes (Delgado et al., 2000). An important role for ERK in TCR signalling is consistent with the impaired development of SP $CD3^{+}$ thymocytes in mutant mice engineered to lack functional Ras and MEK (Alberola-Ila et al., 1996). However, ERK activity has also been detected in T cells that have not been stimulated through the TCR (Roose et al., 2003). In this case, basal ERK activity is dependent on LAT and SLP-76 and is associated with repression of recombination activating genes (RAGs), whose function are no longer required.

In Treg cells, which are hyporesponsive to TCR/CD28 stimulation, impaired ERK activation is associated with decreased expression of cyclins and elevated expression of kip1, which is a cyclin-dependent kinase inhibitor (Li et al., 2005). This finding is consistent with the ability of the MEK1 inhibitor PD98059 to induce a decrease in cyclin expression levels in smooth muscle (Ravenhall et al., 2000). Alternatively, ERK signalling in T cells has also be associated with the inhibition of cyclin activity, resulting in the inability to respond to IL-2 (Chen et al., 1999). Treg cell hyporesponsiveness may also be attributable to low IL-2 production, due to impaired ERK activation (Hickman et al., 2006). Also, this MAPK has been shown to negatively regulate JAK-Signal transducer and activator of transcription (STAT) signalling (Krasilnikov et al., 2003), and may therefore modulate the transduction of signals generated by the IL-2R.

Evidence for the role of ERK in IL-2 production by T cells is also unclear. A decrease in TCR-mediated ERK activation has been observed in elderly

people (Whisler et al., 1996), and is associated with impaired IL-2 expression (Liu et al., 1997). Likewise, inhibition of ERK activity with PD98059 impairs IL-2 production by activated T cells (Koike et al., 2003; Matsuda et al., 1998), and ERK1/2 mediates enhanced IL-2 production caused by p38 MAPK inhibition in human T cells (Kogkopoulou et al., 2006). However, ERK1-deficient T cells maintain the ability to produce IL-2 after stimulation (Nekrasova et al., 2005), which suggests that ERK2 may be the dominant ERK molecule involved in IL-2 expression, or that ERK2 may be able to compensate for ERK1 function when ERK1 activity is absent or impaired. Lastly, it is unclear how ERK1/2 is involved in CD25 expression. Bitegye et al. report that inhibition of MEK1/2 with PD98059 causes upregulation or downregulation of CD25 expression induced by immobilised anti-CD3 monoclonal antibody (mAb) in different T cell clones (Bitegye et al., 2002). ERK is also required for the cytotoxic function of most $CD8^+$ T cells (Lilic et al., 1999), and thus clearly plays an important, albeit unclear, role in T cell activation.

1.3.5.2. The Role Of p38 MAPK In T Cell Activation

p38 MAPK is activated by synergistic TCR and CD28 signals in thymocytes and splenic T cells (Zhang et al., 1999). Recently, non-canonical activation of p38 MAPK was described, in a study that identified p38 MAPK as a substrate for lck, fyn, and ZAP-70 (Salvador et al., 2005), demonstrating a non-cascade mechanism of p38 MAPK activation in T cells. Like ERK, the role of p38 MAPK in T cell activation is complex. Several groups have reported impaired proliferation of T cells following p38 MAPK inhibition (Haeryfar and Hoskin, 2001;

Ward et al., 1997; Zhang et al., 1999). Consistent with these findings, Perchonock et al. have observed enhanced T cell proliferation associated with constitutive p38 MAPK activation (Perchonock et al., 2006). However, p38 MAPK-independent proliferation of $CD4^+$ T cells has also been demonstrated (Rincon et al., 1998).

Blockade of p38 MAPK activity with SB203580 impairs CD3/CD28 stimulation-induced production of IL-2 by T cells (Ward et al., 1997; Wu et al., 2003), as well as IL-2 gene transcription in Jurkat T cells activated with ionophore plus phorbol ester (Matsuda et al., 1998). Even atypical stress-induced IL-2 production appears to be p38 MAPK-dependent in T cells (Loomis et al., 2003; Yu et al., 2004). However, an opposite role for p38 MAPK has also been reported. Inhibition of p38 MAPK can result in elevated IL-2 production by CD3/CD28-stimulated T cells (Kogkopoulou et al., 2006), and still others suggest that p38 MAPK is not involved in IL-2 production at all (Rincon et al., 1998). Since IL-2 is transcribed by NFAT (Rao et al., 1997), some of this controversy may arise from the dual ability of p38 MAPK to both promote and oppose NFAT transcriptional activity (Gomez del Arco et al., 2000; Wu et al., 2003). A more complete understanding of the mechanism that dictates the effect of p38 MAPK on the regulation of NFAT transcriptional activity may clarify the role of p38 MAPK in IL-2 production.

The influence of p38 MAPK in CD25 expression is also unclear. For example, in T cells that do not express adaptor in lymphocytes of unknown function, X (ALX), constitutive p38 MAPK activity is associated with increased

CD25 expression, the latter of which is blocked by pharmacological inhibition of p38 MAPK (Perchonock et al., 2006). Yet pharmacological inhibition of p38 MAPK in T cells also reportedly enhances CD25 expression (Bitegye et al., 2002). T cell death and activation appear to be mediated by p38 MAPK in a T cell subset-dependent manner. p38 activity is linked to $CD8^+$ T cell death, which cannot be overcome by the addition of exogenous IL-2 (Merritt et al., 2000). Also, cytokine production is not affected by a dominant negative p38 MAPK mutation in Th2 cells, whereas IFNγ production is impaired in Th1 transgenic mice that lack functional p38 MAPK (Rincon et al., 1998).

1.3.5.3. The Role Of JNK In T Cell Activation

JNK expression is induced by T cell activation, and an early study in Jurkat T cells demonstrated rapid JNK activation following CD3/CD28 stimulation (Su et al., 1994). However, JNK activation is different in primary cells. TCR signalling alone is sufficient to induce expression of JNK in primary T cells, but this MAPK is only activated if costimulation is also provided (Weiss et al., 2000). Here, the authors demonstrate slow kinetics of JNK activation in primary T cells, which is dependent on an extensive time interval (24h - 48h) of CD3/CD28 stimulation. However, Rivas et al. demonstrate that JNK activation can occur in the absence of costimulation, as long as CD3 stimulation is strong enough (Rivas et al., 2001). JNK activation is thought to be Vav-dependent, and mediated by the Rho-like GTPases Rac1 and Rac2 (Crespo et al., 1996; Li et al., 2000). Although JNK is clearly stimulated following T cell activation, it is unclear how JNK contributes to this process.

IL-2 production is not dramatically altered in JNK1- or JNK2-deficient T cells (Dong et al., 1998; Yang et al., 1998). However, the involvement of JNK in IL-2 expression may be dependent on the strength of the T cell-activating stimulus. Splenic T cells from $JNK1^{-/-}$ mice produce less IL-2 than WT controls in response to so-called suboptimal (1 µg/ml anti-CD3 mAb plus 0.1 µg/ml anti-CD28 mAb) T cell stimulation, whereas optimal (10 µg/ml anti-CD3 mAb plus 0.1 µg/ml anti-CD28 mAb) T cell stimulation results in identical IL-2 production by both mutant and WT cells (Sabapathy et al., 2001). JNK1-deficient T cells do not proliferate as robustly as WT cells in response to CD3/CD28 stimulation; however, proliferation is restored to the WT level by the addition of exogenous IL-2 to the mutant cells (Sabapathy et al., 2001). This suggests that JNK1 predominantly modulates IL-2 expression, but not IL-2R signalling or cell cycle progression. JNK2-deficient T cells have also been shown to exhibit impaired IL-2 production compared to WT cells, which can be improved with stronger CD3/CD28 stimulation, but never equals that of WT cells (Sabapathy et al., 1999a). Interestingly, Conze et al. report that JNK2 acts as a negative regulator of T cell activation since JNK2-deficient T cells are hyperresponsive to CD3/CD28 stimulation, and undergo more proliferation and produce greater amounts of IL-2 than normal T cells (Conze et al., 2002).

Expression of CD25 by $JNK1^{-/-}$ T cells in response to CD3/CD28 stimulation is impaired compared to WT cells (Conze et al., 2002). Pharmacological inhibition of JNK1 and JNK2 with the SP600125 compound also results in reduced CD25 expression (Conze et al., 2002), and CD25 expression

is associated with JNK activity in thymocytes (Na et al., 1999). JNK is also believed to play a roll in regulating cell cycle in T lymphocytes, since the cyclin-dependent kinase inhibitor p21 associates with JNK1 (Patel et al., 1998). Here, the authors report that p21 dissociation from JNK1 correlates with JNK activation and entry into S-phase, suggesting that JNK activity promotes cell growth.

Collectively, the literature concerning the specific roles of MAPKs in different aspects of T cell activation reaches no consensus. ERK, p38 MAPK, and JNK are all activated following T cell stimulation, and have been suggested to function as convergence points for the TCR and costimulatory signalling pathways. Figure 1.3 summarises the information presented here on the role of MAPKs in T cell activation. Though important, the functions of these MAPKs in T cell activation remain unclear. However, the inconsistencies in the literature concerning the role of MAPKs in T cell activation can largely be accounted for by differences in experimental methods and cell types examined.

1.4. GPI-Anchored Proteins

Many proteins are associated with the cell surface by GPI anchors. These glycolipids are posttranslationally linked to the carboxy terminus of their target proteins before they are exported to the surface. The GPI anchors are synthesised in a multi-step process at the membrane of the endoplasmic reticulum. Newly made GPI anchors are transferred onto their target proteins, which are shuttled to the cell membrane via the secretory pathway (Kinoshita et al., 1997). More than 60 have been identified (Kinoshita et al., 1995), including adhesion molecules, ectoenzymes, complement regulators, and cell surface

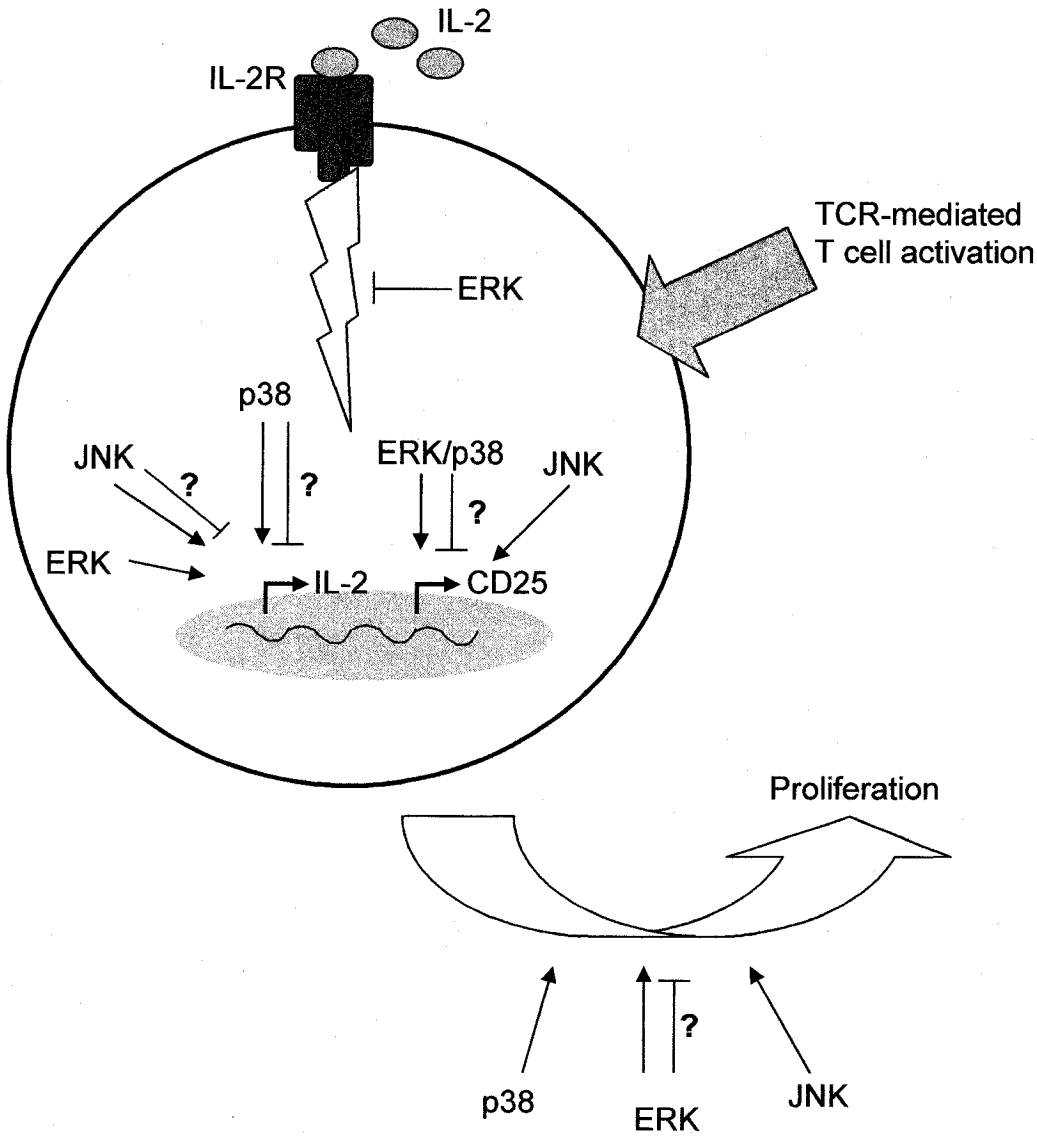


Figure 1.3. The Role Of MAPKs In T Cell Activation. Conflicting evidence suggests that ERK promotes or inhibits CD25 expression, and negatively regulates JAK/STAT signalling in the IL-2R pathway. IL-2 production is dependent on ERK2, and proliferation may be enhanced or impaired by ERK signalling due to its effects on cyclin expression. Likewise, it is unclear how p38 MAPK (p38) affects CD25 expression or IL-2 production. p38 reportedly promotes T cell proliferation, but mediates $CD8^+$ T cell death. Also, there is evidence to suggest that proliferation and IL-2 production are p38-independent. JNK promotes T cell proliferation, and CD25 expression is JNK1-dependent. The effect of JNK on IL-2 production may be dependent on the strength of the T cell-activating stimulus; however, JNK2 has also been shown to negatively regulate IL-2 expression.

receptors. The diverse list of important functions ascribed to GPI-anchored proteins is impressive, considering that the GPI anchor merely tethers these proteins to the outer leaflet of the cell membrane. Regardless, GPI-anchored proteins clearly play an important physiological role, as mutations that ablate the production of these proteins are embryonic lethal (Kawagoe et al., 1996). GPI-anchored proteins are commonly present in lipid rafts, which are membrane microdomains that are critical for the process of signal transduction (Shaw, 2006). Relatively little is known about the signalling properties of GPI-anchored proteins. Indeed, the absence of a transmembrane domain complicates the issue of how GPI-anchored protein signalling occurs, though the theories on GPI-anchored protein signal transduction involve physical association with transmembrane proteins, as well as lipid rafts and their associated signalling molecules (Horejsi et al., 1998). Despite their abundant and ubiquitous expression, surprisingly few medical conditions are associated with GPI-anchored proteins, or the lack thereof.

1.4.1. GPI-Anchored Proteins In Health And Disease

Certainly the most media-worthy GPI-anchored protein-associated human disease in recent years is the degenerative brain disorder Creutzfeldt-Jakob Disease, in large part due to the attention received by its notorious bovine counterpart, Mad Cow Disease. This condition is due to the presence of aberrant GPI-anchored prion proteins, which precipitate the formation of fatal amyloid plaques in the brain. To date, only one human disease is clearly based on a GPI-anchored protein deficiency. Paroxysmal nocturnal haemoglobinuria (PNH) is a

clonal haematopoietic disorder (Kawagoe et al., 1996) that presents clinically as intravascular haemolysis and can also present subclinically, or in association with other bone marrow disorders (Parker et al., 2005). The genetic basis for this disorder is mutation of the phosphatidylinositol glycan class A (PIG-A) gene. Because the PIG-A gene maps to the X chromosome (Xp22), PNH is considered to be X-linked, though it is not a heritable condition. PNH always arises from somatic PIG-A gene mutations in haematopoietic stem cells that are clonally propagated. The PIG-A gene encodes a protein that plays a critical role in GPI anchor biosynthesis (Kinoshita et al., 1997), thus at the cellular level PNH is a disease characterised by the absence of GPI-anchored proteins. CD55 (decay accelerating factor) and CD59 (membrane attack complex inhibitor) are GPI-anchored proteins that regulate the activity of complement. In the absence of CD59 specifically, erythrocytes are susceptible to complement-mediated membrane lysis (Meri et al., 1990). Haemoglobin from the lysed red blood cells is excreted in the urine, which is characteristically dark red in PNH patients. However, coloured urine is merely one clinical sign for this condition. Excessive clotting is a major complication that arises in PNH, and can be fatal (Parker et al., 2005).

It is estimated that 5% to 15% of PNH patients develop leukaemia, and these cancer cells are believed to arise from clonal expansion of cells that harbour the PNH mutation (Harris et al., 1999). Because PNH develops through clonal expansion of only a limited number of progenitor cells, the PIG-A gene mutation might also impart a growth advantage to PNH cells. Preferential growth

of PNH cells has been attributed to an inherent loss of sensitivity to apoptosis since PNH cells do not exhibit a greater proliferative capacity than normal cells (Chen et al., 2002). The absence of GPI-anchored proteins and mutations in the PIG-A gene have been examined in the context of ionising radiation-, serum starvation-, and Fas-induced apoptosis (Brodsky et al., 1997; Kulkarni and Bessler, 2002; Ware et al., 1998), and has been ruled out as a cause for an inherent resistance to apoptosis in PNH cells. However, GPI-anchored protein stimulation has been associated with the induction of cell death as well as hyporesponsiveness to mitogenic stimulation (Cashman et al., 1990; Chappel et al., 1996; Flood et al., 1985; Fujita et al., 1997; Haque et al., 1990; Hueber et al., 1994; Lue et al., 1991; Tomonari, 1988). Therefore, GPI-anchored proteins may play a role in the negative regulation of cellular proliferation.

Studies on GPI-deficient T cells demonstrate dramatically impaired T cell activation and suggest the involvement of GPI-anchored proteins in this process through the ability of GPI-anchored proteins to promote or amplify TCR signalling. Indeed, activation of the src kinases lck and fyn is impaired following TCR stimulation in GPI-negative T cell lines (Romagnoli and Bron, 1997), and lck activation is defective in CD3-stimulated T cells from PNH patients (Romagnoli and Bron, 1999). However, stimulation of T cells with mitogenic mAbs specific for GPI-anchored proteins plus costimulatory molecules, in the absence of TCR triggering, demonstrates that these molecules can also transmit *bona fide* signals that trigger T cell activation.

Several studies report that mAb-mediated stimulation of GPI-anchored proteins on T cells results in T cell activation. Mobilisation of intracellular Ca^{2+} can be induced by antibody crosslinking of Thy-1, Ly-6, CD48, and CD59, and there is evidence to support the role of Qa-2, CD59, Thy-1, and prions in the provision of signal 1 for T cell activation (Haeryfar et al., 2003; Loertscher and Lavery, 2002). Interestingly, the GPI-anchored proteins CD73, CD48, and Thy-1 can costimulate CD3 signals (Haeryfar and Hoskin, 2004; Moran and Miceli, 1998; Resta and Thompson, 1997; Robinson et al., 1989). It remains unclear how GPI-anchored proteins can participate in signalling at all, since these proteins do not possess a mechanism to communicate with the cytosol. However, this process, which certainly involves src kinases, may be dependent on the GPI anchor itself since Qa-2 loses the ability to activate T cells when engineered to express a transmembrane domain (Robinson et al., 1989). Likewise, H-2 molecules, which normally span the membrane, acquire the ability to activate T cells when linked to the cell membrane by a GPI anchor (Robinson et al., 1989). Although important, the GPI moiety itself is not sufficient to confer signalling ability to a protein, as stimulation of the GPI-anchored protein chorioembryonic antigen plus CD28 does not result in T cell activation (Loertscher and Lavery, 2002). Moreover, CD73 is at least one example of a GPI-anchored protein that can trigger biochemical signals independently of its GPI-anchor (Resta and Thompson, 1997).

It remains unclear whether the generation of GPI-associated signals is directly related to the GPI-anchored proteins themselves, or their influence on

lipid raft dynamics. Indeed, sphingomyelinase$^{-/-}$ T cells, which do not possess membrane rafts due to low cholesterol content, exhibit impaired T cell activation (Nix and Stoffel, 2000). Despite the numerous studies on GPI-anchored protein signalling, many issues remain unresolved, and it is unclear how this process can mediate T cell activation. Several theories on how the GPI-anchored protein Thy-1 can induce T cell activation, which is a topic of my research, have been proposed and will be discussed in the next section.

1.5. Thy-1

Thy-1 is a GPI-anchored protein that has long served as a T cell marker in mice. However, since its discovery, many other functions have been ascribed to this molecule, ranging from induction of apoptosis to T cell activation (Haeryfar and Hoskin, 2004). The role of Thy-1 in T cell activation has been the subject of numerous studies and fodder for much debate. Thy-1 is considered by many to be the prototypical GPI-anchored protein, and may very well serve as the ideal model for studies on GPI-anchored protein function. However, as outlined below, despite more than four decades of Thy-1 research, many issues surrounding the role of T cell-associated Thy-1 remain to be resolved.

1.5.1. Thy-1: A Brief History

The discovery of Thy-1 was reported in 1963 by Reif, who found that alloantiserum raised in MHC-compatible C3HeB/Fe mice against AKR mouse thymocytes could lyse those thymocytes (Reif, 1963). The two allelic forms of Thy-1, namely Thy-1.1 and Thy-1.2, account for the alloreactivity of C3HeB/Fe serum against AKR thymocytes. Thy-1 was originally considered a differentiation

marker whose expression was restricted mostly to the brain and thymus (Reif and Allen, 1964). Also referred to by the greek symbol θ, Thy-1 later was accepted as a marker for lymphocytes derived from the thymus (Raff, 1971), though Thy-1 is also present on the fibroblasts and brain cells of all species in which Thy-1 expression has been examined (Pont, 1987). Thy-2 and Thy-3 were later described, and differ from Thy-1 in terms of molecular weight (MW), expression, and, in the case of Thy-2, function. Thy-2 is a 150 kDa alloantigen present at low levels on thymocytes and brain tissue but is not expressed in lymph nodes, and may be involved in antigen presentation (Siadak and Nowinski, 1981). Thy-3 is a 53 kDa glycoprotein that is expressed on DP thymocytes and T cells in a Thy-1-dependent manner. Thy-3 physically associates with Thy-1, and is believed to either function in a similar manner to Thy-1, or to promote Thy-1 function (Aurrand-Lions et al., 1997).

1.5.2. Thy-1 Expression And Tissue Distribution

The Thy-1 gene in mice encodes a 25-kDa protein, the sequence of which contains 111 or 112 amino acids (Pont, 1987). Variations in the observed molecular mass of Thy-1 is due to glycoslation. In fact, nearly a third of the molecular mass of Thy-1 is contributed by carbohydrate moieties. The two allelic variations of Thy-1, Thy-1.1 and Thy-1.2, vary solely by one amino acid at position 89. An arginine residue occupies this position in Thy-1.1, while glutamic acid is present at this site in Thy-1.2 (Isobe et al., 1985). All mouse strains are Thy-1.2^{+}, except the AKR/J and PL strains, which express Thy-1.1. Orthologs of Thy-1 have been identified in multiple species, such as squid, frogs, chickens,

mice, rats, dogs, and humans (Pont, 1987; Williams and Gagnon, 1982). In mice, Thy-1 is expressed on brain cells, fibroblasts, thymocytes, peripheral T cells, myoblasts, epidermal cells and keratinocytes (Pont, 1987; Reif and Allen, 1964). However, in humans, Thy-1 can only be found on endothelial cells, bone marrow cells, haemopoietic cells, and early thymocytes (Dalchau et al., 1989; Mason et al., 1996; Ritter et al., 1983).

Although antisera against Thy-1 were originally employed as a tool for differentiating between B cells and T cells, Thy-1 expression is also now known to be inducible on B cells (Louie et al., 1995). In fact, in the lymphoid compartment alone, many cell types can express Thy-1. For example, expression of Thy-1 is associated, at least indirectly, with natural killer (NK) cell and mast cell function (Draberova et al., 1996; Shah et al., 1986). Splenic (Ishii et al., 2005), bone marrow-derived (S. Furlong, personal communication), and dermal DCs (Tamaki et al., 1996), as well as dendritic epidermal cells (Takashima et al., 1988) also express Thy-1. The fact that thymocyte development is not impaired in Thy-1-deficient mice (Page et al., 1997) is a surprising observation, since immunity is clearly impaired in these animals (Beissert et al., 1998). However, in the absence of a T cell deficiency, altered function in the other aforementioned Thy-1-expressing immune cells could account for the immunological abnormalities caused by the absence of Thy-1.

1.5.3. Thy-1-Mediated T Cell Activation

Serum from rabbits immunised with mouse brain, as well as anti-Thy-1 mAbs, were found to induce the proliferation of T cells more than 15 years after

the initial discovery of Thy-1 (Gunter et al., 1984; Norcross and Smith, 1979), indicating the involvement of Thy-1 in the process of T cell activation. However, both stimulatory and inhibitory roles have been proposed for Thy-1 in this process. For example, thymocytes from Thy-1-deficient mice are hyperresponsive to TCR stimulation (Hueber et al., 1997), which suggests that Thy-1 might negatively regulate TCR signalling. The majority of studies on Thy-1, however, support a mitogenic role for Thy-1 in T cell activation.

mAb-induced Thy-1 signalling in T cells induces proliferation, IL-2 expression, and expression of the IL-2R (Gunter et al., 1984; Kroczek et al., 1986). However, purified T cells do not respond to anti-Thy-1 mAb treatment (Norcross and Smith, 1979). Because the presence of B cells promoted mAb-mediated Thy-1-induced T cell activation, the anti-Thy-1 antibodies may have required Fc receptor crosslinking or costimulation triggered by B cell surface molecules. Interactions between the anti-Thy-1 antibodies and the B cells may be important, since B cells failed to improve the response of T cells to Thy-1-specific $F(ab')_2$ fragments (Norcross and Smith, 1979), and some anti-Thy-1 antibodies are only mitogenic to T cells when immobilised or crosslinked (Marmor and Julius, 2000; Pont, 1987). However, the requirement for costimulation explains the inability of anti-Thy-1 antibodies to activate T cells in the absence of B cells. We have shown that anti-Thy-1 mAb-induced T cell activation is dependent on costimulation provided by CD28 (Haeryfar et al., 2003). Signal transduction via Thy-1 and CD28 are sufficient to activate T cells, since anti-Thy-1 and anti-CD28 mAb-coated plastic beads trigger T cell proliferation (Haeryfar et al., 2003).

Because CD28 irrefutably delivers signal 2, bead-induced T cell activation in our lab clearly establishes that Thy-1 stimulation generates signal 1.

Functional studies also support the ability of Thy-1 to provide signal 1. Kojima et al. report that, like TCR signalling, Thy-1 stimulation can trigger Fas/FasL-mediated T cell cytotoxicity (Kojima et al., 2000). Perforin-mediated cytotoxicity, which is induced by normal T cell stimulation, is not achieved by triggering Thy-1 or with low-level antigen stimulation of the TCR (Haeryfar et al., 2003; Kojima et al., 2000). The authors conclude that although Thy-1 signalling is likely functionally analogous to a TCR-derived signal 1, Thy-1 signals only provide relatively low levels of stimulation to the T cell.

Interestingly, we have also shown that treatment with a blocking anti-Thy-1 mAb during anti-CD3 mAb-induced stimulation impairs IL-2 production, IL-2R expression, and T cell effector function (Haeryfar et al., 2005). These findings suggest that Thy-1 provides costimulation in the context of TCR stimulation. To this end, beads coated with anti-Thy-1 mAbs enhance the ability of bead-immobilised anti-CD3 and anti-CD28 mAbs to trigger T cell activation (Haeryfar et al., 2005; Haeryfar and Hoskin, 2004). Indeed, human CD7, which is accepted as the functional homolog of murine Thy-1 based on gene sequence and tissue distribution (Firer et al., 1995; Schanberg et al., 1991), can provide costimulation for T cell activation (Stillwell and Bierer, 2001). Currently, it is believed that Thy-1 provides signal 1 in the presence of strong costimulation and in the absence of TCR stimulation, but may also costimulate TCR signals under some circumstances (Haeryfar and Hoskin, 2004).

1.5.4. Thy-1 Signal Transduction

Like all GPI-anchored proteins, it is unclear how Thy-1 can trigger intracellular signalling events. Despite the absence of any obvious signalling motifs or even a transmembrane domain, antibody-mediated Thy-1 stimulation results in Ca^{2+} flux (Kojima et al., 2000) and Thy-1 physically associates with fyn as well as G proteins (Thomas and Samelson, 1992), which may mediate Thy-1 signals. Thy-1 immunoprecipitates with CD45 (Volarevic et al., 1990), and p100 (Lehuen et al., 1995), both of which are transmembrane proteins that may act as conduits through which Thy-1 could signal. The TCR/CD3 complex, which also consists of transmembrane proteins and interacts with Thy-1 (Gunter et al., 1987), likely plays an integral role in Thy-1 signal transduction. Indeed, the capacity of Thy-1 to activate T cells is compromised in the absence of the TCR/CD3 complex. Human Jurkat T cells transfected to express murine Thy-1.2 still mobilise intracellular Ca^{2+} but lose the ability to produce IL-2 in response to Thy-1 stimulation when a functional TCR/CD3 complex is not expressed (Gunter et al., 1987). CD59 signalling is also impaired when the TCR/CD3 complex is not present (Deckert et al., 1995). These data are consistent with the theory that TCR signals can be triggered by Thy-1 stimulation, simply by virtue of the abundant expression of Thy-1 on the cell surface (Killeen, 1997). Because Thy-1 inhabits lipid rafts, Thy-1 stimulation may also trigger intracellular signalling through the mobilisation of rafts, thereby concentrating or juxtaposing key signalling molecules.

Our lab has employed selective pharmacological inhibitors to identify signalling molecules involved in Thy-1-induced T cell activation. This study revealed that Thy-1-induced T cell activation in the context of costimulation provided by APCs is dependent on tyrosine kinases, ERK1/2, p38 MAPK, PKC, PI3K, and calcineurin (Haeryfar and Hoskin, 2001). As outlined before, these molecules are all involved in TCR signal transduction, which suggests that Thy-1 transmits a TCR-like signal 1 in response to antibody stimulation. However, a subsequent functional study carried out in our lab suggests that although similar, TCR and Thy-1 signals certainly are not equivalent. Stimulation of T cells with anti-CD3 mAbs, in the context of costimulation provided by DCs, triggers cytolysis of P815 mastocytoma target cells in a reverse antibody-dependent cell-mediated cytotoxicity assay. Similar antibody-mediated stimulation of Thy-1 induces the expression of cytotoxic effector molecules and even T cell – P815 cell conjugate formation, but does not promote target cell lysis (Haeryfar et al., 2003). This report suggests that although Thy-1 signalling likely is dependent on components of the TCR signalling pathway, signals mediated by Thy-1 and the TCR must diverge at some point. Alternatively, Thy-1 may only initiate a partial TCR signal.

1.6. Guiding Principles, Objectives And Goals

To date, the theories outnumber the facts surrounding Thy-1 signal transduction in T cells. The controversy over the role of Thy-1 in T cell biology is largely attributable to three main issues. Firstly, we do not know which ligand stimulates Thy-1 on T cells under physiological conditions. It is also puzzling how

this GPI-anchored protein, which does not span the lipid bilayer of the plasma membrane, can communicate a signal from the cell surface to the cytosol. Lastly, because we do not know how or where T cells might be stimulated by a Thy-1 ligand in a physiological setting, it is difficult to assess whether antibody-mediated Thy-1-induced T cell activation is a relevant model for the study of Thy-1 signal transduction. Indeed, the hyperresponsiveness of thymocytes from Thy-$1^{-/-}$ mice (Hueber et al., 1997) and elevated incidence of leukaemia in PNH patients (Harris et al., 1999) who do not express GPI-anchored proteins suggests a possible negative regulatory role for Thy-1 in cellular proliferation, rather than a stimulatory one. Also, because the immunological ligand for Thy-1 remains unidentified, it is unclear if T cells would even experience Thy-1 stimulation in the context of costimulation under physiological conditions.

The GPI anchor is critical to the signalling capacity of GPI-anchored proteins (Robinson et al., 1989). Despite the fact that Thy-1 is not expressed on human T cells, studies on T cell-associated Thy-1 in mice may reveal new information on the function of Thy-1 in other cells, or on GPI-anchored protein signalling in general. Recent work from our lab implicates MAPKs in Thy-1-mediated T cell activation (Haeryfar and Hoskin, 2001); however, the role of these signalling molecules in TCR-mediated T cell activation is poorly understood, and is therefore even more obscure in the context of Thy-1-induced T cell responses. Also, the majority of studies on Thy-1 signalling take advantage of the presence of accessory cells or stimulatory antibodies directed against other surface molecules for costimulation. Little is known about the downstream

events that contribute to a *bona fide* Thy-1 signal in the absence of other stimuli. This may account for the lack of information on a definitive physiological role of Thy-1, beyond anti-Thy-1 antibody-induced T cell activation.

The objective of this work is to study T cell-associated Thy-1 signalling in the presence and absence of CD28-mediated costimulation in order to test the hypothesis that MAPKs are key elements of the Thy-1 signal transduction pathway in T cells.

The goals of this work are: *I) To clarify the role of ERK1/2, p38 MAPK, and JNK in Thy-1/CD28 stimulation-induced T cell activation; II) To identify components of the Thy-1 signal transduction pathway; III) To describe Thy-1 signal transduction in the absence of costimulation.*

Chapter 2. Materials And Methods

2.1. Mice

Female (6-8 weeks old) C57BL/6 mice were purchased from Charles River Canada (Lasalle, PQ, Canada). Mice were housed in the Carleton Animal Care Facility of Dalhousie University. Standard rodent chow and water were 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. All mice used in experiments were 8-12 weeks of age.

2.2. Culture Medium

Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich, Oakville, ON, Canada) was supplement with 5% heat-inactivated (at 56°C for 30 min) fetal calf serum (FCS), 2 mM $_{L}$-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4) (all from Invitrogen Canada Inc., Burlington, ON, Canada) to make complete RPMI medium (cRPMI).

2.3. Cell Lines

EL4 (ATCC# TIB-39) mouse lymphoma and IL-2-dependent cytotoxic lymphoid line-2 (CTLL-2; ATCC# TIB-214) mouse T lymphocyte cell lines were purchased from the American Tissue Culture Collection (Manassas, VA, USA). The 1H3.1 mouse T cell hybridoma (Yu et al., 1991) was kindly provided by Dr. Kenneth West (Dalhousie Univeristy, Halifax, NS, Canada). The C6 rat glioma cell line was kindly provided by Dr. Kenneth Renton (Dalhousie University). C6,

EL4, and 1H3.1 cell lines were cultured in cRPMI at 37°C in a 5% CO_2 humidified atmosphere. CTLL-2 cells were maintained in HEPES-free cRPMI containing 10% heat-inactivated FCS and 50 U/ml recombinant murine IL-2.

2.4. Reagents

PD98059, SL327, SB203580, ML3403, (L)-form JNK Inhibitor I, SP600125, z-VAD-fmk, and wortmannin were purchased from EMD Biosciences, Inc. (San Diego, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Brefeldin A (BfA) were from Sigma-Aldrich. Recombinant murine IL-2 was purchased from PeproTech (Rocky Hill, NJ, USA) and herbimycin A was from Life Technologies (Burlington, ON, Canada). Cyclosporine A (CsA), in the concentrated Sandimmune I.V. formulation, was from Novartis Pharmaceuticals Canada Inc. (Dorval, PQ, Canada). KN-93, H-89 and U73122 were purchased from BioMol Int. (Plymouth Meeting, PA, USA).

2.5. Antibodies

Anti-Thy-1 (clone G7), phycoerythrin (PE)-conjugated anti-CD25 (clone 3C7), and fluorescein isothiocyanate (FITC)-conjugated anti-Thy-1.2 (clone 30-H12) antibodies were purchased from BD Biosciences (San Jose, CA, USA). Rat IgG_{2c}, anti-CD28 (clone 37.51.1), PE-conjugated anti-TCRβ (clone H57-597), FITC-conjugated anti-CD25 (clone PC61.5.3), FITC-conjugated rat IgG_{2a}, FITC-conjugated rat IgG_{2b}, FITC-conjugated rat IgG_1,and PE-conjugated hamster IgG antibodies were purchased from Cedarlane Laboratories Ltd. (Burlington, ON, Canada). Rat IgG, and human IgG_1 antibodies were from Jackson ImmunoResearch (West Grove, PA, USA), while hamster IgG, anti-IL-2 (clone

JES6-1A12), FITC-conjugated anti-FoxP3 (clone FJK-16s), and FITC-conjugated anti-CD4 (clone L3T4) antibodies were from eBioscience (San Diego, CA, USA). Anti-phospho-vasodilator-stimulated phosphoprotein (VASP) (Ser157), anti-cyclic adenosine monophosphate response element-binding protein (CREB; clone 48H2), anti-phospho-CREB (Ser133), and anti-phospho-SAPK/JNK (Thr183/Tyr185; clone G9) antibodies were from Cell Signaling Technology (Danvers, MA, USA). Anti-VASP (clone H-90), anti-phospho-ERK (clone E-4), anti-ERK (clone K-23), anti-JNK (clone F-3), anti-actin (clone I-19), anti-CD25 (clone M-19), anti-p38 MAPK (clone C-20), horseradish peroxidase (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 antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-phospho-p38 MAPK (Thr180/Tyr182) antibodies were from Biosource Int. (Camarillo, CA, USA), Alexa Fluor® 488-conjugated goat anti-human IgG antibodies were purchased from Molecular Probes (Eugene, OR, USA), and anti-asialo GM1 antibodies were purchased from Wako Chemicals USA, Inc. (Richmond, VA, USA). The Thy-1-Fc fusion protein consisting of the binding domain of Thy-1.1 plus the Fc region of human IgG_1 (Leyton et al., 2001) was kindly provided by Dr. Lisette Leyton (University of Chile, Santiago, Chile). In some experiments supernatants from GK1.5 and 3.155 hybridomas were used as a source of anti-CD4 and anti-CD8 antibodies, respectively.

2.6. T Cell Isolation

Mice were sacrificed by cervical dislocation and spleens were removed under aseptic conditions. Spleens were homogenised to yield a single-cell suspension of leukocytes and erythrocytes. Erythrocytes were eliminated by osmotic shock with 4 ml of 0.2% NaCl for 20 s, and isotonicity was restored with 4 ml of 1.6% NaCl. $CD3^+$ cells were isolated by passing the leukocytes through T cell-enrichment immunocolumns (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The $CD3^+$ cell population collected from each immunocolumn was washed three times with phosphate-buffered saline (PBS; pH 7.2) and further purified by a two-step treatment with anti-asialo GM1 antibodies plus complement to remove contaminating NK cells. The cells were resuspended in a 1:50 dilution of anti-asialoGM1 monoclonal antibody in PBS for 30 min at ambient temperature. After three washes with PBS, the cells were resuspended in a 1:12 dilution of Low-Tox rabbit complement (Cedarlane Laboratories Ltd.) in PBS for 45 min at 37°C. The cells were then washed, resuspended in 10 ml PBS, decanted from the cell debris generated by NK cell lysis, and resuspended in cRPMI for cell counting. Cell viability was determined by Trypan Blue dye exclusion. For some experiments, whole T cells were then treated with anti-CD4 (GK1.5) or anti-CD8 (3.155) mAb at a final hybridoma supernatant dilution of 1:2 followed by complement to yield predominantly $CD8^+$ or $CD4^+$ T cell populations, respectively.

$CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were isolated from whole leukocytes by magnetic cell sorting (MACS) using a $CD4^+CD25^+$ regulatory T cell isolation

kit (Miltenyi Biotec Inc., Auburn, CA, USA). 2 x 10^8 leukocytes were resuspended in 400 µl MACS buffer (0.5% bovine serum albumin [BSA] and 2 mM ethylenediaminetetraacetic acid [EDTA] in PBS) plus 50 µl of biotin-antibody cocktail, and incubated at 4°C for 20 min. The biotin-antibody cocktail is designed for the negative selection of $CD4^+$ cells and contains biotinylated antibodies specific for CD8, CD11b, CD45R, CD49b, and Ter-199. An additional 300 µl MACS buffer plus 100 µl anti-biotin antibody-conjugated microbeads and 50 µl PE-conjugated anti-CD25 antibodies were then added to the cells. After gentle mixing, the cells were incubated for a further 30 min at 4°C, and washed in 10 ml MACS buffer. The cells were resuspended in 500 µl MACS buffer and passed though a column in which microbead-associated cells were magnetically retained. The column was washed twice with 1 ml MACS buffer. The negatively selected $CD4^+$ T cells were resuspended in 950 µl MACS buffer plus 50 µl anti-PE antibody-conjugated microbeads, and incubated at 4°C for 30 min. The cells were then resuspended in 500 µl MACS buffer and passed through a second column. The column was washed three times with 500 µl MACS buffer. Microbead-associated $CD4^+CD25^+$ cells were magnetically retained in the column while $CD4^+CD25^-$ cells were washed out of the column and were collected in the outflow. The $CD4^+CD25^+$ cells were obtained by forcing 1 ml of MACS buffer through the column with a sterile plunger in the absence of the magnetic field. To ensure high purity, the microbead-associated $CD4^+CD25^+$ cell population was passed through a third column in 500 µl MACS buffer. After three washes with 500 µl MACS buffer, the column was removed from the magnet and

$CD4^+CD25^+$ cells were collected in 1 ml MACS buffer that was forced through the column with a sterile plunger. The cells were then resuspended in cRPMI for cell counting and cell viability determination by Trypan Blue dye exclusion.

2.7. Trypan Blue Dye Exclusion

Cells were diluted in PBS containing 0.1% Trypan Blue (Sigma-Aldrich), mounted on a haemacytometer, and visualised on a light microscope. Trypan Blue is a negatively charged vital dye that does not interact with the plasma membrane of viable cells. Final cell concentrations were adjusted to ensure that the correct number of viable cells were used in each assay.

2.8. Flow Cytometry

Cells were washed once with 1 ml PBS and twice with 1 ml immunofluorescence (IF) buffer (1% BSA, 0.2% NaN_3, in PBS). The cells were mixed with fluorochrome-conjugated antibodies or isotype-matched fluorochrome-conjugated control antibodies at a concentration of 10 µg/ml in a final volume of 100 µl IF buffer and incubated on ice for 45 min. The cells were washed twice with 1 ml IF buffer and resuspended in PBS containing paraformaldehyde (PFA; 1% [w/v]). For indirect labelling, the cells were mixed with Thy-1-Fc or human IgG_1 at a concentration of 10 µg/ml in a final volume of 100 µl IF buffer and incubated on ice for 45 min. The cells were then washed three times with 1 ml IF buffer and mixed with Alexa Fluor® 488-conjugated goat anti-human IgG antibodies at a concentration of 10 µg/ml in a final volume of 100 µl IF buffer and incubated on ice for a further 45 min before being washed 3 times in IF buffer, and resuspended in PBS containing 1% PFA. For intracellular

staining of FoxP3, the cells were first permeabilised and fixed with the FoxP3 Staining Buffer Set (eBioscience), and labelled with FITC-conjugated anti-FoxP3 or FITC-conjugated rat IgG_{2a}. All flow cytometry data were acquired on a FACSCalibur flow cytometer with CellQuest software (version 3.3), both from Becton Dickinson (Mississauga, ON, Canada). Flow cytometry data were analysed using FCS Express software (version 1.0 or 2.0; De Novo Software, Thornhill, ON, Canada).

2.9. Antibody Adsorption Onto Carboxylate Microspheres

Approximately 5 x 10^6 polybead® carboxylate 10.0 µm microspheres (Polysciences, Inc., Warrington, PA, USA) were combined with anti-Thy-1, anti-CD28, or isotype control antibodies in 1 ml of PBS in 24-well polystyrene plates or 1.5 ml polypropylene micro tubes (Sarstedt, Inc., Montreal, PQ, Canada). Plates were incubated for at least 4 h at 37°C in a 5% CO_2 humidified atmosphere, whereas tubes were rocked or rotated end-over-end for at least 4 h at ambient temperature. The beads were then collected by centrifugation (5 x 10^3 – 1 x 10^4 x *g*, 10 min), washed three times in PBS, resuspended in cRPMI, and counted.

2.10. T Cell Stimulation

2.5 x 10^5 T cells in a final volume of 200 µl/well in flat-bottom 96-well tissue culture plates (Sarstedt, Inc.) were activated by antibody-coated carboxylate or Mouse CD3/CD28 T Cell Expander Dynabeads® (Dynal Biotech, Oslo, Norway) at a cell-to-bead ratio of 1:1 or 2:1, respectively. Larger numbers of T cells were activated in a final volume of 1 ml in flat-bottom 24-well plates or

1.5 ml microtubes. Alternatively, T cells were incubated overnight in anti-Thy-1 mAb-coated 24-well or 96-well tissue culture plates.

2.11. ^{3}H-Thymidine Incorporation Assay

T cells were pulsed with 0.5 µCi or 0.2 µCi methyl ^{3}H-thymidine ([^{3}H]TdR) (MP Biomedicals, Irvine, CA, USA). The cells were harvested onto fibreglass filter mats 6 h later using a Titertek® cell harvester (both from Skatron Instruments, Sterling, VA, USA). [^{3}H]-TdR incorporation was measured in counts per minute (cpm) with a Wallac 1410 liquid scintillation counter (Wallac Oy, Turku, Finland).

2.12. Preparation Of Total Cellular Lysates

Cells were pelleted by centrifugation at 500 x *g*, 5 min, and lysed with ice-cold lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 50 mM Na_2HPO_4, 0.25% sodium deoxycholate [w/v], 0.1% Nonidet P-40 [w/v], 5 mM EDTA, and 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid [EGTA]) containing freshly added protease and phosphatase inhibitors (5 µg/ml leupeptin, 5 µg/ml pepstatin, 10 mM NaF, 1 mM phenylmethyl sulfonyl fluoride, 1 mM dithiothreitol (DTT), 100 µM Na_3VO_4, 10 µM phenylarsine oxide, and 10 µg/ml aprotinin). Lysates were clarified by high speed centrifugation (10,000 x *g*, 10 min, 4°C), and protein quantification was performed using the Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, Hercules, CA, USA) to determine total protein concentration for each sample. Protein concentrations were equalised and one half volume 3x 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 0.01% bromophenol blue [w/v]) was added to each sample. The samples were then boiled for 5 min and frozen at -80°C until further required.

2.13. SDS-PAGE

Frozen samples were thawed and resolved in parallel with prestained protein markers (Bio-Rad Laboratories) in Tris-HCl acrylamide gels. Resolving gels were made to 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 a 4% acrylamide stacking gel (125 mM Tris-HCl [pH 6.8], 0.1% SDS [w/v], 0.1% APS [w/v] and 0.3 % TEMED [v/v]). Samples were 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 [v/v]).

2.14. Western Blot Analysis

Polyacrylamide gels were transferred onto nitrocellulose membranes in electrotransfer buffer (25 mM Tris base [pH 8.3], 192 mM glycine and 20% methanol [v/v]) at 400 mA for 1 h. Membranes were then immediately placed in a blocking solution of Tris-buffered saline (TBS)-Tween-20 (TBST; 20 mM Tris-HCl [pH 7.6], 200 mM NaCl, 0.05% Tween-20 [v/v]) containing 5% fat-free milk (w/v) for 1h. Membranes were then washed 3 times with fresh TBST and incubated in the appropriate primary antibody (typically a 1:1000 dilution of stock antibody in blocking solution) for 1 h at ambient temperature or overnight at 4°C with gentle rocking. Membranes were washed 6 times with fresh TBST and incubated in the appropriate HRP-conjugated secondary antibody (typically a 1:1000 dilution of

stock antibody in blocking solution) for 1 h at ambient temperature. Membranes were again washed six times with fresh TBST and reacted with enhanced chemiluminescence (ECL) reagents (GE Healthcare, Baie d'Urfe, Quebec, Canada) for 1 min. Excess ECL reagent was removed and the membranes were used to expose x-ray film. Film was processed in a Kodax X-OMAT 1000A automated x-ray developer. As per antibody product sheets, blocking solution contained 3% or 5% BSA (w/v) and/or 0.1% Tween-20 (v/v), and membranes were washed with TBS or TBS containing 0.1% Tween-20 (v/v).

2.15. MTT Assay

The MTT assay was carried out as a second measure of cell viability (Mosmann, 1983). T cells (2.5 x 10^5 per well) were cultured in the presence of MAPK inhibitors or dimethyl sulfoxide (DMSO) vehicle alone for 24 h in round-bottom 96-well tissue culture plates in a final volume of 200 µl. A 20 µl aliquot of MTT (5 mg/ml in PBS) was added to each well for the final 3 h of culture, after which the cells were pelleted by centrifugation (1,400 x *g*, 5 min) and the supernatants were removed. Cell pellets were solubilised in DMSO and transferred to flat-bottom 96-well tissue culture plates. Absorbance at 490 nm was recorded using an EL311 microplate autoreader (BioTek Instruments, Inc., Winooski, VT, USA).

2.16. IL-2 Quantification By Enzyme-Linked Immunosorbant Assay (ELISA)

Cell supernatants were collected and assayed for IL-2 with an ELISA kit from BD Biosciences, according to the manufacturer's instructions. Briefly, high-binding 96-well polystyrene plates were coated overnight at 4°C with detection

antibody in coating buffer (0.1 M Na_2CO_3, pH 9.5). The plates were then washed with wash buffer (PBS, 0.05% Tween-20), and blocked with assay diluent (PBS, 10% FCS [v/v]). The plates were then washed 3 more times and culture supernatants and recombinant murine IL-2 standards were added to the plates for 2 h, washed 5 times, and probed with biotinylated anti-IL-2 detection antibodies plus avidin-conjugated HRP in assay diluent. The plates were washed 7 times, and substrate reagents (equal parts tetramethylbenzidine and H_2O_2) were added to the plates for 30 min. Stop solution (1M H_2SO_4) was added, generating a final colour change, and absorbance readings at 450 nm were recorded with a wavelength correction for 570 nm using an EL_X800 UV universal microplate reader (Bio Tek Instruments, Inc.) and KCjunior software (version 1.17; Bio Tek Instruments, Inc.). Absorbance levels were analysed using SOFTmax® PRO software (version 4.3; Molecular Devices Corp., Sunnyvale, CA, USA) to determine IL-2 concentrations from the culture supernatants.

2.17. RNA Isolation

Cells were lysed in 1 ml TRIZOL® reagent (Invitrogen) and incubated at ambient temperature for 5 min. The TRIZOL/cell lysate mixture was then vigorously mixed with 200 µl chloroform, incubated at ambient temperature for a further 3 min, and centrifuged for 15 min at 12,000 x *g*, 4°C. The aqueous phase was transferred to a clean micro tube, mixed with 500 µl ice-cold isopropanol by gentle vortexing, and incubated at ambient temperature for a further 10 min. The samples were then centrifuged for 10 min at 12,000 x *g*, 4°C to pellet the RNA. The RNA pellet was resuspended in ice-cold 75% ethanol ([v/v] in water

containing 0.1% diethylpyrocarbonate), gently vortexed, and centrifuged at 7,500 x *g*, for 10 min, 4°C. The RNA pellet was then dried and resuspended in 35 µl pyrogen-free water.

2.18. Real-Time Polymerase Chain-Reaction (PCR)

Approximately 1 µg of RNA from each sample was reacted with 200 U Moloney murine leukaemia virus reverse transcriptase in 20 µl first-strand buffer containing 10 µM DTT, 500 µM deoxynucleotide triphosphates, and 150 ng/µl random hexanucleotide primers (all purchased from Invitrogen) at 37°C for 1 h to generate cDNA. Reverse transcription was halted by heating the samples at 95°C for 10 min. PCR reactions were then carried out using Brilliant® SYBR® Green quantitative PCR reagents (Stratagene, La Jolla, CA, USA). A 2-µl volume of each cDNA sample was mixed with 10 µl 2X Master Mix, 0.5 µl ROX reference dye (diluted 1:1000 in pyrogen-free water), 125 nM forward and reverse primers for murine IL-2 or murine RNA polymerase (RNA Pol) II in a final volume of 20 µl. PCR reactions were conducted in duplicate tubes. Negative controls did not contain cDNA. Forty amplification cycles for IL-2 and RNA Pol II were carried out in an MxP3000 quantitative PCR machine (Stratagene) with the following conditions: 95°C for 30 s (denaturation), 57°C for 30 s (annealing), and 72°C for 60 s (synthesis). The IL-2 and RNA Pol II primer sequences were selected because they span introns, which allows contamination with genomic DNA to be detected. RNA Pol II primer sequences were generated using Primer Bank (http://pga.mgh.harvard.edu/primerbank). Amplicon sizes and primer sequences were as follows:

IL-2 (170 bp) (F): 5'-TGA TGG ACC TAC AGG AGC TCC TGA G-3'

(R): 5'-GAG TCA AAT CCA GAA CAT GCC GCA G-3'

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

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

The relative gene expression data collected with MxPro software (Stratagene) were then analysed using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). The C_T value is the number of cycles required for an amplification curve to reach an arbitrarily assigned detectable level. C_T values were entered into the following equation:

Fold increase = $2^{-\Delta\Delta C_T}$

$-\Delta\Delta C_T = -(\Delta C_{T,q} - \Delta C_{T,cb})$;

$\Delta C_{T,q}$ = ΔC_T for T cells activated with anti-Thy-1/anti-CD28 antibody-coated beads in the presence of vehicle or SB203580;

$\Delta C_{T,cb} = \Delta C_T$ for T cells exposed to isotype control antibody-coated beads;

$\Delta C_T = C_T$ (for IL-2) - C_T (for RNA Pol II).

Calculated values were adjusted to reflect fold increase relative to T cells activated in the presence of vehicle.

2.19. Induction Of Treg Cells

Twenty four-well polystyrene plates were coated with 5 µg anti-Thy-1 antibodies in a sterile 0.05 M Tris-HCl solution (pH 9.5) at a final volume of 1 ml

per well, and incubated at 37°C in a 5% CO_2 humidified atmostphere overnight. The plates were then washed with cRPMI. $CD4^+CD25^-$ T cells obtained by MACS isolation and added to the antibody-coated wells in cRPMI at a final concentration of ~2 x 10^6 cells per ml, per well, and incubated overnight at 37°C in a 5% CO_2 humidified atmosphere. The cells were then aspirated with a pipette or with a 10 ml syringe and 22G1 needle and resuspended in 850 µl MACS buffer plus 50 µl PE-conjugated anti-CD25 antibodies, and incubated at 4°C for 30 min. The cells were then washed in 10 ml MACS buffer, resuspended in 950 µl MACS buffer plus 50 µl anti-PE antibody-conjugated microbeads, and incubated at 4°C for a further 30 min. $CD25^+$ cells were then isolated by positive selection using the MACS magnet and cell isolation columns as previously described. As required, dead cells were excluded from antibody-induced $CD4^+CD25^+$ cell preparations using a dead cell removal kit (Miltenyi Biotec), or by extensive washing with cRPMI.

2.20. Immunoprecipitation

Brain, thymus, and spleen, as well as superficial cervical, axillary, brachial, and inguinal lymph nodes were homogenised in ice-cold PBS and depleted of erythrocytes by osmotic shock. Tissue homogenates were then lysed in ice-cold lysis buffer containing freshly added protease and phosphatase inhibitors. Lysates were clarified by high speed centrifugation (10,000 x *g*, 10 min, 4°C). Protein assays were performed to determine total protein concentration in each sample. After protein concentrations were equalised, 1 ml of each sample was combined with 1 µg/ml Thy-1-Fc and mixed end-over-end overnight at 4°C. Thy-

1-Fc-associated proteins were precipitated overnight with 20 µl of Protein A/G-PLUS Agarose immunoprecipitation bead slurry with end-over-end mixing. Beads were then pelleted by centrifugation (500 x *g*, 5 min, 4°C) and washed 3 times with ice-cold PBS containing 1M NaCl. The samples were treated with 20 µl 3x SDS-PAGE sample buffer, boiled for 5 min, and frozen at -80°C until further required.

2.21. Mass Spectrometry

Polyacrylamide gels were stained in 5 volumes of Brilliant Blue R dye (2.5 mg/ml, methanol:H_2O:glacial acetic acid [9:9:1]) for 4 h at ambient temperature. Gels were destained in methanol:H_2O:glacial acetic acid (9:9:1) for up to 8 h, with multiple changes of destaining solution. Protein bands were cut, stored in distilled water, and submitted to the Atlantic Research Centre Mass Sectrometry Facility (Dalhousie University) for reverse phase – high pressure liquid chromatography – mass spectrometry – mass spectrometry analysis and subsequent database searching.

2.22. Immunohistochemistry

C57BL/6 mouse brain tissue was fixed in the Universal Molecular Fixative (UMFix™, Sakura Finetek USA, Inc., Torrance, CA, USA) reagent for 12 h and sectioned by Mrs. Patricia Colp (Histology Research Services, Dalhousie University). Tissue sections cut at a thickness of 5 µm were stained with the Thy-1-Fc chimeric protein (12.5 µg/ml) and Alexa Fluor® 488-conjugated goat anti-human secondary antibodies (10 µg/ml) in PBS. Negative controls were not stained with Thy-1-Fc. Images were captured on a Nikon Eclipse E600

epifluorescent microscope using Act-1 software from Nikon (Mississauga, Ontario, Canada).

2.23. Determination Of DNA Fragmentation

The [^{3}H]TdR release assay was used to measure DNA fragmentation (Matzinger, 1991). Cells were incubated for 4 h in fresh cRPMI containing 5 µCi/ml [^{3}H]TdR in a 37°C, 5% CO_2, humidified atmosphere. After extensive washing, the cells were added to flat-bottom 96-well tissue culture plates at a final concentration of 2.5 x 10^5 cells/ml, and a final volume of 200 µl, in the presence of anti-Thy-1 antibodies or isotype-matched control antibodies. After 24 h cellular DNA was harvested onto fibreglass filtermats with a Titertek® cell harvester (Skatron Instruments). Radioactivity for each sample was measured in cpm by liquid scintillation counting. Percent DNA fragmentation was then calculated as (1-[$cpm_{experimental}/cpm_{control}$]) x 100, where $cpm_{experimental}$ and $cpm_{control}$ were from anti-Thy-1 mAb-treated cells and isotype control antibody-treated cells, respectively.

Alternatively, genomic DNA was isolated from anti-Thy-1 mAb- or isotype control antibody-treated cells using a DNeasy® Tissue Kit (Qiagen Inc., Mississauga, ON, Canada) and resolved in parallel with a 100-base pair (bp) molecular weight (MW) standard (Invitrogen Canada Inc.) in a 1.5% (w/v) agarose gel made in tris-acetic acid-EDTA (TAE) buffer (40 mM tris, 20 mM acetic acid, 1 mM EDTA). Samples were electrophoresed at 100 V for 1 h in TAE buffer, stained with ethidium bromide, and visualised with UV light.

2.24. Statistical Analyses

Data were analysed by one-way analysis of variance (ANOVA) with a Bonferroni post-test correction using GraphPad InStat version 3.00 for Windows 95 (GraphPad Software, San Diego California USA). Differences were considered statistically significant when *p* values were less than 0.05.

Chapter 3. Results: MAPKs Modulate IL-2 Production, CD25 Expression, And IL-2R Signalling In T Cells Stimulated With Microbeads Coated With Anti-Thy-1 And Anti-CD28 mAbs

More than two decades have passed since the original observations that antibody stimulation of Thy-1 on T cells results in IL-2 expression and cell proliferation (Gunter et al., 1984; Kroczek et al., 1986). However, remarkably little is known about the signalling pathway(s) that govern this process. In most studies on antibody-induced Thy-1 signalling, T cells are activated in the presence of accessory cells that express myriad costimulatory molecules. Because of the immense number of possible combinations of costimulatory events that could accompany Thy-1 signalling, this mixed cell system is not ideal for characterising individual signalling pathways.

In recent years, our laboratory has examined the differences between Thy-1- and CD3-induced T cell activation (Haeryfar and Hoskin, 2001). One area of focus was the role of MAPKs in these processes. At the time, a mixed cell system was being employed, and thus it remains difficult to interpret results from this study. Our lab has since adopted mAb-coated cell-sized beads to provide signal 1 (*e.g.*, Thy-1 stimulation) in the context of CD28 costimulation (Haeryfar et al., 2003). Here, I have used pharmacological inhibitors to study the role of ERK1/2, p38 MAPK, and JNK in IL-2 production, CD25 expression, and IL-2R signalling following activation of T cells with microbeads coated with anti-Thy-1 and anti-CD28 mAbs.

3.1. T Cells Obtained From Splenocytes With T Cell Enrichment Immunocolumns Are TCRαβ- And Thy-1.2-Positive

Because the presence of non-T cells would confound my studies on Thy-1-mediated T cell activation, it was important to establish a method for isolating pure T cells. I adopted a T cell isolation protocol utilising commercial T cell-enrichment immunocolumns coupled with antibody- and complement-mediated depletion of NK cells. The previous work on MAPK in Thy-1-induced T cell activation used T cell cultures that were typically 81% $CD3^+$ (Haeryfar and Hoskin, 2001). As shown in Figure 3.1A, the vast majority (~95%) of the cells recovered by my T cell isolation procedure stained positive for TCRαβ. High T cell purity was also reflected in Figure 3.1B, which shows that nearly all cells (~99%) were Thy-1.2^+.

3.2. Microbeads Coated With Anti-Thy-1 And Anti-CD28 mAbs Induce Maximal T Cell Activation At 48 h

TCR-driven [^{3}H]TdR incorporation of highly purified T cells can be measured as early as 24 h, and as late as 96 h post-stimulation (C. Doucette, personal communication). A leading theory on the role of Thy-1 in immunity is that it generates a TCR-like signal in the context of robust costimulation (Haeryfar and Hoskin, 2004). However, Thy-1-induced T cell activation has always been examined at 48 h in our lab (Haeryfar et al., 2003; Haeryfar and Hoskin, 2001). Because it was unclear whether other time points might be more appropriate in terms of detecting maximal T cell activation, I performed a time course analysis of Thy-1/CD28 costimulation-induced T cell activation with mAb-coated microbeads (Figure 3.2A). Minimal [^{3}H]TdR incorporation was detected at

24 h and 72 h while [^{3}H]TdR incorporation was virtually absent at 96 h. Thy-1/CD28-mediated T cell [^{3}H]TdR incorporation was maximal at 48 h. The [^{3}H]TdR incorporation assay protocol that I was following involved pulsing the cell cultures with [^{3}H]TdR for only 6 h. I wanted to address the possibility that Thy-1/CD28-induced [^{3}H]TdR incorporation could be taking place outside of the discreet 6 h window of time during which the cell cultures were exposed to [^{3}H]TdR. Therefore, I performed a 96-h time course experiment in which the cells were pulsed for 6 h, 24 h, or in a cumulative fashion, ending every 24 h. Figure 3.2.B shows that similar levels of [^{3}H]TdR incorporation were attained for the 6-h pulse at 48 h, the 24-h pulse at 48 h, and the cumulative pulses at 48 h, 72 h, or 96 h. Because only minimal [^{3}H]TdR incorporation was detected for all pulsing conditions that did not span the 6-h pulse ending at 48 h, and there were no statistically significant differences between the levels of [^{3}H]TdR incorporation for the pulsing conditions mentioned above ($p > 0.05$ for 6-h pulse at 48 h, compared to the 24-h pulse ending at 48 h, or cumulative pulses ending at 48 h, 72 h, or 96 h, by Bonferroni multiple comparisons test), 6-h [^{3}H]TdR pulsing at 48 h was employed for all subsequent experiments examining Thy-1/CD28-driven [^{3}H]TdR incorporation. Although Thy-1/CD28-induced T cell activation was consistently maximal at 48 h, there was variation in the amount of T cell activation measured. For example, in Figure 3.2A, maximal T cell activation yielded 51,359 ± 1,289 cpm, while 26,403 ± 5,507 cpm was measured for Thy-1/CD28-induced T cell activation (6-h pulse) at 48 h in Figure 3.2B. Differences in the intensity of Thy-1/CD28-induced T cell activation may have been due to variability among the

mice used, but may also have been due to differences in antibody adsorption onto the microbeads.

3.3. Microbeads Coated With Anti-Thy-1 And Anti-CD28 mAbs Induce MAPK Phosphorylation

MAPK play important roles in virtually all facets of T cell biology, from cell development to cell death (Dong et al., 2002). Our lab has previously demonstrated that inhibition of ERK1/2 or p38 MAPK affects Thy-1-induced T cell activation (Haeryfar and Hoskin, 2001). However, because of possible non-specific effects by pharmacological inhibition and the fact that Thy-1/CD28 costimulation has never been shown to directly activate MAPK, I carried out a time course experiment that examined the phosphorylation status of ERK1/2, p38 MAPK and JNK in T cells following stimulation by microbeads coated with anti-Thy-1 and anti-CD28 antibodies. Figure 3.3 shows that ERK1/2, p38 MAPK and JNK were all phosphorylated in a time-dependent manner. Phosphorylation of ERK1/2 was evident by 30 min post-stimulation, and was maximal between 4 h and 8 h. Interestingly, ERK2 (lower band) was phosphorylated more rapidly and more extensively than ERK1 (upper band). Phosphorylation of p38 MAPK and JNK was more modest than that of ERk1/2, was not evident until 4 h post-stimulation, and was maximal at 8 h. Phosphorylation of p38 MAPK and JNK returned to control levels ("medium" lane) after 24 h; however, phospho-ERK1 and phospho-ERK2 were still present even at this late time point.

3.4. Inhibition Of ERK1/2, p38 MAPK, Or JNK Attenuates Thy-1/CD28-Mediated T Cell Activation

Our publication on MAPK in Thy-1-induced T cell activation is the sole evidence that these signalling molecules might play a role in this process (Haeryfar and Hoskin, 2001). At the time, the role of JNK was not examined, so I undertook the task of evaluating the role of ERK1/2, p38 MAPK, and JNK in Thy-1-induced T cell activation using the microbead-stimulation system. I observed Thy-1-induced T cell activation in the presence of the MEK1 inhibitor PD98059 (Dudley et al., 1995), the p38 MAPK inhibitor SB203580 (Lee et al., 1999), and the JNK inhibitor SP600125 (Bennett et al., 2001a). Inhibition of MEK1, (which prevents activation of ERK1/2), p38 MAPK, or JNK resulted in a dose-dependent impairment of Thy-1/CD28-induced [^{3}H]TdR incorporation (Fig. 3.4). Differences among experimental values were statistically significant ($p < 0.0001$ by ANOVA). At the 10 μM dose, PD98059, SB203580, and SP600125 all significantly inhibited T cell activation ($p < 0.001$ compared to T cells activated in the presence of drug vehicle alone). Pretreatment with 10 μM PD98059 reduced Thy-1/CD28-induced [^{3}H]TdR incorporation from 68,013 ± 6,336 cpm (T cells activated in the presence of drug vehicle alone) by 32% to 46,400 ± 5,182 cpm, and pretreatment with 10 μM SB203580 reduced [^{3}H]TdR incorporation by 45%. At a concentration of 10 μM, SP600125 reduced [^{3}H]TdR incorporation by 75%, and 5 μM SP600125 reduced [^{3}H]TdR incorporation by 23%, which was also statistically significant ($p < 0.05$ compared to T cells activated in the presence of drug vehicle alone).

3.5. Attenuation Of T Cell Activation By MAPK Inhibitors Is Not Due To Decreased Cell Viability

Because MAPK play a role in cell survival (Krens et al., 2006), it was possible that the observed effects of MAPK inhibition on T cell activation were due to a reduction in T cell viability. A number of approaches were therefore taken to address the possibility that the MAPK inhibitors were exerting cytotoxic effects on the Thy-1/CD28-activated T cells. The MTT assay is a colorimetric assay designed to monitor mitochondrial reductase activity in cells as a measure of cell survival (Mosmann, 1983). T cells were treated with 10 μM PD98059, SB203580, or SP600125, drug vehicle or medium alone, for 24 h, and cell viability was assessed by MTT assay. Figure 3.5A shows that none of the MAPK inhibitors caused a statistically significant reduction in absorbance at 490 nm, relative to the vehicle control ($p > 0.05$ for PD98059, SB203580, and SP600125). The DMSO vehicle alone also did not significantly affect T cell viability ($p > 0.05$ relative to medium alone). Also, I found that 10 μM MAPK inhibitors, which significantly inhibited [^{3}H]TdR incorporation in mAb-stimulated primary T cells, did not dramatically affect the uptake of [^{3}H]TdR over a 6-h period in EL4 lymphoma cells. Basally proliferating EL4 cells were pretreated with 10 μM PD98059, SB203580 or SP600125, drug vehicle alone or medium alone for 30 min, and were pulsed with [^{3}H]TdR for 6 h. As shown in Figure 3.5B, overall differences were not significant by ANOVA ($p > 0.05$), and none of the MAPK inhibitors significantly affected [^{3}H]TdR uptake compared to the vehicle alone ($p > 0.05$ for PD98059, SB203580, and SP600125). Lastly, T cell viability was evaluated by Trypan Blue dye exclusion. Trypan Blue is a negatively charged dye that is

excluded by the intact plasma membrane of viable cells. T cells were incubated in the presence of drug vehicle or 10 μM of the MAPK inhibitors for 24 h. Cells were collected and resuspended in Trypan Blue dye, and both viable (white) and non-viable (blue) cells were enumerated using a light microscope. The cell viability data is summarised in Table 3.1. After 24 h, T cells exposed to vehicle alone were 81 ± 6% viable. PD98059-, SB203580-, and SP600125-treated cells were 78 ± 3%, 80 ± 11%, and 79 ± 6 % viable, respectively. Differences were not significant by ANOVA ($p > 0.05$) and none of the MAPK inhibitors caused a statistically significant change in cell viability compared to the vehicle control ($p > 0.05$ for PD98059, SB203580, and SP600125).

3.6. Alternate MAPK Inhibitors Also Attenuate Thy-1/CD28-Mediated T Cell Activation

The specificity of pharmacological inhibitors is often scrutinised in the literature. Alternate inhibitors of MEK1, p38 MAPK, and JNK were therefore used in [^{3}H]TdR incorporation assays to support the putative role of ERK1/2, p38 MAPK and JNK in Thy-1/CD28-induced T cell activation. T cells were exposed to SL327 (a MEK1 inhibitor), ML3403 (a p38 MAPK inhibitor), (L)-JNK1 (a peptide inhibitor of JNK), or the drug vehicle alone before addition of microbeads coated with anti-Thy-1 and anti-CD28 mAbs. Figure 3.6A shows that SL327 caused a dose-dependent decrease in T cell activation. Differences were found to be statistically significant ($p = 0.005$ by ANOVA), and 500 nM SL327 significantly reduced [^{3}H]TdR incorporation by 51% ($p < 0.01$). ML3403 and (L)-JNK1 also impaired Thy-1/CD28-induced [^{3}H]TdR incorporation in a dose-dependent fashion (Fig. 3.6B and 3.6C, respectively; $p < 0.0001$ by ANOVA). All doses

tested for ML3403 and (L)-JNK1 resulted in a significant decrease in Thy-1/CD28-induced [^{3}H]TdR incorporation compared to vehicle control-treated cells ($p < 0.05$ for 1 μM ML3403, $p < 0.001$ for all other doses of ML3403 and (L)-JNK1). At the highest doses shown, ML3403 and (L)-JNK1 inhibited [^{3}H]TdR incorporation by 76% ($p < 0.001$) and 74% ($p < 0.001$), respectively. These data give further credence to the role of MAPK in Thy-1-induced T cell activation.

3.7. Inhibition Of ERK1/2, p38 MAPK, Or JNK Modulates Thy-1/CD28-Induced IL-2 Production

IL-2 is produced following T cell costimulation, and drives cell division (Smith, 1988). It was therefore possible that MAPK inhibition was interfering with Thy-1-induced T cell activation at the level of IL-2 production. I pretreated T cells with MAPK inhibitors prior to microbead stimulation, and measured IL-2 concentrations in culture supernatants after 24 h by ELISA. Figure 3.7A shows that inhibition of MAPKs significantly altered IL-2 production ($p < 0.0001$ by ANOVA). At concentrations of 10 μM and 5 μM, PD98059 significantly inhibited Thy-1/CD28-induced IL-2 production compared to the vehicle control ($p < 0.001$ and $p < 0.05$, respectively). Unexpectedly, blockade of p38 MAPK activity by SB203580 enhanced IL-2 levels ($p < 0.001$ for all concentrations). Like PD98059, pretreatment with SP600125 at concentrations of 10 μM and 5 μM resulted in significant inhibition of Thy-1/CD28-induced IL-2 production compared to vehicle control ($p < 0.001$). Because the effects of MAPK inhibition on IL-2 production were evident before substantial [^{3}H]TdR incorporation had taken place (i.e., at 24 h after exposure to mAb-coated beads), I decided to add the MAPK inhibitors 24 h after exposure to mAb-coated microbeads and observe their effect on [^{3}H]TdR

incorporation at 48 h. As shown in Figure 3.7B, there was no significant difference in the effect on [^{3}H]TdR incorporation whether p38 MAPK or JNK inhibitors were added prior to, or 24 h after, bead stimulation ($p > 0.05$). This suggested that p38 MAPK and JNK likely play important roles in T cell activation after IL-2 is produced. However, there was a difference between [^{3}H]TdR incorporation values, depending on when ERK1/2 was inhibited. There was significantly more [^{3}H]TdR incorporation when PD98059 was added to cultures 24 h after bead stimulation, compared to when the inhibitor was added prior to bead stimulation ($p < 0.001$). These data suggest that the role for ERK1/2 in Thy-1-induced T cell activation predominantly takes place during the first 24 h.

3.8. IL-2-Dependent T Cell Growth Is Impaired By Inhibition Of p38 MAPK Or JNK

CTLL-2 cells were generated by using IL-2 to propagate T cells activated in a tumour-lymphocyte culture (Gillis and Smith, 1977). This T cell line has the convenient property of being IL-2-dependent, and is commonly used for the study of IL-2R signalling. To determine if inhibition of ERK1/2, p38 MAPK, or JNK interfered with IL-2R signalling, I pretreated CTLL-2 cells with MAPK inhibitors and measured IL-2-driven [^{3}H]TdR incorporation after 48 h. Inhibition of p38 MAPK or JNK resulted in a significant impairment of CTLL-2 cell proliferation compared to the vehicle control ($p < 0.001$ for all concentrations; Figure 3.8A). However, inhibition of ERK1/2 did not dramatically alter IL-2-induced T cell growth. At a concentration of 10 μM, PD98059 reduced CTLL-2 cell proliferation by only 10% ($p < 0.01$ compared to vehicle control), but 5 μM and 1 μM concentrations had no effect ($p > 0.05$) To establish that p38 MAPK- and JNK-

mediated inhibition of CTLL-2 cell growth was not due to altered expression of the IL-2R, I measured expression of CD25 by flow cytometry after addition of MAPK inhibitors and IL-2. Figure 3.8B shows that the effect of MAPK inhibition on CD25 expression was minimal and could not account for the observed impairment of [^{3}H]TdR incorporation.

3.9. Exogenous IL-2 Restores [^{3}H]TdR Incorporation By Thy-1/CD28-Stimulated T Cells Treated With Inhibitors Of ERK1/2 Or JNK

Experiments with CTLL-2 cells suggested that p38 MAPK and JNK were important in IL-2R signalling. In order to confirm these results in primary T cells, an IL-2 addback experiment was performed on freshly isolated T cells. Here, I carried out a [^{3}H]TdR incorporation assay with T cells pretreated with MAPK inhibitors, with or without exogenous IL-2 added to the cultures. Figure 3.9 shows that the addition of exogenous IL-2 greatly enhanced Thy-1/CD28-induced T cell activation. Interestingly, although T cells stimulated by anti-Thy-1 mAb-coated microbeads were not activated, adding IL-2 to these cultures resulted in significantly more [^{3}H]TdR incorporation. Despite inhibition of ERK1/2 or JNK, exogenous IL-2 greatly enhanced T cell activation. However, the effect of p38 MAPK inhibition on [^{3}H]TdR incorporation could not be completely overcome by addition of exogenous IL-2, which failed to restore [^{3}H]TdR incorporation in SB203580-treated cells beyond the level seen in Thy-1/CD28-stimulated cells without exogenous IL-2. These data suggest that in primary T cell cultures activated by Thy-1/CD28 costimulation, p38 MAPK mediates IL-2R signalling.

3.10. Inhibition Of JNK Blocks Thy-1/CD28-Induced CD25 Expression

Inhibition of MAPK did not have a profound effect on CD25 expression by CTLL-2 cells. Initial attempts to measure CD25 expression in primary T cells by flow cytometry were confounded by the autofluorescent nature of the antibody-coated microbeads present in the cell cultures. I therefore carried out Western blot experiments to determine if Thy-1/CD28-induced CD25 expression in primary T cells was altered by MAPK inhibition. T cells from untreated, isotype control Ab-treated, and anti-CD28 mAb-treated cells did not express CD25 (Figure 3.10A). Stimulation with anti-Thy-1 mAb resulted in moderate CD25 expression, while costimulation with Thy-1- and CD28-specific mAbs induced robust expression of CD25. Inhibition of MEK1 or p38 MAPK did not substantially alter Thy-1/CD28-induced CD25 expression; however, inhibition of JNK impaired expression of CD25. I previously showed that IL-2 signalling induced CD25 expression (Figure 3.8B). Because inhibition of JNK impaired IL-2 production following Thy-1/CD28 costimulation, it was unclear if blocking JNK activity directly affected CD25 expression of if reduced CD25 expression was related to impaired IL-2 production. To address this, I reevaluated CD25 expression in Thy-1-activated T cells that had been pretreated with SP600125 in the presence or absence of IL-2. Figure 3.10B shows that addition of exogenous IL-2 only partially restored CD25 expression in SP600125-treated T cells. These data suggest that JNK signalling plays a direct role in CD25 expression.

3.11. Inhibition Of p38 MAPK Enhances Thy-1/CD28-Induced IL-2 Expression

Haeryfar et al. showed that inhibition of p38 MAPK enhanced Thy-1-induced T cell activation in a mixed-cell system (Haeryfar and Hoskin, 2001). The observation that inhibition of p38 MAPK impaired [^{3}H]TdR incorporation but enhanced IL-2 levels in culture supernatants following T cell activation via Thy-1 and CD28 was, therefore, quite puzzling. Elevated amounts of IL-2 in the culture supernatants could have been due to decreased uptake/usage of IL-2 by the T cells, or a direct effect on IL-2 expression. Because p38 MAPK regulates NFAT activity (Wu et al., 2003) and NFAT is known to bind the IL-2 gene promoter (Rooney et al., 1995), I examined IL-2 expression by Western blot and real-time PCR to see if, and how, inhibition of p38 MAPK affected expression of the IL-2 gene. Intracellular IL-2 levels were analysed in activated T cells by Western blot after treating them with BfA, a fungal macrocyclic lactone that shuts down Golgi trafficking (Sciaky et al., 1997). Figure 3.11A reveals that intracellular IL-2 was virtually undetectable by Western blot in Thy-1/CD28-activated T cells; however, addition of BfA to the cell cultures allowed IL-2 to be detected. When p38 MAPK was inhibited by pretreatment with SB203580, intracellular IL-2 expression was elevated beyond that observed in T cells activated in the absence of SB203580. To determine if p38 MAPK inhibition was affecting IL-2 expression at the level of mRNA synthesis, I carried out real-time PCR amplification of IL-2 mRNA from T cells activated via Thy-1 and CD28 in the presence of SB203580 or drug vehicle alone (Figure 3.11B). Induction of IL-2 mRNA expression was determined using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001), and is expressed relative to fold

induction in the cells pretreated with vehicle. Blockade of p38 MAPK with SB203580 resulted in three times more IL-2 message being produced after Thy-1/CD28 costimulation than in vehicle-treated control cells. Although preliminary, these data suggest that p38 MAPK regulates IL-2 mRNA expression in Thy-1/CD28-stimulated T cells. However, based on these data alone we can not discriminate between enhanced mRNA stability and increased transcription.

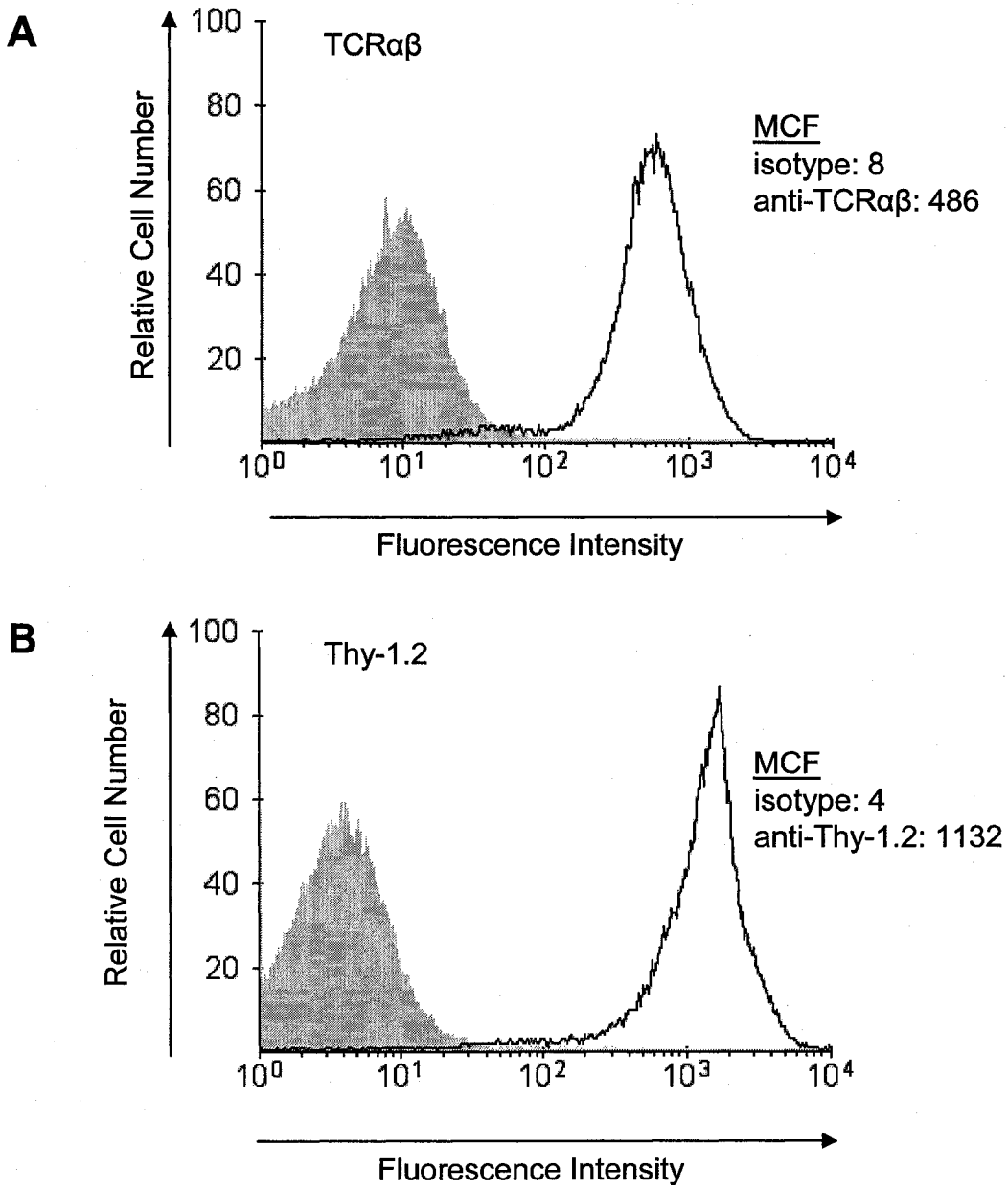


Figure. 3.1. T Cells Obtained From Splenocytes With T Cell Enrichment Immunocolumns Are TCRαβ- And Thy-1.2-Positive. Spleen leukocytes were passed through a $CD3^+$ T cell enrichment column and depleted of NK cells by treatment with anti-asialoGMI antibody plus complement. The cells were then stained with (a) PE-conjugated anti-TCRαβ (clone H57-597) mAb (open peak) or PE-conjugated hamster IgG (shaded peak); (b) FITC-conjugated anti-Thy-1.2 (clone 30-H12; open peak) or FITC-conjugated rat IgG2b (shaded peak); and analysed by flow cytometry. T cells were consistently ~95% TCRαβ-positive and ~99% Thy-1.2-positive. Mean channel fluorescence (MCF) values are shown. Results are from individual representative experiments (n=3).

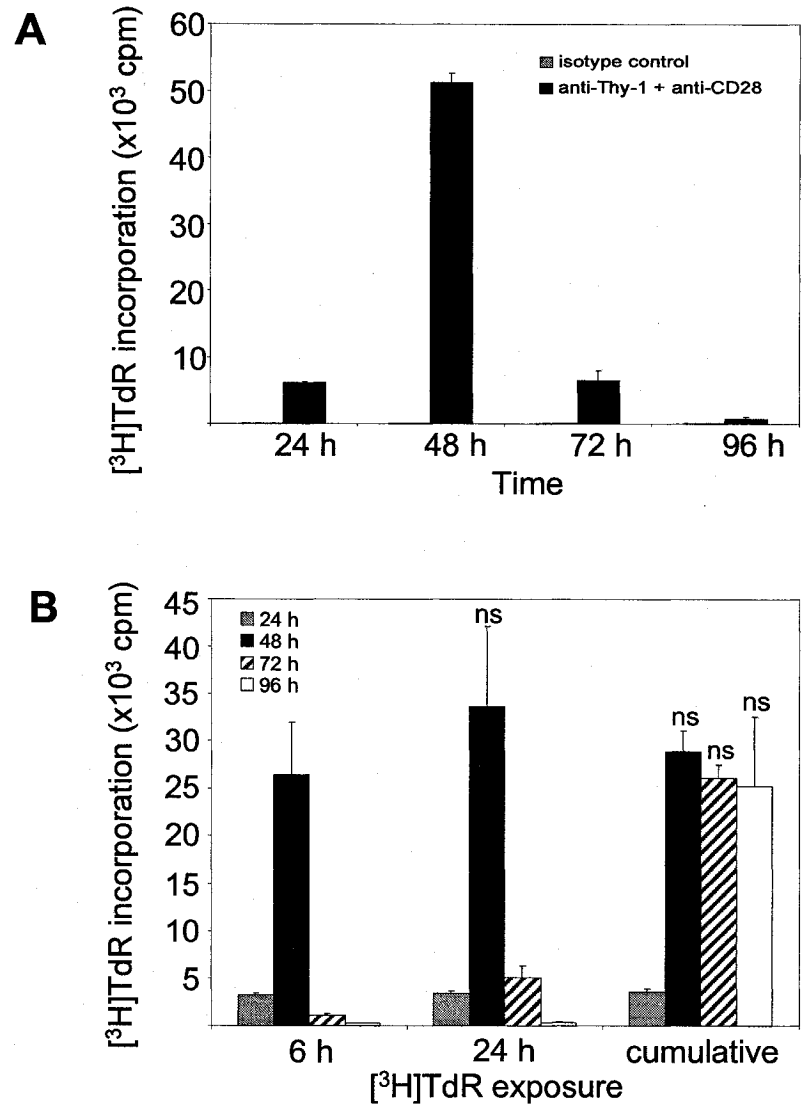
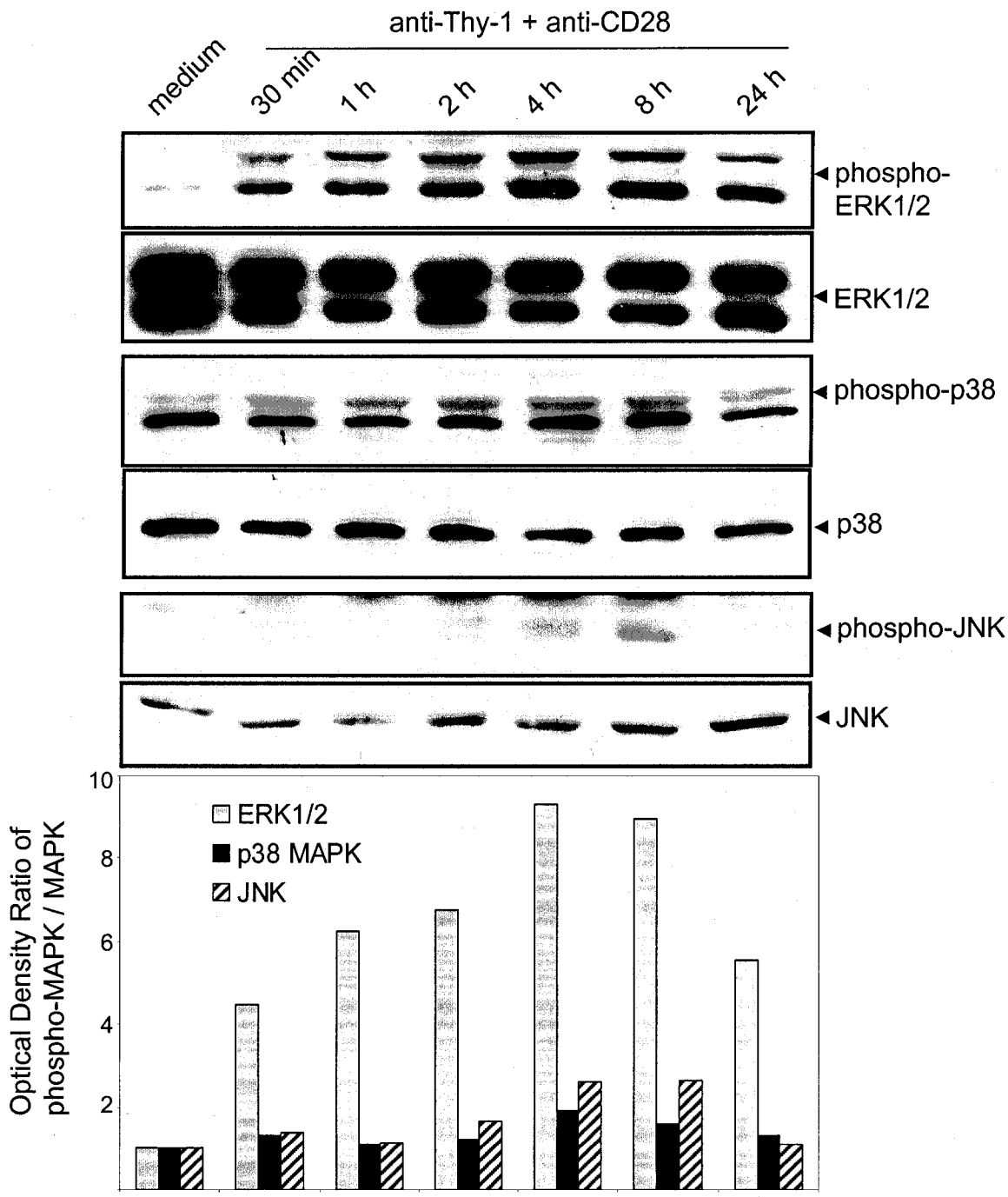


Figure. 3.2. Microbeads Coated With Anti-Thy-1 And Anti-CD28 mAbs Induce Maximal T Cell Activation At 48 h. (A) T cells and microbeads coated with anti-Thy-1 mAb (clone G7) and anti-CD28 mAb (clone 37.51.1) (black bars) or isotype control antibodies (grey bars) were mixed at a 1:1 ratio in a 96-well flat-bottom tissue culture plate. T cell activation was measured after 24 h, 48 h, 72 h, and 96 h of culture in quadruplicate wells by [^{3}H]TdR incorporation. Data from one representative experiment are shown as mean cpm ± SD (n=2). (B) T cells and microbeads coated with anti-Thy-1 mAb (clone G7) and anti-CD28 mAb (clone 37.51.1) were mixed at a 1:1 ratio in a 96-well flat-bottom tissue culture plate, and were pulsed with 0.5 µCi [^{3}H]TdR for 6 h, 24 h, or the duration of the time course (cumulative) and T cell activation was measured at 24 h (grey bars), 48 h (black bars), 72 h (hatched bars), and 96 h (white bars) post-stimulation in quadruplicate wells. Data from one experiment are shown as mean cpm ± SD. ns = not significant, compared to 6-h pulse at 48 h.

Figure. 3.3. Microbeads Coated With Anti-Thy-1 And Anti-CD28 mAbs Induce MAPK Phosphorylation. T cells and microbeads coated with anti-Thy-1 mAb (clone G7) and anti-CD28 mAb (clone 37.51.1) were mixed at a 1:1 ratio for 30 min, 1 h, 2 h, 4 h, 8 h, and 24 h. The negative control consisted of T cells that were exposed to medium alone for 5 min. T cells lysates were prepared and Western blotting was performed with phospho-specific antibodies for ERK1/2, p38 MAPK, and JNK to detect MAPK phosphorylation. Blots were stripped and reprobed with antibodies specific for ERK1/2, p38 MAPK, and JNK to indicate total protein loading. Results are representative of two different experiments. Optical density ratios were calculated by comparing the density of individual phospho-MAPK bands with the corresponding total MAPK band. Data were normalised to the optical density ratio for each MAPK in negative control cells.


anti-Thy-1 + anti-CD28
medium
30 min
1 h
2 h
4 h
8 h
24 h
phospho-ERK1/2
ERK1/2
phospho-p38
p38
phospho-JNK
JNK
Optical Density Ratio of phospho-MAPK / MAPK
10
8
6
4
2
ERK1/2
p38 MAPK
JNK

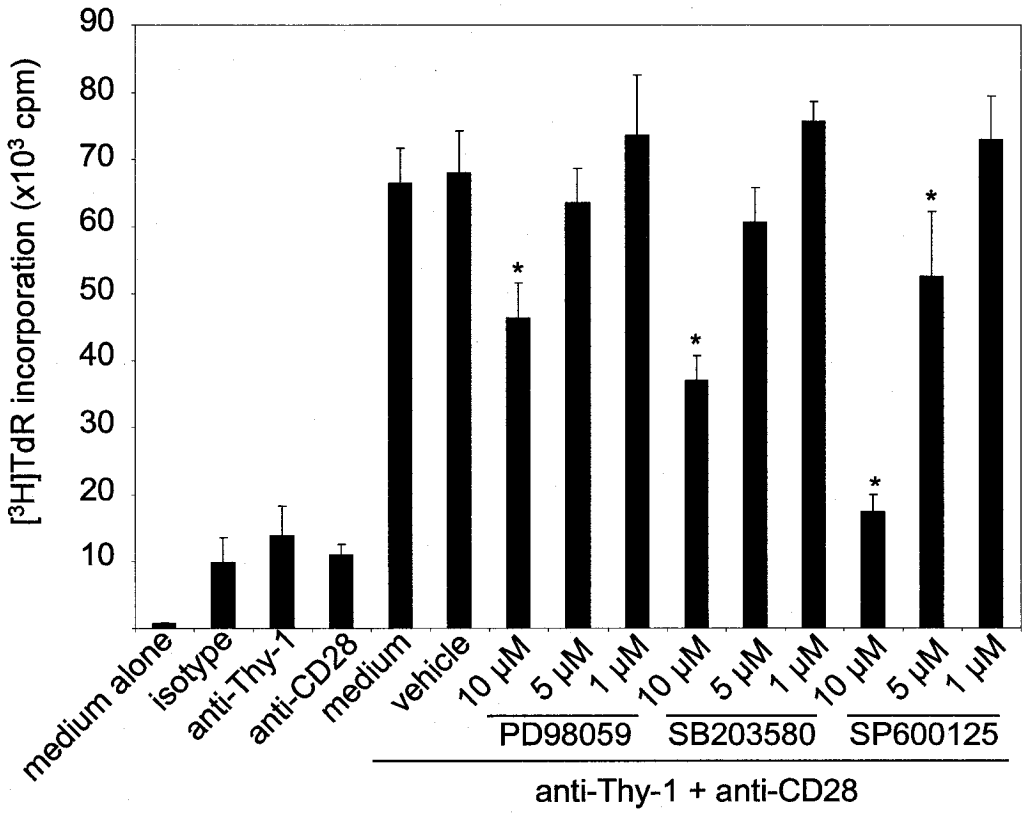


Figure. 3.4. Inhibition Of ERK1/2, p38 MAPK, Or JNK Attenuates Thy-1/CD28-Mediated T Cell Activation. T cells were pretreated with PD98059 (a MEK1 inhibitor), SB203580 (a p38 MAPK inhibitor) or SP600125 (a JNK inhibitor) at 10 µM, 5 µM, or 1 µM, with the drug vehicle alone, or medium alone for 30 min. T cells were then exposed to microbeads coated with anti-Thy-1 mAb (clone G7) and/or anti-CD28 mAb (clone 37.51.1) or isotype control antibodies at a 1:1 ratio, or medium alone. T cell activation was measured after 48 h in quadruplicate wells by [^{3}H]TdR incorporation. Data from one representative experiment are shown as mean cpm ± SD (n=3). * denotes $p < 0.05$, compared to vehicle control.

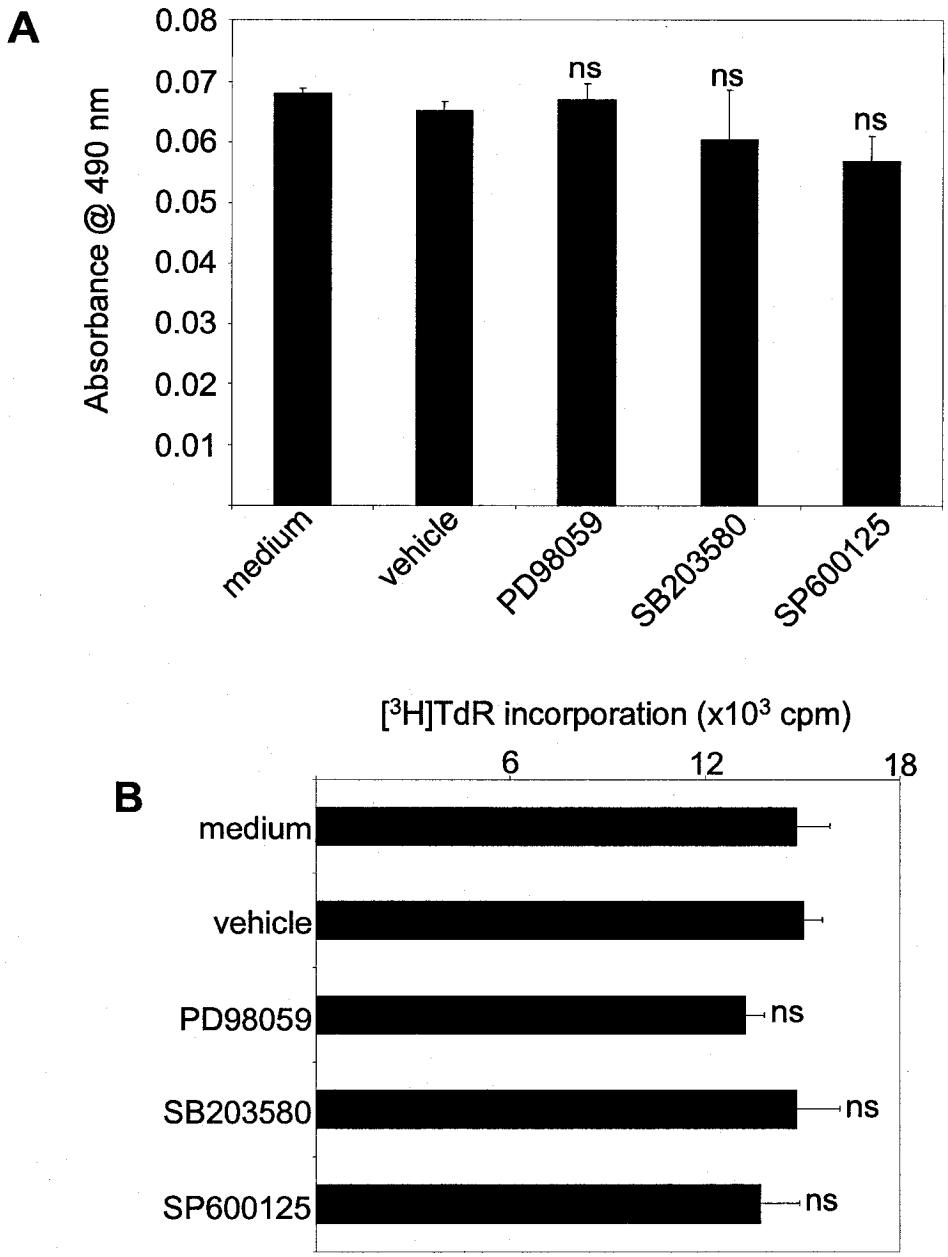


Figure. 3.5. Attenuation Of Thy-1/CD28-Induced T Cell Activation By MAPK Inhibitors Is Not Due To Decreased Cell Viability. (A) T cells were exposed to 10 μM PD98059 (a MEK1 inhibitor), SB203580 (a p38 MAPK inhibitor), SP600125 (a JNK inhibitor), the drug vehicle alone, or medium alone for 24 h. Mitochondrial reductase activity was then measured by MTT assay in quadruplicate wells as an indicator of T cell viability. Data are presented as absorbance @ 490 nm ± SD. (B) EL4 mouse lymphoma cells were pretreated with 10 μM PD98059, SB203580, SP600125, or vehicle for 30 min, and [^{3}H]TdR incorporation was measured in quadruplicate wells 6 h later. Data from one experiment are presented as mean cpm ± SD (n=3). ns = not significant, compared to vehicle control.

Table 3.1. MAPK Inhibitors Are Not Toxic To T Cells. T cells were exposed to 10 μM PD98059 (a MEK1 inhibitor), SB203580 (a p38 MAPK inhibitor), SP600125 (a JNK inhibitor), or vehicle for 24 h. The cells were then stained with Trypan Blue vital dye and counted on a light microscope. Data are presented as mean values ± SEM from three experiments.

Treatment	**% viable ± SEM**
Vehicle	81 ± 6
PD98059	78 ± 3
SB203580	80 ± 11
SP600125	79 ± 6

Figure 3.6. Alternate MAPK Inhibitors Also Attenuate Thy-1/CD28-Mediated T Cell Activation. T cells were pretreated with (A) SL327 (a MEK1 inhibitor), (B) ML3403 (a p38 MAPK inhibitor) or (C) the peptide inhibitor of JNK, (L)-JNKI1, at the indicated concentrations or vehicle alone for 30 min. The cells were then exposed to microbeads coated with anti-Thy-1 mAb (clone G7) and anti-CD28 mAb (clone 37.51.1) at a 1:1 ratio and T cell activation was measured in quadruplicate wells after 48 h by [^{3}H]TdR incorporation. Data from individual experiments are presented as mean cpm ± SD (n=2). * denotes $p < 0.05$, compared to vehicle control.

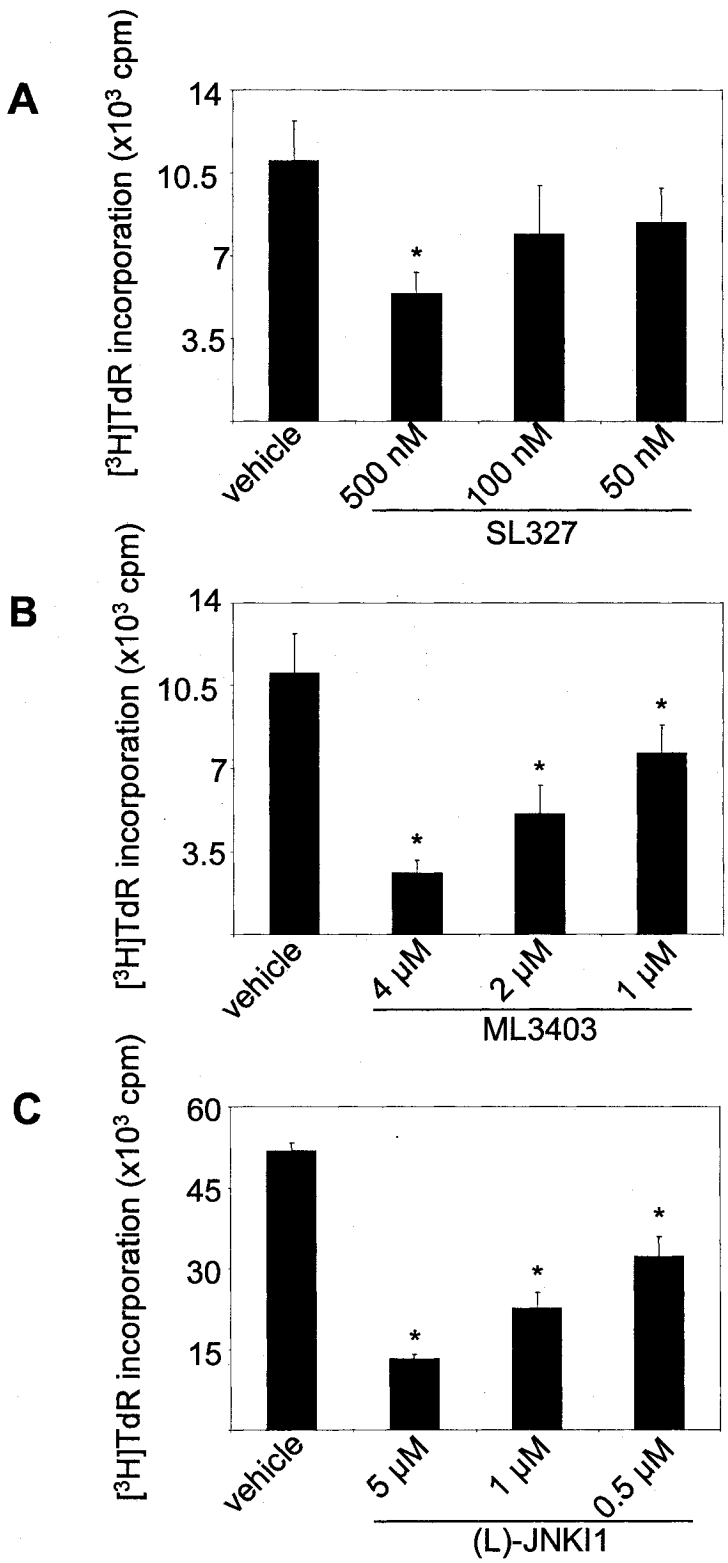
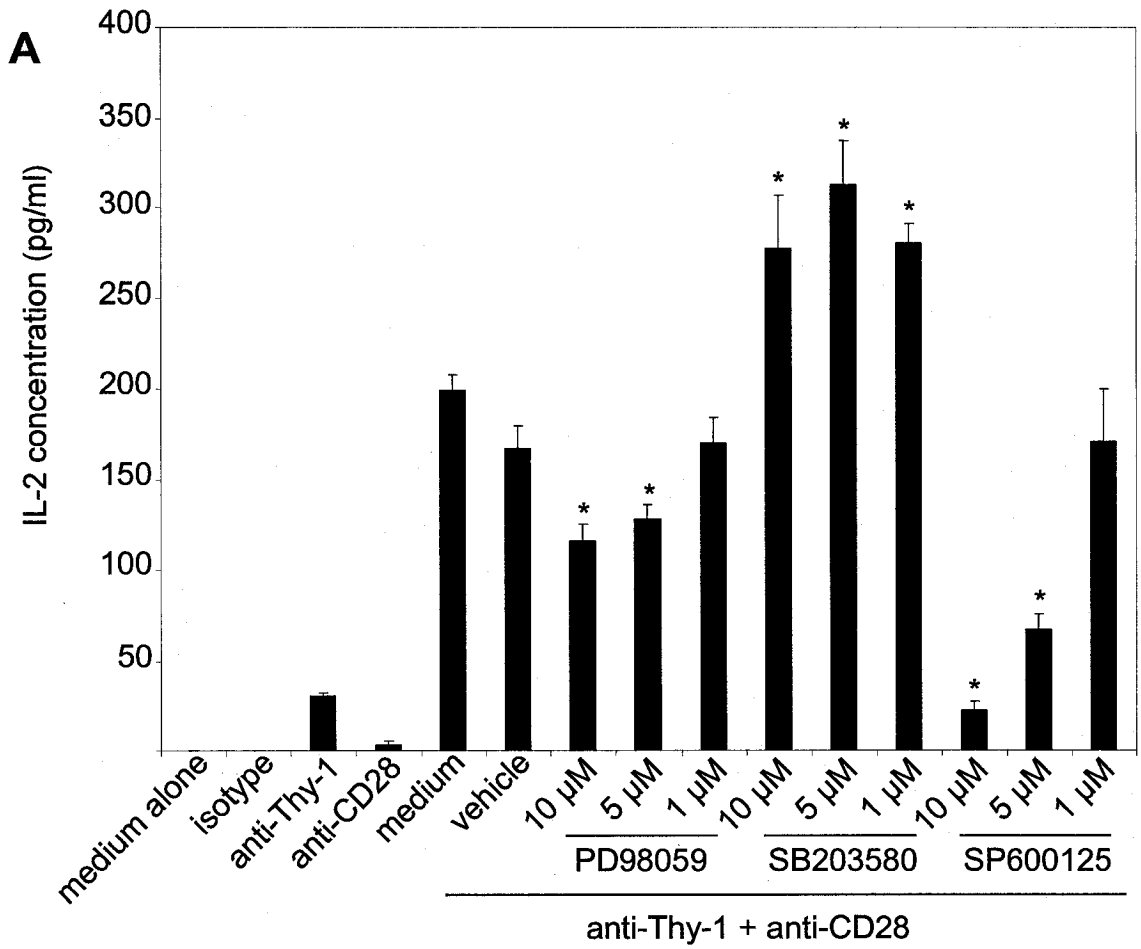

A
[3H]TdR incorporation (x10^3 cpm)
14
10.5
7
3.5
vehicle
500 nM
100 nM
50 nM
SL327
B
[3H]TdR incorporation (x10^3 cpm)
14
10.5
7
3.5
vehicle
4 μM
2 μM
1 μM
ML3403
C
[3H]TdR incorporation (x10^3 cpm)
60
45
30
15
vehicle
5 μM
1 μM
0.5 μM
(L)-JNKI1

Figure. 3.7. Inhibition Of ERK1/2, p38 MAPK, Or JNK Modulates Thy-1/CD28-Induced IL-2 Production. (A) T cells were pretreated with PD98059 (a MEK1 inhibitor), SB203580 (a p38 MAPK inhibitor) or SP600125 (a JNK inhibitor) at 10 μM, 5 μM, or 1 μM, or with the drug vehicle (DMSO) alone, or medium alone for 30 min and then exposed to microbeads coated with anti-Thy-1 mAb (clone G7) and/or anti-CD28 mAb (clone 37.51.1), or isotype control antibodies (bead: cell ratio of 1:1) or medium. IL-2 production was measured after 24 h by sandwich ELISA. Data from one representative experiment are shown as mean IL-2 concentration (pg/ml) ± SD (n>3). (B) T cells were pretreated with 10 μM PD98059, SB203580 or SP600125, or vehicle, 30 min prior to, or 24 h after, exposure to microbeads coated with anti-Thy-1 mAb (clone G7) and anti-CD28 mAb (clone 37.51.1). [^{3}H]TdR incorporation was measured in quadruplicate wells 48 h after addition of beads. Data from one representative experiment are shown as mean cpm ± SD (n=2). * denotes $p < 0.05$, compared to vehicle control.


A
IL-2 concentration (pg/ml)
400
350
300
250
200
150
100
50
medium alone
isotype
anti-Thy-1
anti-CD28
medium
vehicle
10 μM
5 μM
1 μM
10 μM
5 μM
1 μM
10 μM
5 μM
1 μM
PD98059
SB203580
SP600125
anti-Thy-1 + anti-CD28

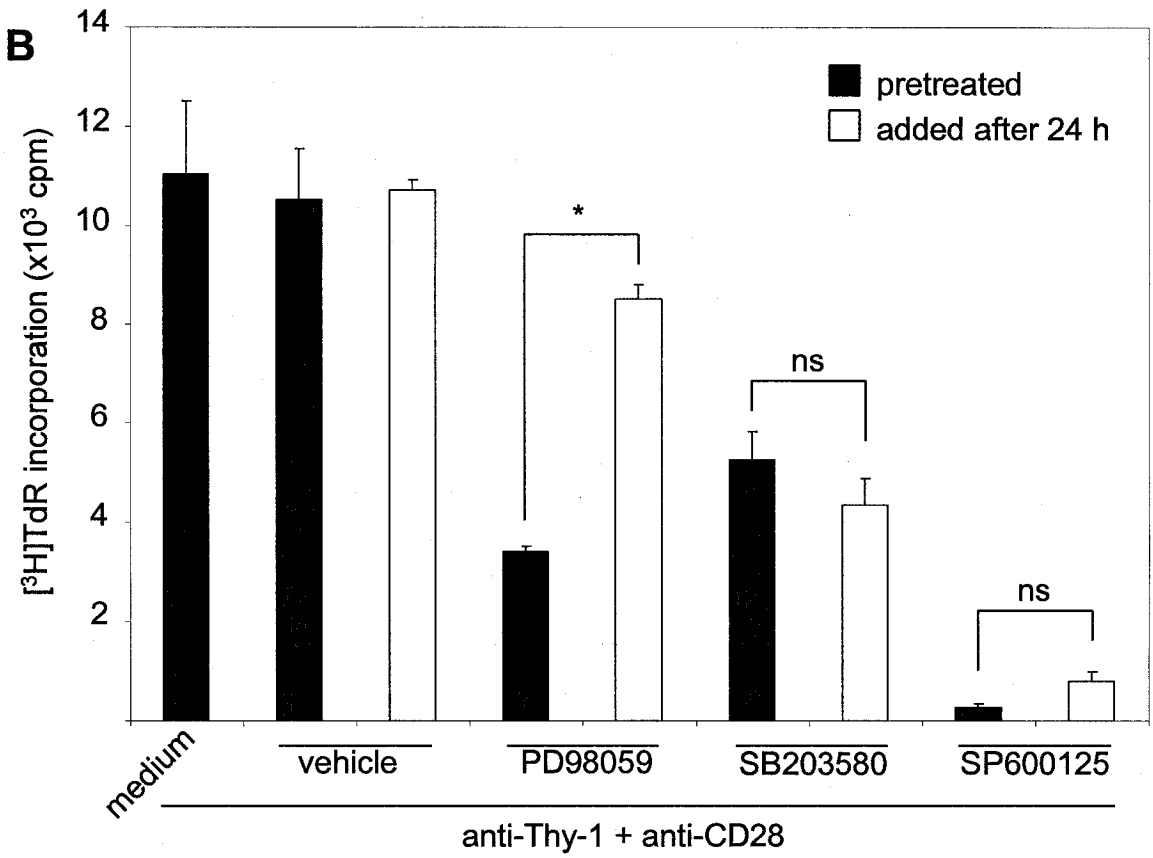
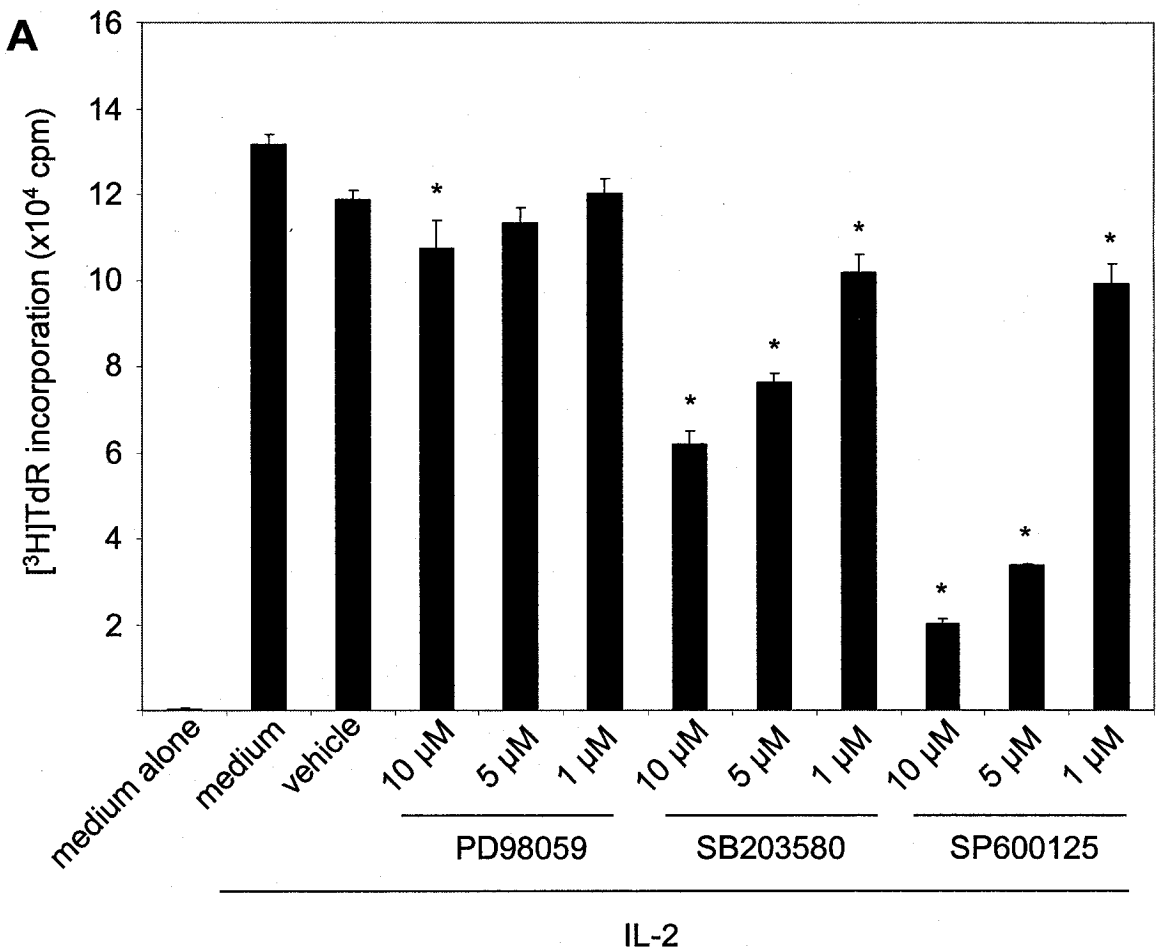
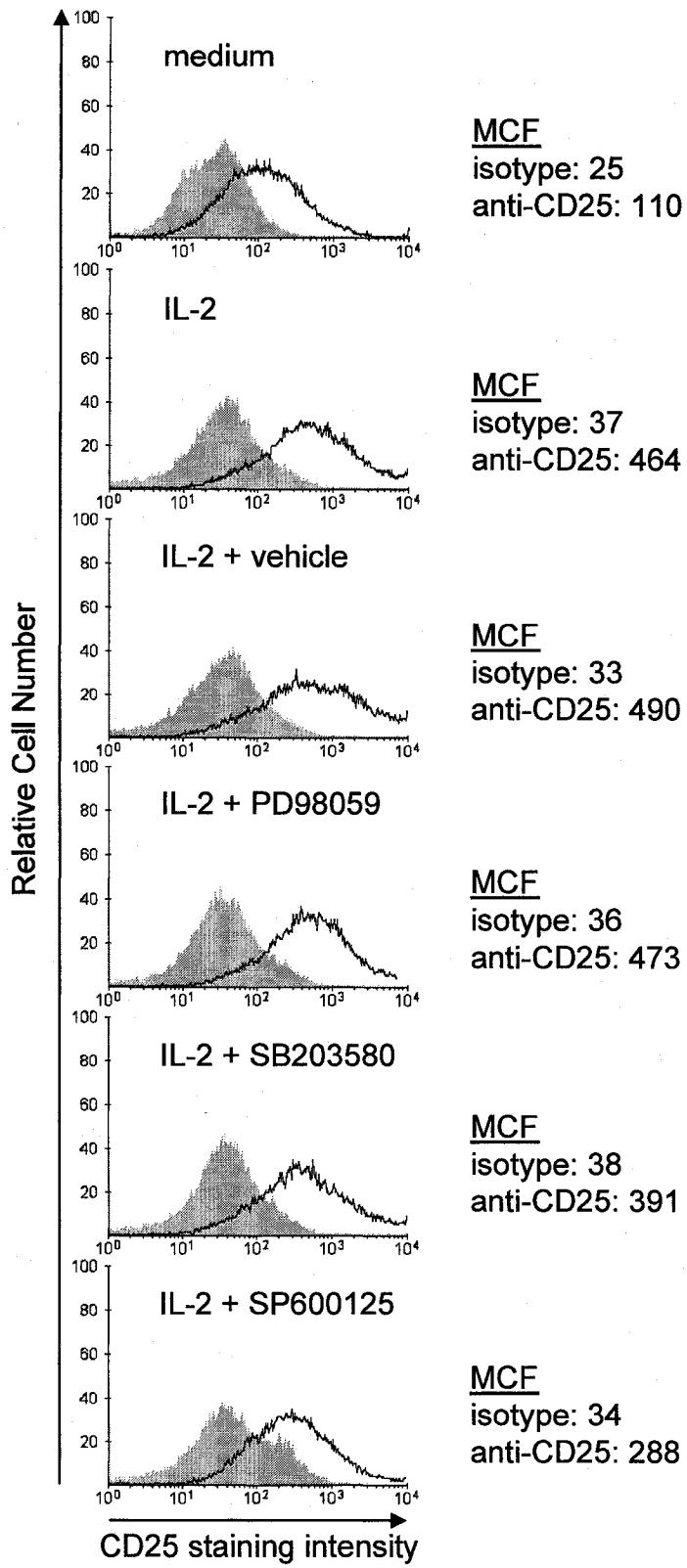

B
14
12
10
8
6
4
2
[3H]TdR incorporation (x10³ cpm)
pretreated
added after 24 h
*
ns
ns
medium
vehicle
PD98059
SB203580
SP600125
anti-Thy-1 + anti-CD28

Figure 3.8. IL-2-Dependent T Cell Growth Is Impaired By Inhibition Of p38 MAPK Or JNK. CTLL-2 cells were starved of IL-2 for 3 h and pretreated with PD98059 (a MEK1 inhibitor), SB203580 (a p38 MAPK inhibitor) or SP600125 (a JNK inhibitor) at 10 μM, 5 μM, or 1 μM, or with drug vehicle for 30 min before addition of 50 U/ml IL-2. (A) Proliferation was measured in quadruplicate wells after 48 h by [^{3}H]TdR incorporation. Data from one representative experiment are shown as mean cpm ± SD (n=3). (B) CTLL-2 cells were stained with PE-conjugated anti-CD25 (open peaks) or isotype control antibodies (shaded peaks) and analysed by flow cytometry. Mean channel fluorescence (MCF) values are shown. Results are from one representative experiment (n=3). * denotes $p < 0.01$, compared to vehicle control.


A
16
14
12
10
8
6
4
2
[3H]TdR incorporation (x10^4 cpm)
*
medium alone
medium
vehicle
10 μM
5 μM
1 μM
10 μM
5 μM
1 μM
10 μM
5 μM
1 μM
PD98059
SB203580
SP600125
IL-2

B


medium
MCF
isotype: 25
anti-CD25: 110
IL-2
MCF
isotype: 37
anti-CD25: 464
IL-2 + vehicle
MCF
isotype: 33
anti-CD25: 490
IL-2 + PD98059
MCF
isotype: 36
anti-CD25: 473
IL-2 + SB203580
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anti-CD25: 288
Relative Cell Number
CD25 staining intensity

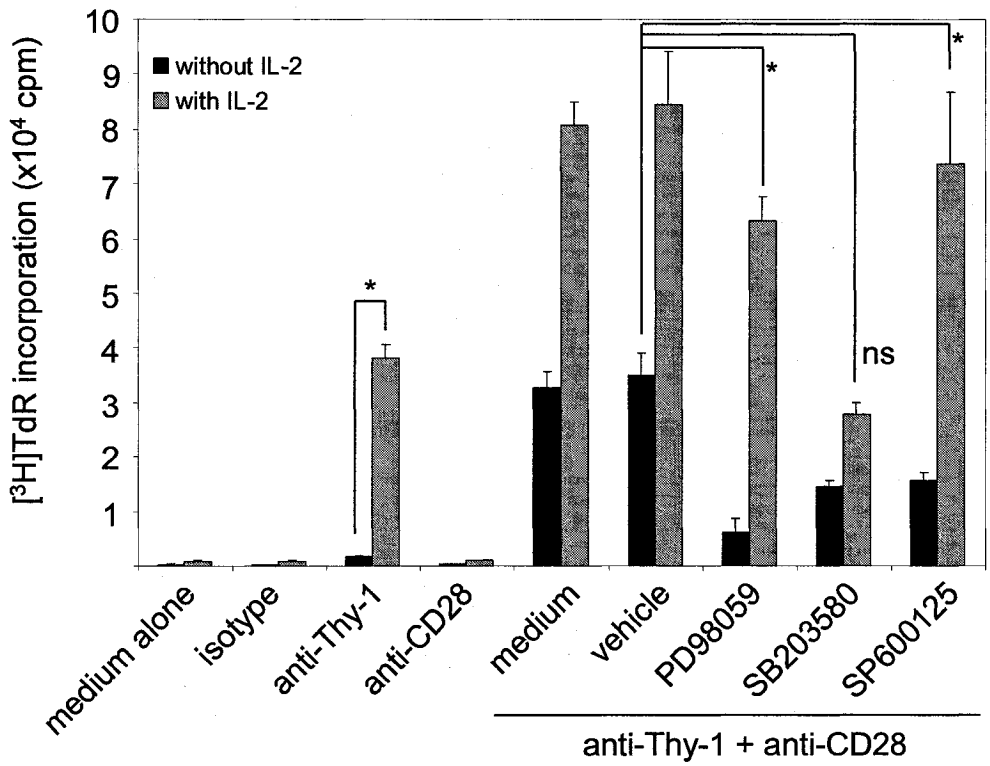


Figure 3.9. Exogenous IL-2 Restores [^{3}H]TdR Incorporation By T Cells Treated With Inhibitors Of ERK1/2 Or JNK. T cells were pretreated with 10 μM PD98059 (a MEK1 inhibitor), SB203580 (a p38 MAPK inhibitor) or SP600125 (a JNK inhibitor), or with drug vehicle, or medium for 30 min. Cells were then exposed to microbeads coated with anti-Thy-1 mAb (clone G7) and/or anti-CD28 mAb (clone 37.51.1) or isotype control antibodies at a 1:1 ratio, or medium alone with or without 50 U/ml IL-2. T cell activation was measured in quadruplicate wells after 48 h by [^{3}H]TdR incorporation. Data from one representative experiment are shown as mean cpm ± SD (n=2). * denotes $p < 0.001$, ns = not significant.

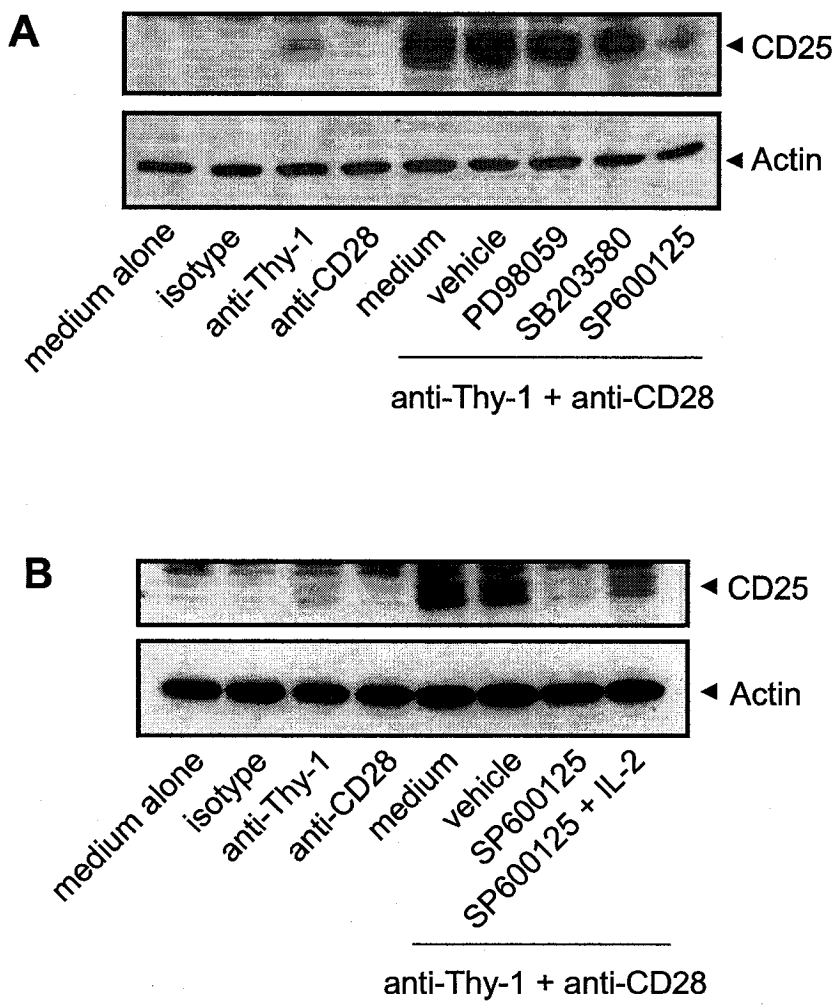


Figure 3.10. Inhibition Of JNK Blocks Thy-1/CD28-Induced CD25 Expression In Primary T Cells. (A) T cells were pretreated with 10 μM PD98059 (a MEK1 inhibitor), SB203580 (a p38 MAPK inhibitor) or SP600125 (a JNK inhibitor), or with drug vehicle or medium, for 30 min and then exposed to medium or microbeads coated with anti-Thy-1 mAb (clone G7) and/or anti-CD28 mAb (clone 37.51.1) or isotype control antibodies at a 1:1 ratio for 24 h. (B) T cells were exposed to medium, vehicle, or microbeads coated with anti-Thy-1 mAb (clone G7) and/or anti-CD28 mAb (clone 37.51.1) or isotype control antibodies at a 1:1 ratio with or without 50 U/ml IL-2 as indicated for 24 h. Thy-1/CD28-stimulated cells were pretreated with 10 μM SP600125 or vehicle for 30 min before bead addition. Cell lysates were prepared and Western blotting was carried out with antibodies specific for CD25. Blots were stripped and reprobed with antibodies for actin to indicate total protein loading. Results from individual representative experiments are shown (n=2).

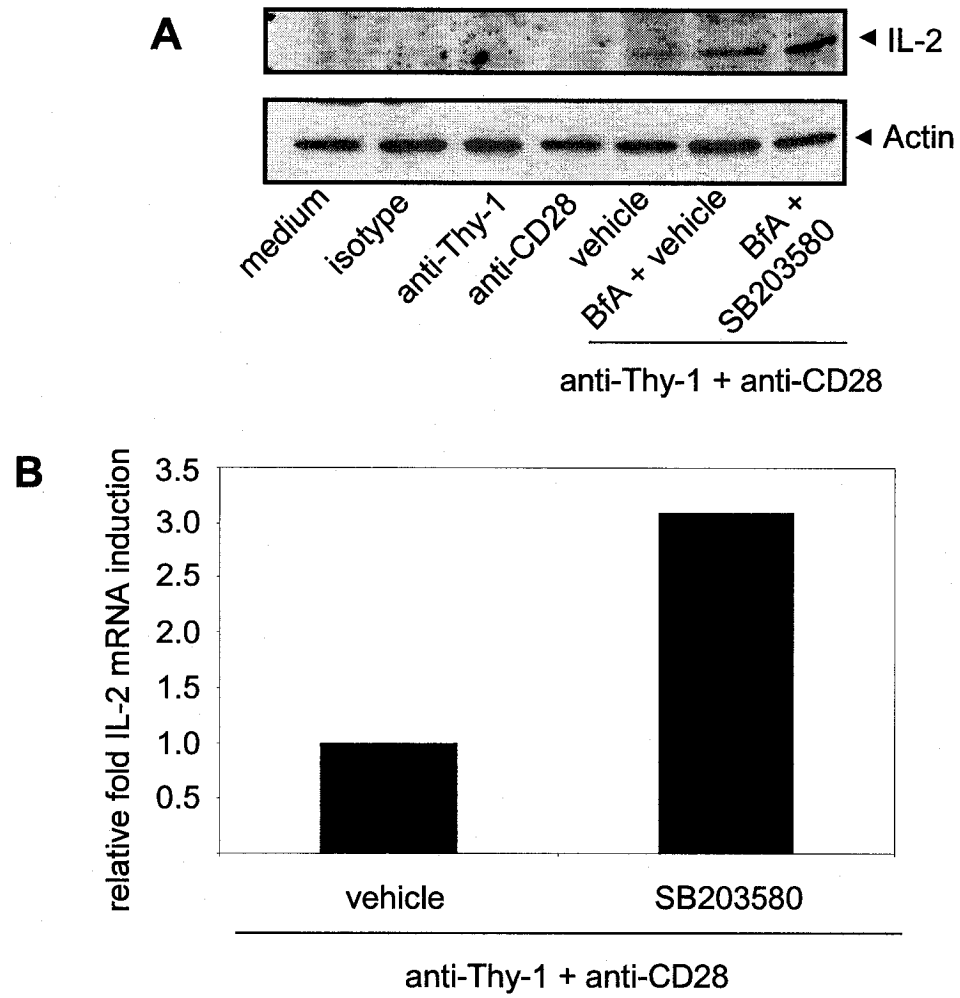


Figure 3.11. Inhibition Of p38 MAPK Enhances Thy-1/CD28-Induced IL-2 Expression. (A) T cells were pretreated with 10 µM SB203580 (a p38 MAPK inhibitor), vehicle, or medium for 30 min and exposed to medium or microbeads coated with anti-Thy-1 mAb (clone G7) and/or anti-CD28 mAb (clone 37.51.1) or isotype control antibodies at a 1:1 ratio for 24 h. During the final 6 h of culture some cells were exposed to 10 µg/ml BfA, or the drug vehicle, as indicated. Cell lysates were prepared and Western blotting was carried out with antibodies specific for IL-2. Blots were stripped and reprobed with antibodies for actin to indicate total protein loading. Results from one representative experiment are shown (n=2). (B) T cells were pretreated with 10 µM SB203580 or vehicle for 30 min and exposed to microbeads coated with anti-Thy-1 mAb (clone G7) and anti-CD28 mAb (clone 37.51.1) or isotype control antibodies at a 1:1 ratio for 18 h. cDNA was generated from total RNA, and real-time PCR was carried out with primers for IL-2 and RNA Pol II. Relative expression of IL-2 mRNA was calculated according to the $2^{-\Delta\Delta C_T}$ method and normalised to the vehicle-treated sample. Data are from one experiment.

Chapter 4. Results: Pharmacological Inhibitors Identify Components Of The Thy-1 Signalling Pathway In T Cells

Amidst the many publications on Thy-1 signalling in T cells, there are no studies examining Thy-1 signalling in the absence of costimulation. Because of this, very little is known about a *bona fide* Thy-1 signalling pathway in T cells. It has been implied that Thy-1 signals through components of the TCR signalling pathway, as Thy-1 stimulation does not activate T cells in the absence of a functional TCR/CD3 complex (Gunter et al., 1987). A more recent study has shown that in the absence of Thy-1, TCR stimulation does not result in productive intracellular signalling (Leyton et al., 1999). Of the important signalling intermediates in the TCR/CD3 pathway, PTK, MAPK, PI3K, calcineurin, PKC (Haeryfar and Hoskin, 2001), and LAT (Leyton et al., 1999) have been implicated in Thy-1 signal transduction in the context of costimulatory signalling.

The discovery of a Thy-1 ligand in the neuronal system has brought about renewed interest in the function of Thy-1, albeit exclusively in neurons. As a result, some advances have recently been made in our understanding of Thy-1 signalling. Specifically, protein kinase A (PKA), VASP, and CREB all appear to be involved in transducing a Thy-1 signal in neurons (Chen et al., 2007). I took advantage of the observation that Thy-1 stimulation induced CD25 expression (Figure 3.10) to evaluate the role of these signalling molecules in the transduction of a Thy-1 signal in purified T cells.

4.1. Thy-1 Stimulation Induces CD25 Expression In T Cells

The first indication that Thy-1 stimulation might induce CD25 expression was that Thy-1 stimulation in the presence of exogenous IL-2 resulted in T cell

activation (Figure 3.9). I subsequently observed that Thy-1 signalling resulted in CD25 expression at 24 h (Figure 3.10). To explore the kinetics of Thy-1-induced CD25 expression, I stimulated T cells with anti-Thy-1 mAb-coated microbeads for 8 h, 16 h, or 24 h. CD25 expression was then examined by Western blot. CD25 expression was induced as early as 8 h, but was clearly maximal after 24 h of stimulation with anti-Thy-1 antibodies (Figure 4.1).

PTKs, which mediate myriad important signalling processes, including T cell activation (Haeryfar and Hoskin, 2001), were found to be involved in Thy-1 induced CD25 expression. Figure 4.2 shows that at all doses assayed, pretreatment with the PTK inhibitor herbimycin A impaired Thy-1-induced expression of CD25, implicating a vital role for PTK in Thy-1 signalling.

4.2. ERK1/2 And JNK Mediate Thy-1-Induced CD25 Expression

In a situation where T cells are costimulated by Thy-1 and CD28 together, I used MAPK inhibitors to show that ERK1/2, p38 MAPK, and JNK play important, but different, roles in T cell activation. Here, I employed the same MAPK inhibitors to determine if ERK1/2, p38 MAPK, or JNK were important in the induction of CD25 expression as a result of Thy-1 signalling. ERK1/2 and JNK were both found to mediate CD25 expression (Figure 4.3A and Figure 4.3C, respectively), while inhibition of p38 MAPK had no effect on Thy-1-induced expression of CD25 (Figure 4.3B).

4.3. PLC And PKC Mediate Thy-1-Induced CD25 Expression

LAT is suggested to be an important component of a Thy-1 signal transduction pathway in T cells (Leyton et al., 1999). The principal function of

LAT in T cell activation is to activate PLCγ (Finco et al., 1998; Zhang et al., 1998), thus promoting the catabolism of PIP_2 to IP_3 plus DAG, which activates PKC (Hodgkin et al., 1998). To determine if PLCγ and PKC also played a role in Thy-1 signalling, I examined Thy-1-induced CD25 expression after Thy-1 stimulation of T cells pretreated with inhibitiors of these signalling molecules. Inhibition of either PLCγ (Figure 4.4A) or PKC (Figure 4.4B) resulted in decreased CD25 expression, indicating that both of these signalling intermediates are important in Thy-1-induced CD25 expression.

4.4. Ca^{2+}/Calmodulin-Dependent Protein Kinase II (CaMKII) And Calcineurin Mediate Thy-1-Induced CD25 Expression

CaMKII is a calmodulin-dependent kinase that is involved in T cell responses to Ca^{2+} signalling, as well as mediating NFκB activation (Dupont and Goldbeter, 1998; Ishiguro et al., 2006). Calcineurin is the calmodulin-dependent phosphatase that dephosphorylates NFAT, allowing it to enter the nucleus and promote gene expression (Crabtree, 1999; Haeseleer et al., 2002). Because calmodulin is activated by Ca^{2+} signalling (Haeseleer et al., 2002) and Thy-1 stimulation is known to induce Ca^{2+} influx (Barboni et al., 1991), I asked whether these two calmodulin-regulated signalling molecules were involved in Thy-1-induced CD25 expression. T cells were pretreated with KN-93, a methoxybenzenesulfonamide compound that competitively inhibits CaMKII (Sumi et al., 1991), or with CsA, an inhibitor of calcineurin (Clipstone and Crabtree, 1992), and activated with anti-Thy-1 mAb-coated microbeads. Figure 4.5A shows that at the highest dose, KN-93 had a modest inhibitory effect on CD25 expression. However, at all concentrations tested, calcineurin completely blocked

Thy-1-induced CD25 expression (Figure 4.5B). These data support a role for CaMKII and, particularly, calcineurin in Thy-1 signalling in T cells.

4.5. PI3K Mediates Thy-1-Induced CD25 Expression

PI3K plays an important role in T cell activation (Ward et al., 1996) and a previous study suggests that PI3K is also involved in Thy-1 signalling (Haeryfar and Hoskin, 2001). To determine if PI3K mediates CD25 expression in purified T cells following Thy-1 stimulation, I pretreated T cells with wortmannin, a potent inhibitor of PI3K (Powis et al., 1994) prior to Thy-1 stimulation, and analysed induction of CD25 expression by Western blot. CD25 expression was dramatically inhibited in T cells that were pretreated with wortmannin (Figure 4.6A). Cell survival is among the functions associated with PI3K. Caspases regulate cell death, and Thy-1 signalling has also previously been shown to induce apoptosis in thymocytes (Hueber et al., 1994). Moreover, Thy-1 signalling is believed to be similar to TCR signalling, which is, at least in part, mediated by caspases (Chun et al., 2002; Misra et al., 2005). Caspases have even been shown to be necessary for anti-CD3 mAb-induced CD25 expression in human T cells (Falk et al., 2004). I therefore pretreated T cells with z-VAD-fmk to inhibit caspase activitation before Thy-1 stimulation, and again looked at CD25 expression by Western blot. Figure 4.6B shows that inhibition of caspases actually resulted in elevated Thy-1-induced CD25 expression. These data suggest that both PI3K and caspases are activated as a result of antibody-mediated Thy-1 triggering, but appear to carry out opposing functions with respect to Thy-1 signal transduction.

4.6. Thy-1 Stimulation Induces Phosphorylation Of VASP And CREB, And PKA Mediates Thy-1-Induced CD25 Expression

Thy-1 signalling in neurons involves VASP, CREB, and PKA (Chen et al., 2007). These molecules are all known to be involved in T cell activation (Boonyaratanakornkit et al., 2005; Krause et al., 2000), but there have been no studies addressing their function in Thy-1 signalling in T cells. Therefore, I stimulated T cells with anti-Thy-1 mAb-coated microbeads for 5 min, 30 min, 1 h, 2 h, 4 h, 8 h or 24 h, and examined VASP and CREB phosphorylation by Western blot (Figure 4.7). VASP and CREB phosphorylation were both most pronounced after 5 min stimulation and returned to background levels by 24 h. These findings implied that the upstream kinase PKA might also be involved in Thy-1 signalling. To investigate this possibility, I pretreated T cells with H-89, a selective inhibitor of PKA (Chijiwa et al., 1990), and induced CD25 expression by stimulating Thy-1 with mAb-coated beads. Figure 4.8 shows that inhibition of PKA activity with H-89 prevented expression of CD25, thereby suggesting that PKA is also an important signalling intermediate in Thy-1 signal transduction.

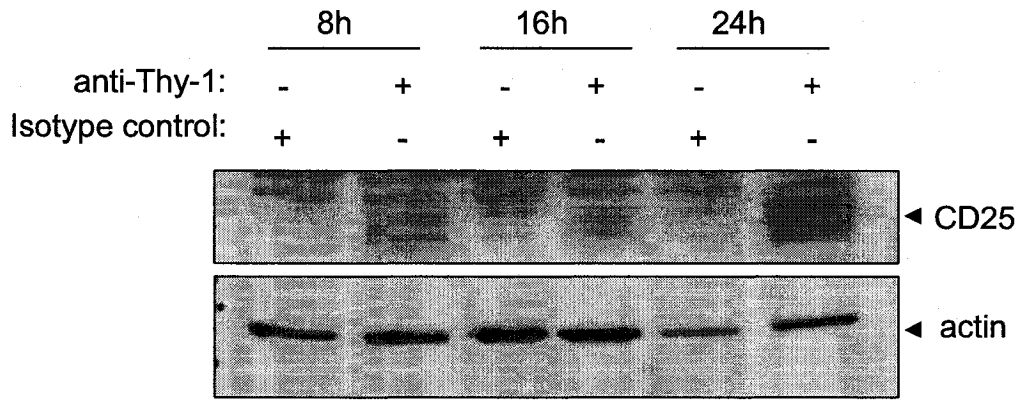


Figure 4.1. Thy-1 Stimulation Induces CD25 Expression In T Cells. T cells were exposed to anti-Thy-1 mAb or isotype control antibody-coated microbeads for 8 h, 16 h, or 24 h. Cell lysates were prepared and Western blotting was carried out with antibodies specific for CD25. Blots were stripped and reprobed with antibodies for actin to indicate total protein loading. Results from one experiment are shown.

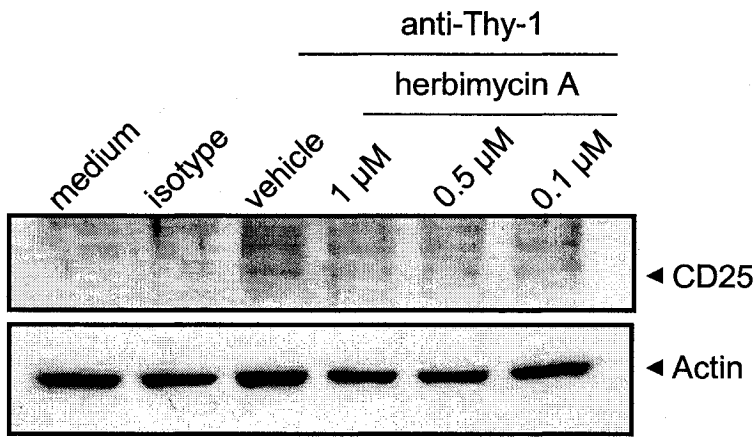
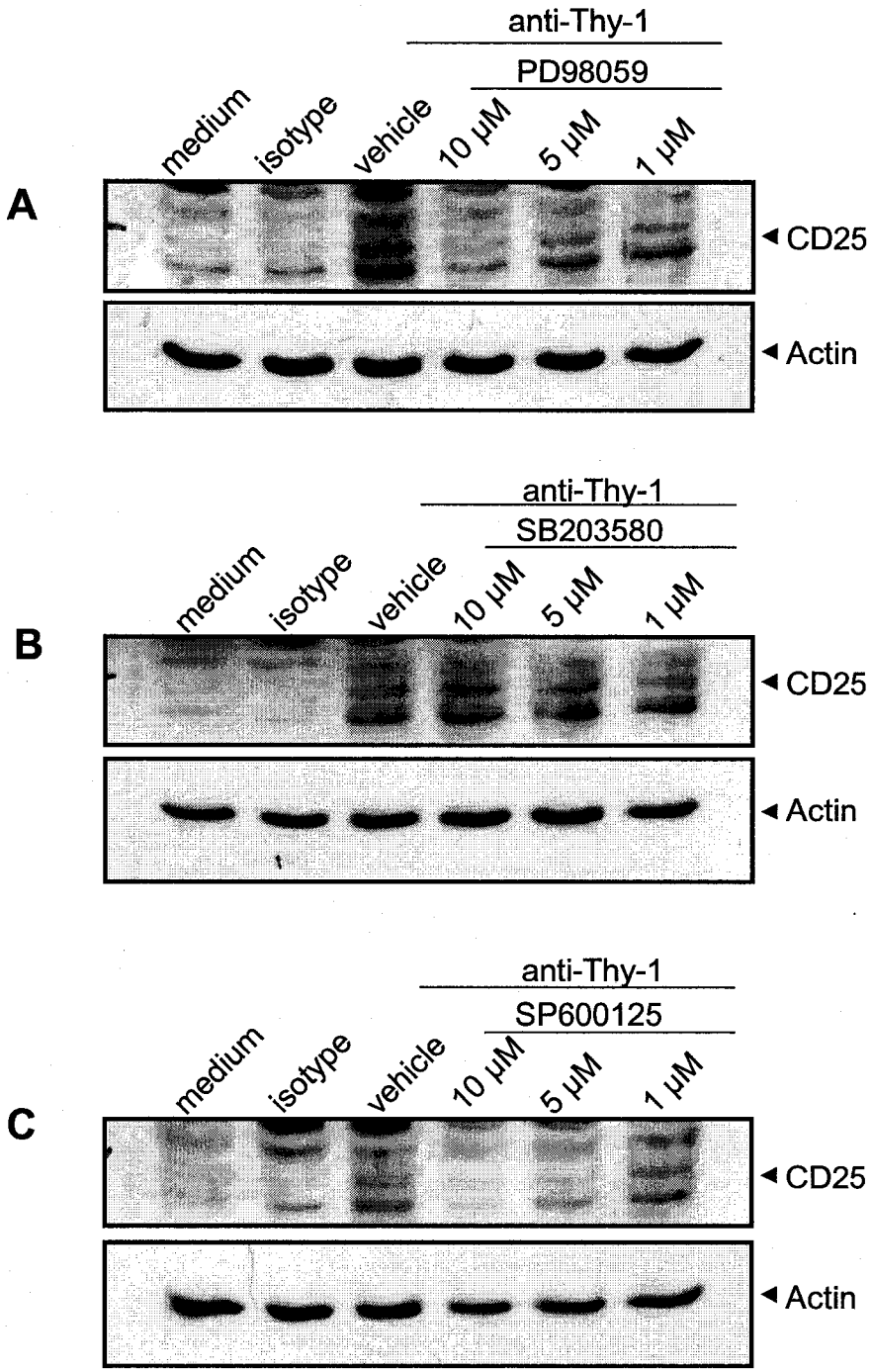


Figure 4.2. PTKs Mediate Thy-1-Induced CD25 Expression In T Cells. T cells were pretreated with herbimycin A (a PTK inhibitor) at the indicated concentrations, or with drug vehicle or medium for 30 min before addition of medium, anti-Thy-1 mAb-coated or isotype control antibody-coated microbeads. Cell lysates were prepared after 24 h and Western blotting was carried out with antibodies specific for CD25. Blots were stripped and reprobed with antibodies for actin to indicate total protein loading. Results from one representative experiment are shown (n=2).

Figure 4.3. ERK1/2 And JNK Mediate Thy-1-Induced CD25 Expression In T Cells. T cells were pretreated with (A) PD98059 (a MEK1 inhibitor), (B) SB203580 (a p38 MAPK inhibitor), or (C) SP600125 (a JNK inhibitor) at the indicated concentrations, vehicle or medium for 30 min before addition of medium, anti-Thy-1 mAb-coated microbeads or isotype control antibody-coated microbeads. Cell lysates were prepared after 24 h and Western blotting was carried out with antibodies specific for CD25. Blots were stripped and reprobed with antibodies for actin to indicate total protein loading. Results from individual representative experiments are shown (n=2).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PD98059
medium
isotype
vehicle
10 μM
5 μM
1 μM
A
CD25
Actin
anti-Thy-1
SB203580
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10 μM
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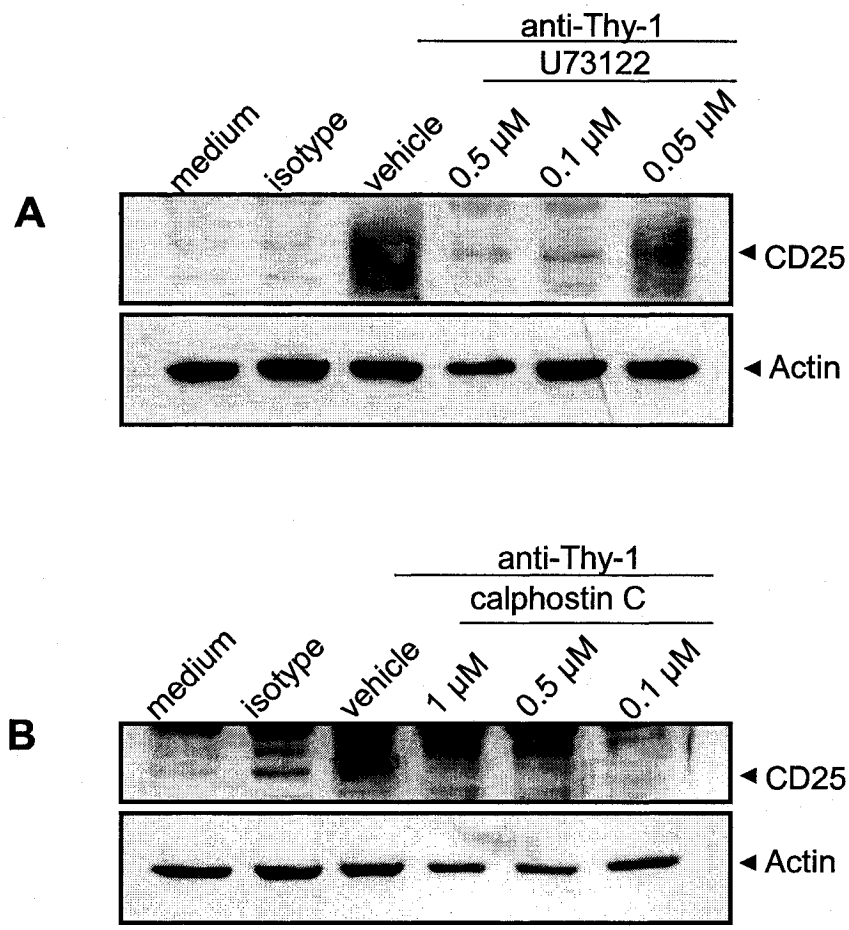


Figure 4.4. PLC And PKC Mediate Thy-1-Induced CD25 Expression In T Cells. T cells were pretreated with (A) U73122 (a PLC inhibitor) or (B) calphostin C (a PKC inhibitor) at the indicated concentrations, or with the drug vehicle or medium alone for 30 min before addition of medium, anti-Thy-1 mAb-coated microbeads or isotype control antibody-coated microbeads. Cell lysates were prepared after 24 h and Western blotting was carried out with antibodies specific for CD25. Blots were stripped and reprobed with antibodies for actin to indicate total protein loading. Results from individual representative experiments are shown (A: n=3; B: n=2).

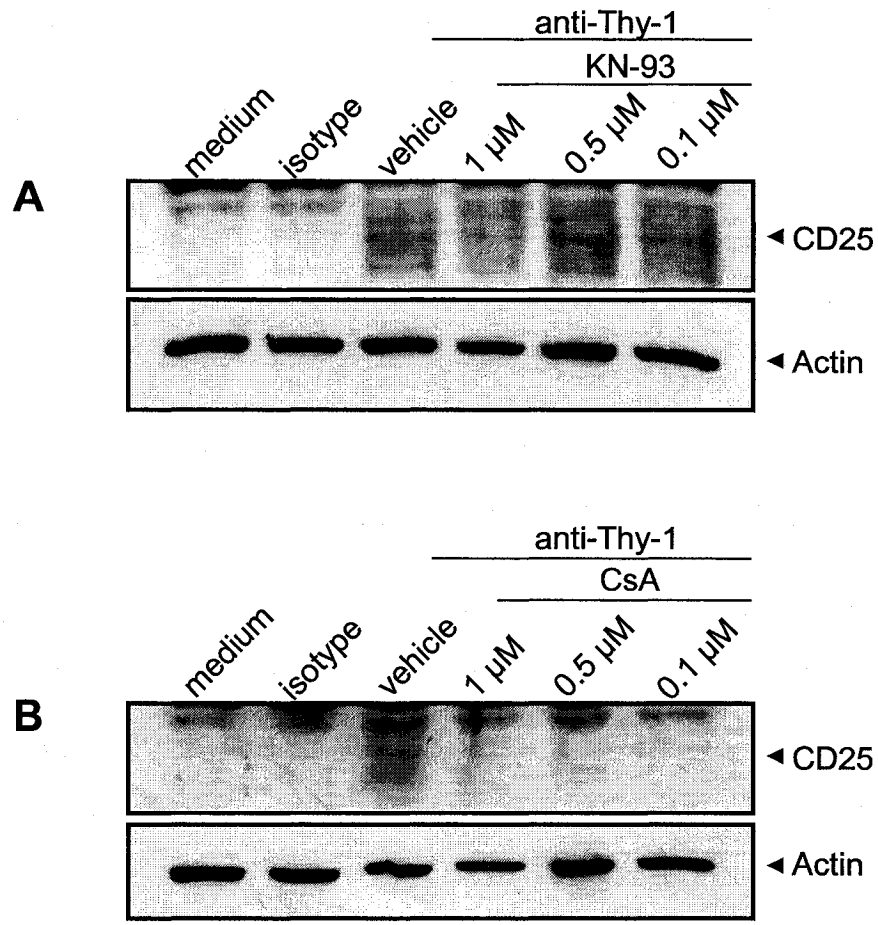


Figure 4.5. CaMKII And Calcineurin Mediate Thy-1-Induced CD25 Expression In T Cells. T cells were pretreated with (A) KN-93 (a CaMKII inhibitor) or (B) CsA (a calcineurin inhibitor) at the indicated concentrations, or with drug vehicle, or medium for 30 min before addition of medium, anti-Thy-1 mAb-coated microbeads or isotype control antibody-coated microbeads. Cell lysates were prepared after 24 h and Western blotting was carried out with antibodies specific for CD25. Blots were stripped and reprobed with antibodies for actin to indicate total protein loading. Results from individual representative experiments are shown (n=2).

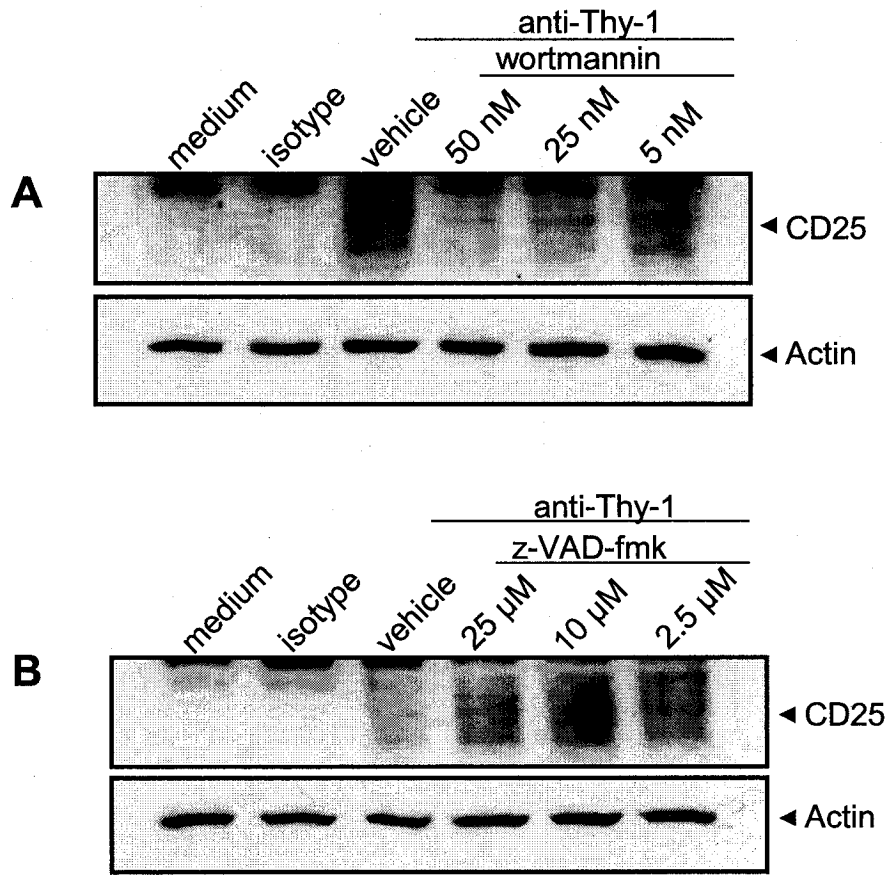


Figure 4.6. PI3K, But Not Caspases, Mediates Thy-1-Induced CD25 Expression In T Cells. T cells were pretreated with (A) wortmannin (a PI3K inhibitor) or (B) z-VAD-fmk (a broad-spectrum caspase inhibitor) at the indicated concentrations, or with drug vehicle or medium for 30 min before addition of medium, anti-Thy-1 mAb-coated microbeads or isotype control antibody-coated microbeads. Cell lysates were prepared after 24 h and Western blotting was carried out with antibodies specific for CD25. Blots were stripped and reprobed with antibodies for actin to indicate total protein loading. Results from individual representative experiments are shown (n=2).

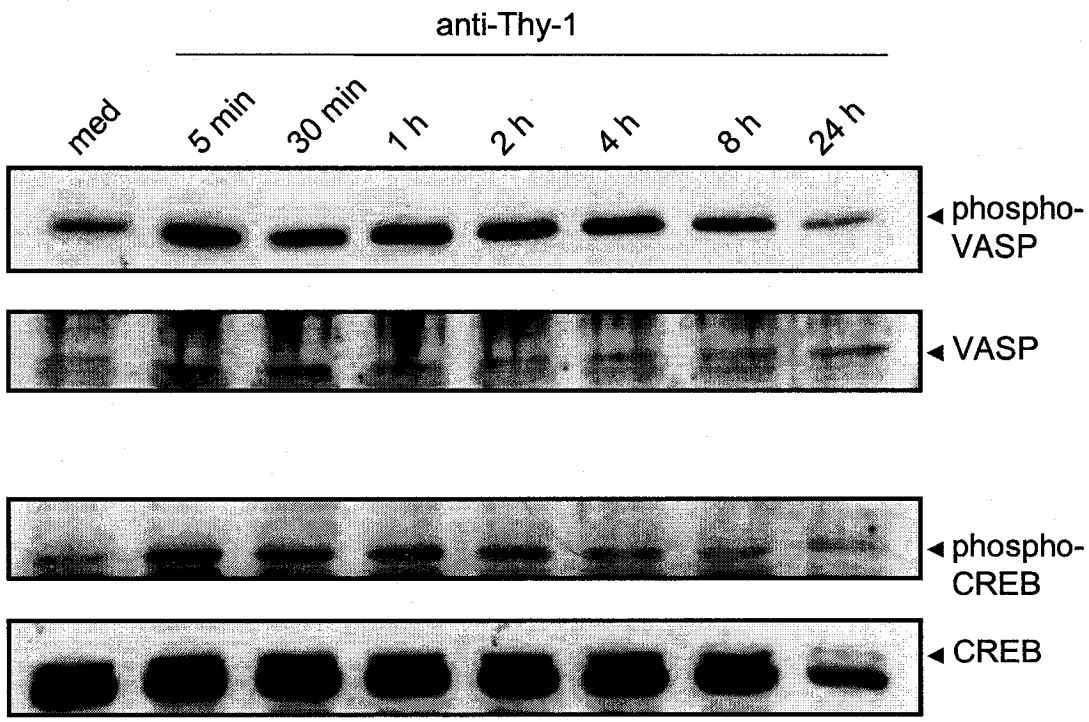


Figure 4.7. Thy-1 Stimulation Induces Phosphorylation Of VASP And CREB In T Cells. T cells were exposed to anti-Thy-1 mAb-coated microbeads for 5 min, 30 min, 1 h, 2 h, 4 h, 8 h, or 24 h. Negative control cells were exposed to medium alone for 5 min. Cell lysates were prepared and Western blotting was carried out with phospho-specific antibodies for VASP and CREB. Blots were stripped and reprobed with antibodies for VASP and CREB to indicate total protein loading. Results from one representative experiment are shown (n=2).

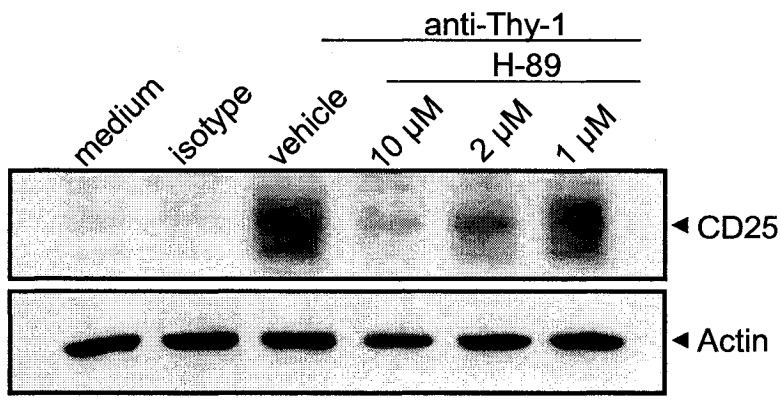


Figure 4.8. PKA Mediates Thy-1-Induced CD25 Expression In T Cells. T cells were pretreated with H-89 (a PKA inhibitor) at the indicated concentrations, with drug vehicle, or medium for 30 min before addition of medium, anti-Thy-1 mAb-coated microbeads or isotype control antibody-coated microbeads. Cell lysates were prepared after 24 h and Western blotting was carried out with antibodies specific for CD25. Blots were stripped and reprobed with antibodies for actin to indicate total protein loading. Results from one representative experiment are shown (n=3)

Chapter 5: Results: Thy-1 Signalling Generates Anergic $CD4^+CD25^+FoxP3^-$ T Cells With Atypical Suppressor Function

The role of Thy-1 in immunity remains a matter of intrigue and debate. Evidence clearly suggests a role for Thy-1 in mediating and/or enhancing T cell activation (Haeryfar and Hoskin, 2004). However, the hyperresponsiveness of Thy-1-deficient thymocytes to TCR stimulation suggests that Thy-1 may also carry out a negative regulatory role in the immune system (Hueber et al., 1997). I observed that Thy-1 signalling in T cells in the absence of costimulation induced CD25 expression (Figure 3.10), but failed to induce IL-2 production (Figure 3.7) or [^{3}H]TdR incorporation (Figure 3.4). Because expression of CD25 and hyporesponsiveness to stimulation are hallmark features of certain subsets of Treg cells (Jonuleit and Schmitt, 2003), I performed preliminary experiments to investigate the possibility that Thy-1 signalling could induce a regulatory phenotype in normal T cells. Unfortunately, mAb-coated microbeads, though useful for stimulating T cells, are difficult to remove from cell cultures. Because the suppressive function of Thy-1-stimulated T cells would ultimately have to be evaluated, a new system for activating T cell via Thy-1 had to be established.

5.1. Plate-Bound Anti-Thy-1 mAb Induces CD25 Expression

Plate-immobilised anti-Thy-1 was evaluated as a tool for triggering CD25 expression on T cells. Plastic tissue culture plates were coated with 5 µg, 10 µg, 20 µg, or 40 µg anti-Thy1- mAb, or medium alone overnight, and T cells were added to the plates the next day. The T cells were collected 24 h later and CD25 expression was analysed by flow cytometry. Figure 5.1 shows that all doses of anti-Thy-1 induced a modest increase in CD25 expression, represented as a

rightward shift in the peak of CD25 fluorescence relative to medium alone. There were no increases in CD25 expression with 10 µg, 20 µg or 40 µg anti-Thy-1 mAb, beyond that observed for 5 µg, substantial enough to warrant continued use of these higher concentrations of anti-Thy-1 mAb. Induction of CD25 was therefore carried out with 5 µg plate-immobilised anti-Thy-1 mAb for all subsequent experiments.

5.2. $CD4^+CD25^-$ Cells Express CD25 Following Anti-Thy-1 Stimulation

I next compared CD25 expression on freshly isolated $CD4^+CD25^+$ Treg cells to CD25 expression on Thy-1-stimulated $CD4^+CD25^-$ T cells. $CD4^+$ T cells were isolated by MACS and further separated into $CD25^-$ and $CD25^+$ populations. $CD4^+CD25^-$ T cells were stimulated with plate-bound anti-Thy-1 mAb, and $CD25^+$ cells were isolated from these cultures the next day. Unstimulated $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ Treg cells, as well as the $CD25^+$ fraction from Thy-1-stimulated $CD4^+CD25^-$ cultures, were analysed for CD25 expression by flow cytometry. I found that both anti-Thy-1 mAb-stimulated $CD4^+CD25^-$ and unstimulated $CD4^+CD25^+$ T cells expressed more CD25 than unstimulated $CD4^+CD25^-$ T cells (Figure 5.2). Interestingly, anti-Thy-1-stimulated $CD4^+CD25^-$ T cells also expressed several fold more CD25 than freshly isolated $CD4^+CD25^+$ Treg cells.

5.3. Thy-1-Stimulated T Cells Do Not Incorporate [^{3}H]TdR In Response To Subsequent CD3/CD28 Stimulation

Because CD25 expression was so much higher in Thy-1-stimulated T cells than in fresh Treg cells, I was concerned that plate-bound anti-Thy-1 stimulation might also be inducing IL-2 production, which could account for elevated

expression of CD25. If IL-2 were being produced, the Thy-1-induced $CD4^+CD25^+$ T cells could simply be a population of activated T cells. I therefore tested the culture supernatants from the anti-Thy-1 mAb-coated plates after 24-h incubations with $CD4^+CD25^-$ T cells for the presence of IL-2. Only 13 ± 1 pg/ml IL-2 was present in the culture supernatants of plate-bound anti-Thy-1 mAb-stimulated $CD4^+CD25^-$ T cells (Table 5.1). By comparison, anti-Thy-1 mAb-coated microbeads were also found to induce very little IL-2 production (31 ± 1 pg/ml) whereas microbeads coated with antibodies against Thy-1 and CD28 induced robust IL-2 production (199 ± 8 pg/ml). Although the Thy-1-stimulated T cells expressed CD25, the absence of IL-2 suggested they were not activated. Plate-bound anti-Thy-1 mAbs also failed to induce [^{3}H]TdR incorporation by the $CD4^+CD25^-$ T cells (data not shown). To further characterise these cells, I examined their responsiveness to traditional costimulation via CD3 and CD28. Here, freshly isolated $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells as well as Thy-1-induced $CD4^+CD25^+$ T cells were exposed to CD3/CD28 T Cell Expander Dynabeads®. These are small iron beads coated with mitogenic mAbs directed against CD3 and CD28. As shown in Figure 5.3, fresh $CD4^+CD25^-$ T cells responded robustly to CD3/CD28 stimulation (52,396 ± 2,551 cpm). However, fresh $CD4^+CD25^+$ and Thy-1-induced $CD4^+CD25^+$ T cells showed only weak activation in response to CD3/CD28 stimulation, with [^{3}H]TdR incorporation values of only 913 ± 226 cpm and 4,241 ± 578 cpm, respectively. These data suggest that Thy-1-induced $CD4^+CD25^+$ T cells are phenotypically similar to fresh $CD4^+CD25^+$ Treg cells.

5.4. $CD4^+CD25^-$ T Cell Activation In Response To CD3/CD28 Stimulation Is Attenuated In The Presence Of Anti-Thy-1-Induced $CD4^+CD25^+$ T Cells

There are many different subsets of Treg cells, with varying expression of Treg cell-associated surface markers. However, the common feature shared by all so-called regulatory cells is the ability to suppress normal immune responses. Therefore, to determine if Thy-1-induced $CD4^+CD25^+$ T cells were functionally similar to Treg cells, I compared the suppressive capacity of both types of cells. Fresh $CD4^+CD25^-$ T cells were stimulated with CD3/CD28 T Cell Expander Dynabeads® in the presence or absence of Thy-1-induced or freshly isolated $CD4^+CD25^+$ Treg cells. As shown in Figure 5.4, the normal response to CD3/CD28 costimulation was 11,626 ± 800 cpm. In the presence of Thy-1-induced or fresh $CD4^+CD25^+$ T cells, the response to costimulation was significantly reduced ($p < 0.001$) by 71% and 51%, respectively. In some experiments, Treg cell-mediated suppression was found to be equal to, or greater than, that of Thy-1-induced $CD4^+CD25^+$ T cells.

5.5. Thy-1-Induced $CD4^+CD25^+$ T Cells Produce IL-2 In Response To CD3/CD28 Stimulation

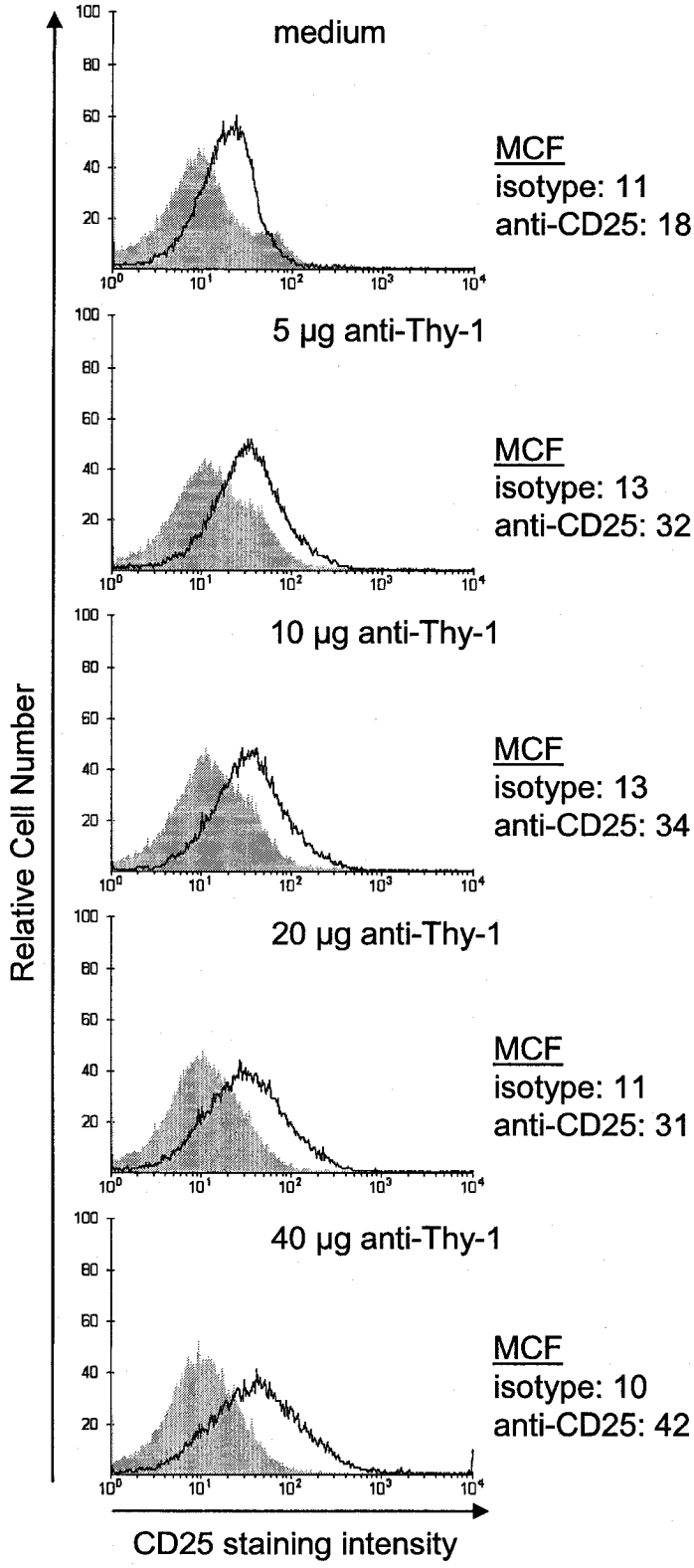
IL-2 is a critical regulator of $CD4^+CD25^+$ T cell-mediated regulatory function. It has been shown to break Treg cell anergy and mediate suppression of IL-2 production by responder T cells (Thornton et al., 2004). I therefore measured IL-2 concentrations in culture supernatants from T cell suppression assays. Unstimulated $CD4^+CD25^-$ and Thy-1-induced $CD4^+CD25^+$ T cells produced no IL-2 (Figure 5.5). IL-2 production by CD3/CD28-stimulated $CD4^+CD25^-$ T cells was 489 ± 31 pg/ml IL-2, which was significantly increased to

851 ± 52 pg/ml in the presence of Thy-1-induced $CD4^+CD25^+$ T cells ($p < 0.001$). Surprisingly, CD3/CD28 stimulation of Thy-1-induced $CD4^+CD25^+$ T cells resulted in the production of 690 ± 8 pg/ml IL-2, which was also significantly greater than the amount of IL-2 produced by CD3/CD28-costimulated $CD4^+CD25^-$ T cells ($p < 0.001$). These results reveal that despite failing to incorporate [^{3}H]TdR in response to CD3/CD28-mAb coated beads and possessing suppressor function, Thy-1-induced $CD4^+CD25^+$ T cells also possess the atypical feature of producing IL-2 in response to CD3/CD28 stimulation.

5.6. Thy-1-Induced $CD4^+CD25^+$ T Cells Do Not Express FoxP3

The transcription factor FoxP3 is the most widely acknowledged marker of Treg cells (Fontenot et al., 2005b; Fontenot and Rudensky, 2005). Freshly isolated $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells, as well as Thy-1-induced $CD4^+CD25^+$ T cells were permeabilised and stained with FITC-conjugated FoxP3 antibodies, and analysed by flow cytometry. As shown in Figure 5.6, freshly isolated $CD4^+CD25^+$ T cells were positive for FoxP3; however, fresh $CD4^+CD25^-$ and Thy-1-induced $CD4^+CD25^+$ T cells were negative for FoxP3. These results demonstrate that although capable of suppressing immune responses, Thy-1-induced $CD4^+CD25^+$ T cells fail to express the hallmark transcription factor that identifies most $CD4^+CD25^+$ Treg cells.

Figure 5.1. Plate-Bound Anti-Thy-1 mAb Induces CD25 Expression. T cells were incubated overnight in plates coated with 5 µg, 10 µg, 20 µg, or 40 µg anti-Thy-1 mAb, or medium alone. Cells were collected and stained with FITC-conjugated anti-CD25 (open peaks) or isotype control antibodies (shaded peaks) and analysed by flow cytometry. Mean channel fluorescence (MCF) values are shown. Results are from one experiment.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isotype: 11
anti-CD25: 31
40 µg anti-Thy-1
MCF
isotype: 10
anti-CD25: 42
Relative Cell Number
CD25 staining intensity

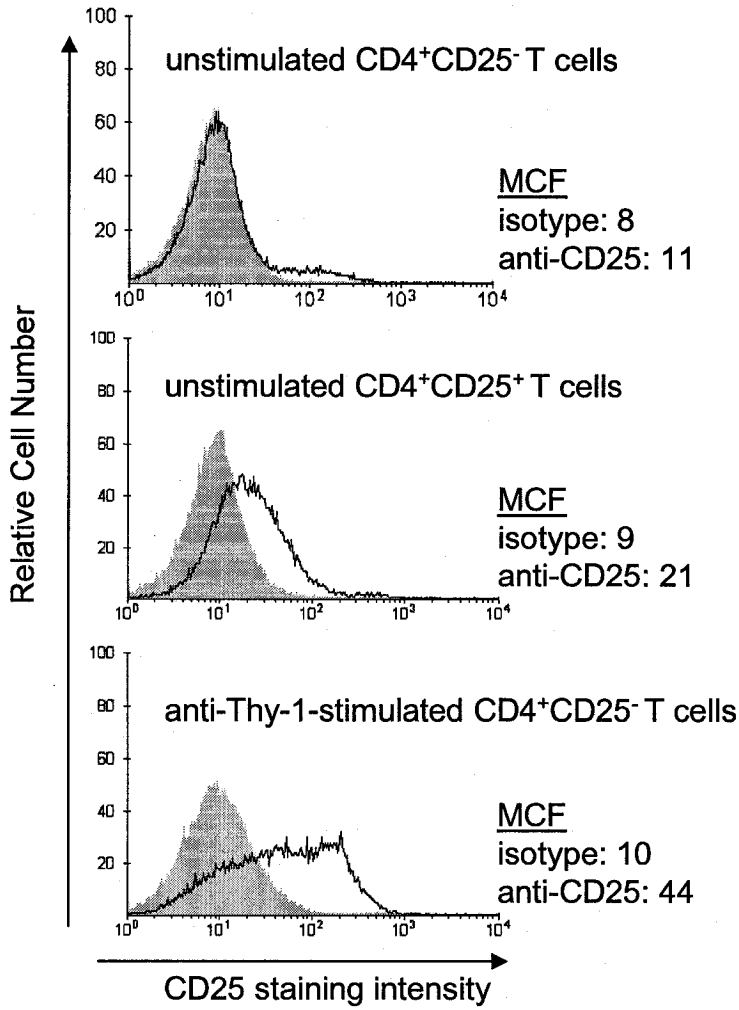


Figure 5.2. $CD4^+CD25^-$ T Cells Express CD25 Following Anti-Thy-1 Stimulation. $CD4^+CD25^-$ T cells were isolated from whole spleen leukocytes and incubated overnight in an anti-Thy-1 mAb-coated tissue culture plate. $CD25^+$ T cells were isolated from the plate using MACS magnets and reagents, and stained with FITC-conjugated antibodies specific for CD25 (open peaks) or isotype control antibodies (shaded peaks) in parallel with natural $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells. CD25 expression was then assessed by flow cytometry. Mean channel fluorescence (MCF) values are shown. Results are from one experiment

Table 5.1. Thy-1 Stimulation In The Absence Of Costimulation Does Not Promote IL-2 Production. T cells were stimulated with plate-bound anti-Thy-1 mAb, microbeads coated with anti-Thy-1 mAb, or microbeads coated with anti-Thy-1 plus anti-CD28 mAb for 24 h. Culture supernatants were collected and IL-2 levels from quadruplicate samples were measured by sandwich ELISA. Data are presented as average IL-2 concentration (pg/ml) ± SD (n=3).

Condition	**IL-2 concentration (pg/ml) ± SD**
Anti-Thy-1/anti-CD28 on beads	199 ± 8
Anti-Thy-1 on beads	31 ± 1
Plate-bound anti-Thy-1	13 ± 1

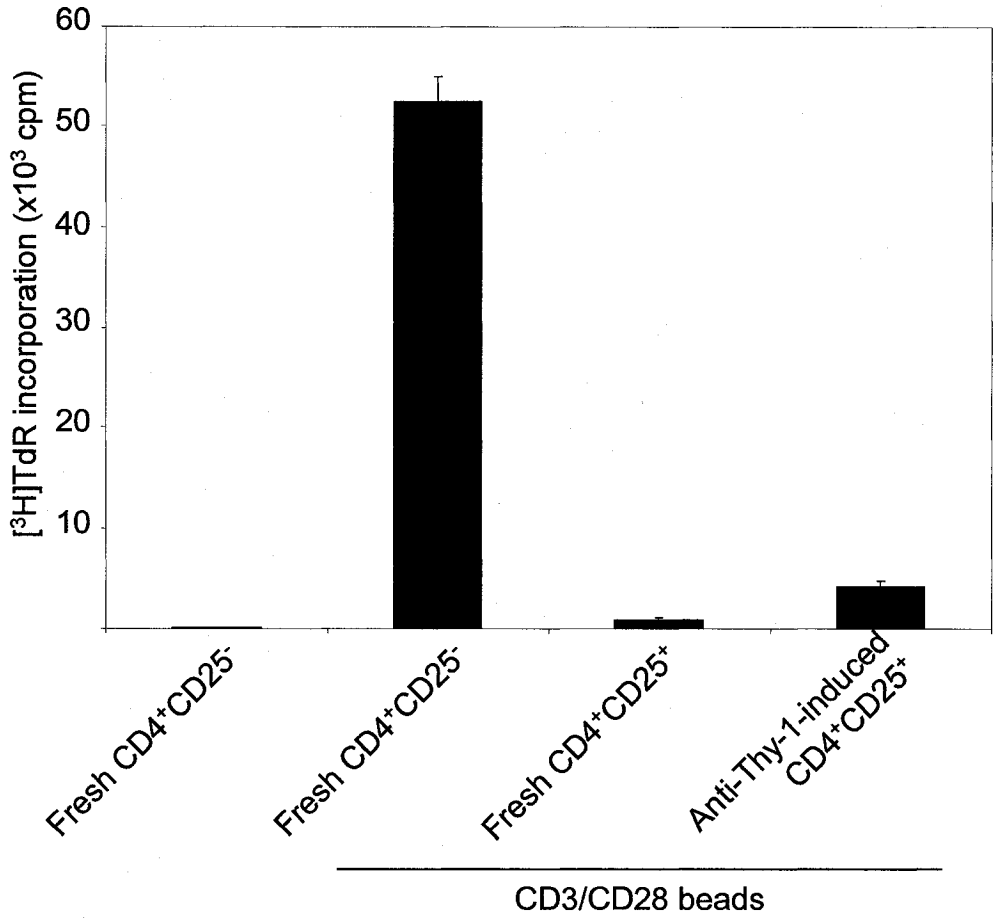


Figure 5.3. Thy-1-Stimulated T Cells Do Not Incorporate [^{3}H]TdR In Response To CD3/CD28 Stimulation. Unstimulated $CD4^+CD25^-$ T cells, unstimulated $CD4^+CD25^+$ T cells, or anti-Thy-1-induced $CD4^+CD25^+$ T cells were exposed to CD3/CD28 T Cell Expander Dynabeads® or medium alone and [^{3}H]TdR incorporation was measured in quadruplicate wells after 48 h. Data from one experiment are presented as average cpm ± SD (n=3).

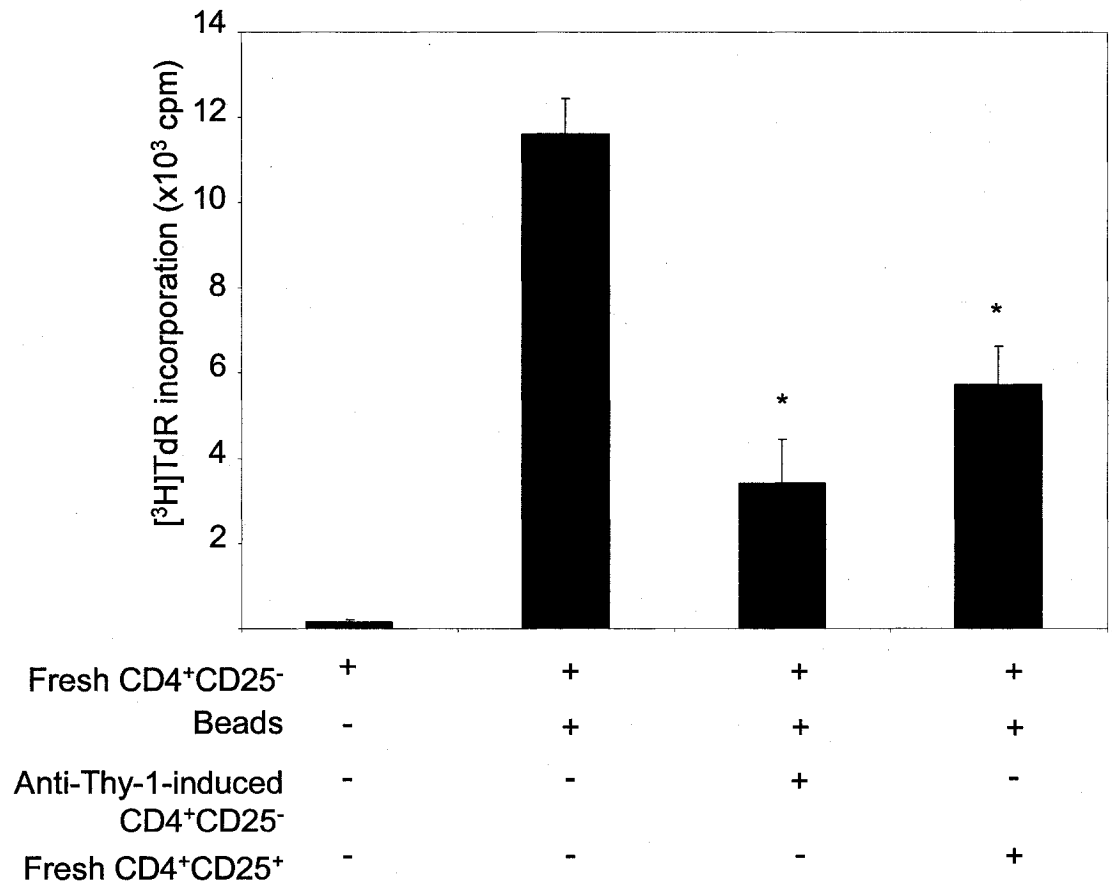


Figure 5.4. $CD4^+CD25^-$ T Cell Activation In Response To CD3/CD28 Stimulation Is Attenuated In The Presence Of Anti-Thy-1-Induced $CD4^+CD25^+$ T Cells. $CD4^+CD25^-$ T cells were incubated in the presence or absence of CD3/CD28 T Cell Expander Dynabeads®, with or without Thy-1-induced or freshly isolated $CD4^+CD25^+$ Treg cells. T cell activation was measured in quadruplicate wells after 48 h by $[^3H]$TdR incorporation. Data from one experiment are presented as average cpm ± SD (n=3). * denotes $p < 0.001$, compared to fresh $CD4^+CD25^-$ stimulated with CD3/CD28 expander beads.

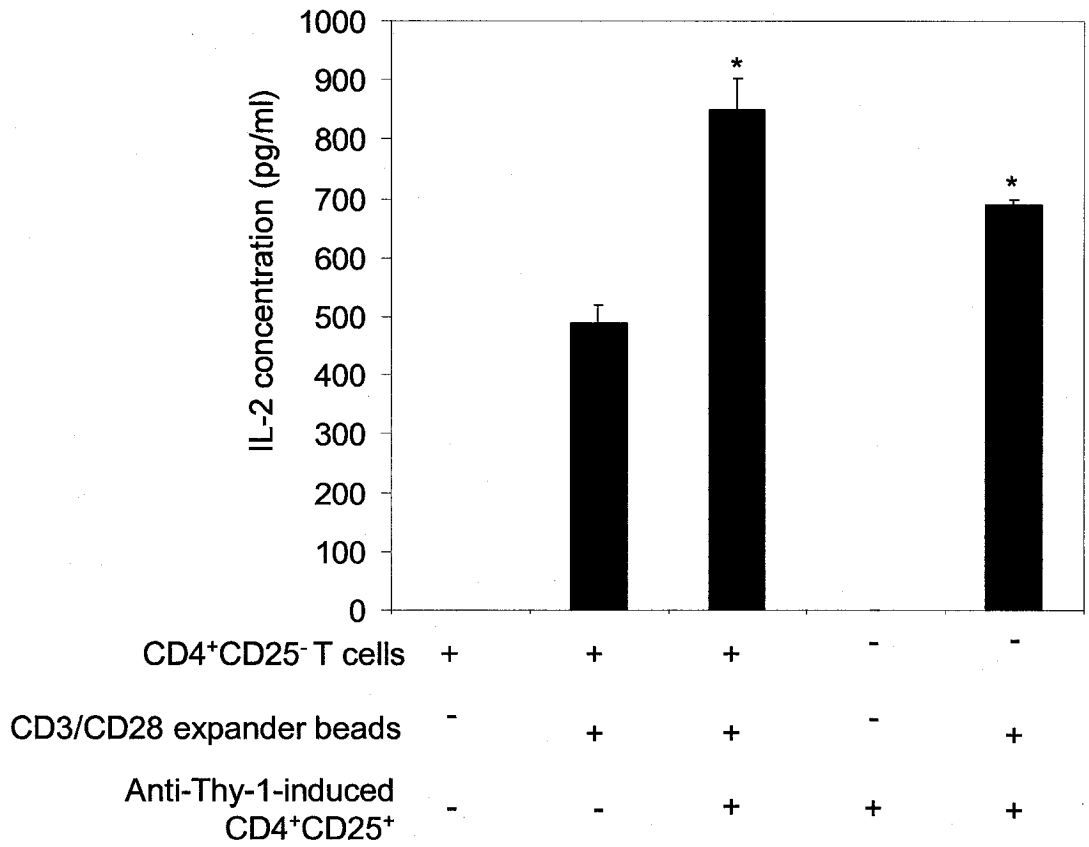


Figure 5.5. Thy-1-Induced $CD4^+CD25^+$ T Cells Produce IL-2 In Response To CD3/CD28 Stimulation. $CD4^+CD25^-$ T cells were combined with CD3/CD28 T Cell Expander Dynabeads® with or without Thy-1-induced or freshly isolated $CD4^+CD25^+$ Treg cells, as indicated. After 24 h, culture supernatants were collected and IL-2 levels were assessed in quadruplicate by sandwich ELISA. Data from one experiment are presented as mean IL-2 concentration (pg/ml) ± SD (n=3). * denotes $p < 0.001$, compared to fresh $CD4^+CD25^-$ stimulated with CD3/CD28 expander beads.

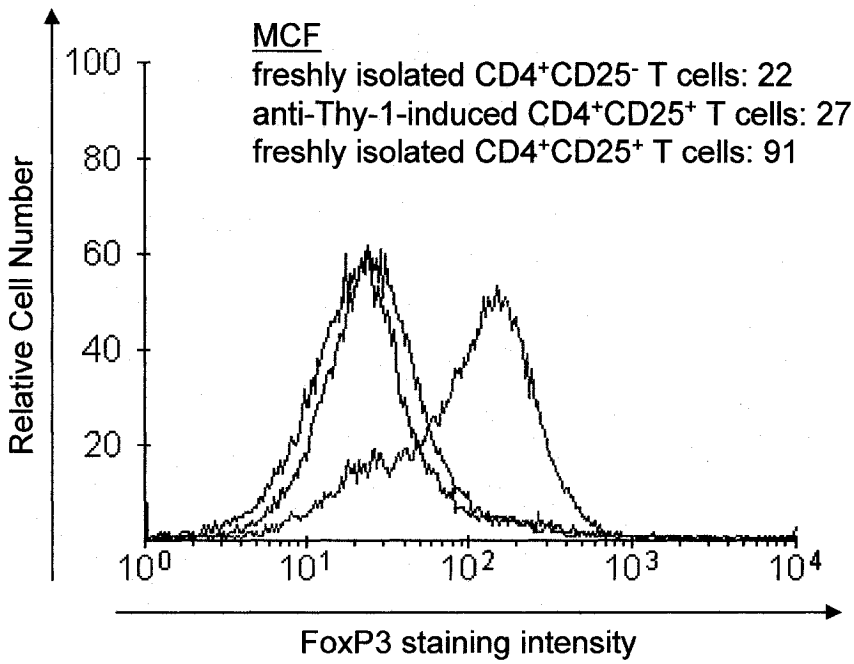


Figure 5.6. Thy-1-Induced $CD4^+CD25^+$ T Cells Do Not Express FoxP3. Thy-1-induced $CD25^+$ T cells (blue peak) were permeabilised and stained with FITC-conjugated anti-FoxP3 and isotype control antibodies in parallel with freshly isolated $CD4^+CD25^-$ (black peak) and $CD4^+CD25^+$ (red peak) T cells. The cells were then analysed by flow cytometry. Mean channel fluorescence (MCF) values are shown. Results are from one representative experiment (n=2).

Chapter 6. Discussion

6.1 MAPKs Regulate Thy-1/CD28-Induced T Cell Activation

Although ERK1/2 and p38 MAPK have previously been implicated in Thy-1-induced T cell activation (Haeryfar and Hoskin, 2001), the role of these MAPKs was examined in non-purified T cells. As a result of the use of impure T cell preparations, the influence of MAPKs on accessory cells, and their ability to costimulate anti-Thy-1-driven T cell activation, confounds those data and makes interpretation of the results difficult. Also, at the time of those experiments, the highly specific JNK inhibitor SP600125 was not available. Therefore, I examined the role of MAPKs in anti-Thy-1-induced T cell activation using highly pure (~95%) T cells, and the most selective pharmacological inhibitors of ERK1/2, p38 MAPK, and JNK presently available, according to my review of the literature. Also, I activated T cells with cell-sized microbeads coated with stimulatory anti-Thy-1 (clone G7) and anti-CD28 mAbs, limiting the number of possible costimulatory interactions that might exaggerate, or even mask, the effects of MAPK inhibition.

Consistent with previous Thy-1 studies carried out in our lab, I observed maximal Thy-1-induced T cell activation at 48 h. But TCR-driven T cell activation is detectable by 24 h, and persists until at least 96 h (C. Doucette, personal communication). Because Thy-1 signalling is analogous to TCR signalling (Haeryfar et al., 2003), I was concerned that I might be missing Thy-1-induced [^{3}H]TdR incorporation by pulsing for only 6 h every 24 h. However, a more comprehensive [^{3}H]TdR-pulse time course revealed that virtually all Thy-1-

induced [^{3}H]TdR incorporation occurred between 42 h and 48 h. This surprising observation suggested that Thy-1/CD28-stimulated T cells may only be completing one round of cell division, or may in fact not be exiting S-phase at all.

Although the PD98059, SB203580, and SP600125 compounds are reportedly highly specific for their respective MAPK targets at the doses I used, a common criticism of pharmacological inhibitors is the possibility for non-specific effects on other cellular factors. To date, the involvement of MAPKs in Thy-1-induced T cell activation has only been demonstrated in inhibitor studies (Haeryfar and Hoskin, 2001). I therefore stimulated T cells with anti-Thy-1/anti-CD28 mAb-coated beads and analysed MAPK activation by Western blot. The results revealed that ERK1/2, p38 MAPK and JNK were all activated in response to Thy-1/CD28 stimulation, with ERK1/2 clearly being the most strongly activated MAPK. Interestingly, ERK2 phosphorylation was more intense than that of ERK1, suggesting that Thy-1 stimulation either favors activation of ERK2, or inefficiently activates ERK1. Because the specific roles of each of these two ERK molecules during T cell activation are not clear, it was difficult to interpret these results. However, the preferential phosphorylation of ERK2 over ERK1 has also been observed in the context of classical CD3/CD28-mediated T cell activation (C. Doucette, personal communication). Also, differences in antibody binding affinity within the Western blot process could account for variations in band intensity, both between blots for different MAPKs, and in the labelling of phospho-ERK1 and phospho-ERK2.

Inhibition of ERK1/2, p38 MAPK, or JNK resulted in impaired anti-Thy-1-induced T cell activation, as determined by [^{3}H]TdR incorporation measurements. Reduced [^{3}H]TdR incorporation was not due to general toxicity of the inhibitors because T cell viability was not affected by these compounds, as determined by MTT assay and Trypan Blue vital dye staining. Also, the MAPK inhibitors did not prevent cytokine-independent [^{3}H]TdR incorporation in EL4 mouse lymphoma cells. Moreover, I observed impaired Thy-1-induced T cell activation when the T cells were pretreated with different, but analogous, MAPK inhibitors (the MEK inhibitor SL327, the p38 MAPK inhibitor ML3403, and the JNK inhibitor (L)-JNKI1), which suggested that the impaired T cell activation was due to the selective inhibition of ERK1/2, p38 MAPK, or JNK. With the exception of PD98059, which inhibits MEK1 activity and therefore ERK1/2 phosphorylation, inhibition of MAPK activity by SB203580 and SP600125 cannot be confirmed by immunoblotting for the phosphorylated form of the MAPKs they target. These inhibitors competitively bind the adenosine triphosphate pocket of p38 MAPK and JNK, respectively, and thus inhibit their ability to phosphorylate substrates (Bennett et al., 2001a; Frantz et al., 1998). The effect of SB203580 and SP600125 on the phosphorylation status of downstream targets of p38 MAPK and JNK would ultimately have to be demonstrated to confirm the activity of these compounds in T cells during Thy-1-induced activation.

Interestingly, impaired Thy-1-induced [^{3}H]TdR incorporation associated with MAPK inhibition was not completely reflective of the collective effects of the MAPK inhibitors on IL-2 production. Although pretreatment with PD98059 and

SP600125 caused reduced IL-2 expression following Thy-1/CD28 stimulation, blockade of p38 MAPK activity with SB203580 promoted IL-2 production. Clearly, inhibition of p38 MAPK impaired other T cell functions; otherwise, elevated IL-2 production would have translated into enhanced T cell activation. Indeed, p38 MAPK-independent costimulatory signalling would explain the enhanced [^{3}H]TdR incorporation previously reported for impure T cells pretreated with p38 MAPK inhibitors prior to Thy-1 stimulation (Haeryfar and Hoskin, 2001). Studies on IL-2R signalling in the IL-2-dependent CTLL-2 cell line, and CD25 expression in primary cells, revealed that SB203580 impaired IL-2R function but did not affect CD25 expression. This was also evident from the inability of exogenous IL-2 to substantially enhance the activation of SB203580-treated primary T cells in response to Thy-1/CD28 stimulation. I initially devised an ELISA experiment to measure IL-2 uptake/usage by CTLL-2 cells, and observed that greater amounts of IL-2 remained in culture when p38 MAPK was inhibited compared to the appropriate control (Figure A.1). However, because SB203580 also prevented CTLL-2 growth, reduced cell numbers could account for low IL-2 usage in this system.

In an ELISA, reduced IL-2 uptake would be indistinguishable from increased IL-2 expression. To determine if IL-2 production was in fact being influenced by SB203580, I carried out a Western blot for IL-2 that was retained intracellularly by pretreatment with BfA. This fungal antibiotic shuts down Golgi trafficking (Sciaky et al., 1997), and is therefore a useful tool for studying cytokine expression. I found that blockade of p38 MAPK activity increased the amount of

IL-2 present in Thy-1/CD28-stimulated T cells. Real-time PCR further determined that IL-2 expression was increased at the level of mRNA expression following p38 MAPK blockade. Intuitively, one might assume that blocking p38 MAPK would result in decreased cytokine expression, considering the important role that this MAPK plays in stabilising mRNA transcripts that contain AU-rich motifs (Frevel et al., 2003), which are present in IL-2 primary transcripts (Chen and Shyu, 1995). However, I found p38 MAPK inhibition to have the opposite effect on IL-2 expression. Kogkopoulou et al. also demonstrated increased IL-2 production following activation of T cells that were treated with a p38 MAPK inhibitor and report that inhibition of p38 MAPK does not affect IL-2 mRNA stability (Kogkopoulou et al., 2006). The authors show that increased IL-2 expression due to p38 MAPK blockade is mediated by ERK1/2. I also found that Thy-1/CD28-induced IL-2 production was mediated by ERK1/2. This MAPK is linked to IL-2 expression by transcription factors that activate the IL-2 promoter. The transcription factor Elk-1 is a major substrate of ERK1/2, and mediates expression of the AP-1 protein c-Fos (Whitmarsh and Davis, 1996). ERK1/2 also promotes expression and function of other AP-1 proteins (Treinies et al., 1999; Young et al., 2002), and the nuclear transit of at least one NFκB family member (Koike et al., 2003). However, ERK5, which is expressed by T cells and promotes IL-2 promoter activity (Garaude et al., 2005), can also be inhibited by PD98059 (Mody et al., 2001). Thus ERK5 may also govern Thy-1/CD28-driven T cell responses.

p38 MAPK can also negatively regulate NFAT function by promoting cytoplasmic translocation of this transcription factor, which also binds the IL-2 promoter. Wu et al. also observed elevated IL-2 production when ConA- or CD3/CD28-stimulated T cells were treated with 10 μM SB203580 (Wu et al., 2003). In this report, the authors demonstrate that MAPKK3 mediates nuclear export of NFAT. Therefore, inhibition of p38 MAPK might drive IL-2 expression by promoting nuclear localisation of NFAT.

SB203580 is an inhibitor of p38α MAPK and p38β MAPK (Cohen, 1997). However, p38δ MAPK is also expressed in T cells (Hale et al., 1999), although its function in T cells is unclear. Even if secondary to that of p38α MAPK or p38β MAPK, or if opposed by these p38 MAPK isoforms, the function of p38δ MAPK would be dominant in the presence of SB203580, and might account for the observed changes in IL-2 expression caused by this p38 MAPK inhibitor. A more extensive study on the function of p38 MAPK with a pharmacological inhibitor that blocks the activity of all p38 MAPK isoforms, such as BIRB796 (Kuma et al., 2005), might confirm or rule out a role for p38δ MAPK in Thy-1/CD28-induced T cell activation.

I found that JNK activity was required for IL-2 expression, as well as CD25 expression. Though CTLL-2 data suggested that JNK also mediates IL-2R signalling, primary T cells stimulated with Thy-1 and CD28 mAbs were able to overcome the growth inhibition caused by the JNK inhibitor when exogenous IL-2 was added. This suggested that in primary T cells, JNK activity is not important for IL-2R signalling. A role for JNK in regulating IL-2 expression has previously

been demonstrated (Matsuda et al., 1998), likely due, at least in part, to its ability to activate c-Jun, since the IL-2 promoter contains binding sites for AP-1 (Jain et al., 1992), which is known to mediate IL-2 expression (Matsuda et al., 1998). NFAT is regulated by JNK and also mediates IL-2 expression (Rooney et al., 1995), while both AP-1 and NFAT are involved in CD25 expression (Schuh et al., 1998). Inhibition of JNK may have also resulted in impaired IL-2 production following Thy-1/CD28 stimulation by direct effects on IL-2 gene transcription or mRNA half-life, since JNK is also involved in regulating IL-2 mRNA stability (Chen et al., 1998).

Because I examined IL-2 production and CD25 expression at the same time following Thy-1/CD28 stimulation, the absence of IL-2 could have explained impaired CD25 expression in T cell cultures that were exposed to the SP600125 JNK inhibitor. STAT5, which communicates IL-2R signals to the nucleus, is also involved in CD25 expression (John et al., 1996). Therefore, it was possible that the effect of JNK inhibition on CD25 expression in my experimental system was indirect, and caused by a reduction in IL-2-induced STAT5 activation. However, even in the presence of exogenous IL-2, CD25 expression could not be restored to control levels in the presence of the JNK inhibitor. In spite of this, exogenous IL-2 clearly established a functionally competent level of CD25 expression, since the T cells were activated in response to Thy-1/CD28 stimulation in the presence of the JNK inhibitor when exogenous IL-2 was present.

I have described the first systematic investigation on the roles of ERK1/2, p38 MAPK, and JNK in Thy-1/CD28-induced T cell activation, which are

summarised in the model shown in Figure 6.1. Presently, no such report has ever been made in the context of Thy-1 or TCR stimulation. With this knowledge of the role of MAPKs in Thy-1/CD28-induced T cell activation, a better understanding of how the MAPKs regulate TCR-driven T cell activation might shed further light on the similarities and differences that exist among Thy-1 and TCR signals.

6.2 Thy-1-Induced CD25 Expression Is Dependent On Components Of The TCR Signalling Pathway

Relatively little is known about the pathway that mediates Thy-1 signalling in T cells. To date, the only quantifiable cellular readouts reported for Thy-1 signalling are thymocyte apoptosis and T cell activation. Unfortunately, at least in the case of T cell activation, this cellular response is not caused by Thy-1 signalling alone. Thy-1-induced primary T cell activation, as reported in the literature, is typically driven by costimulation provided by accessory cells or mAbs that crosslink costimulatory molecules on T cells. Thy-1 stimulation alone does not activate purified T cells (Norcross and Smith, 1979). Moreover, costimulatory molecule interactions must be provided in proper context with Thy-1, as I found that when coated onto separate beads, anti-Thy-1 and anti-CD28 mAbs did not activate T cells (Figure A.2). Molecular readouts for Thy-1 signalling include Ca^{2+} mobilisation, and protein phosphorylation. However, there are no descriptions in the literature of gene expression induced by Thy-1 signalling *per se* in primary T cells. In Western blot experiments designed to monitor Thy-1/CD28-induced CD25 expression, I observed moderate CD25 expression in control T cells that were stimulated with anti-Thy-1 mAb-coated beads alone. CD25 expression was

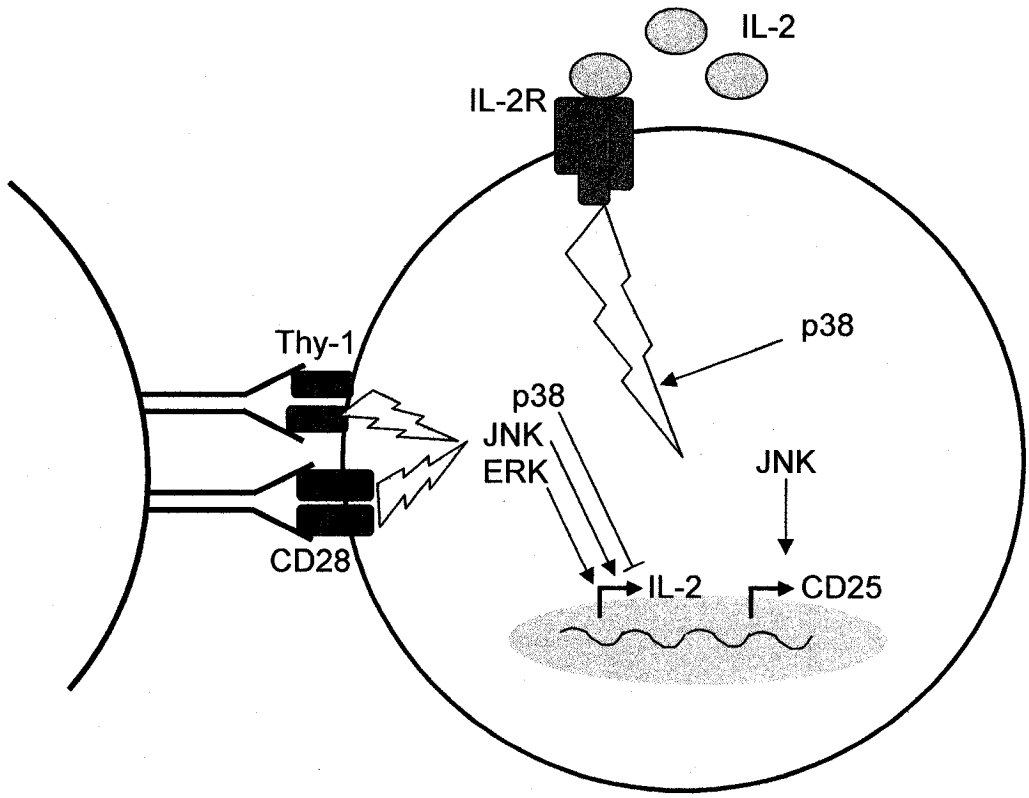


Figure 6.1. Proposed Model Of MAPK-Mediated Regulation Of Thy-1-Induced T Cell Activation. Following Thy-1/CD28 stimulation ERK1/2 (ERK), p38 MAPK (p38), and JNK each regulate different aspects of T cell activation. ERK signalling is required for IL-2 expression, while JNK signalling drives the expression of IL-2 as well as CD25. The role of p38 in Thy-1-induced T cell activation is to promote IL-2R signalling and negatively regulate IL-2 expression.

therefore adopted as a simple readout for Thy-1 signalling in purified primary T cells.

In my hands, Thy-1-induced CD25 expression was maximal at 24 h. Although CD25 is a 55 kDa protein, formerly known as p55, immunoblotting for CD25 resulted in multiple protein bands, as well as smearing. Posttranslational modification of CD25 could account for the range in molecular weight detectable by Western blotting, since murine CD25 contains multiple sites for N-glycosylation. I employed a panel of selective pharmacological inhibitors to identify components of the signalling pathway that mediates CD25 expression in Thy-1-stimulated T cells. The PTK inhibitor herbimycin A prevented Thy-1-induced CD25 expression, demonstrating the requirement for PTKs in this process. Several PTKs could be involved in Thy-1 signalling in T cells. Fyn, in particular, likely plays an important role in CD25 expression, as $fyn^{-/-}$ T cell clones are refractory to Thy-1-mediated activation (Lancki et al., 1995). However, in rats, the PTKs fyn, lck (Garnett et al., 1993), as well as lyn (Narisawa-Saito et al., 1996) have all been shown to coimmunoprecipitate with Thy-1. It is unclear how Thy-1 interacts with cytosolic proteins. PTKs localise to lipid rafts where Thy-1 resides (Ilangumaran et al., 2000), thus the physical association of Thy-1 with PTKs may be a function of other proteins present in lipid microdomains. For example, p100 is a transmembrane protein that interacts with Thy-1 and immunoprecipitates with 57 kDa and 59 kDa proteins that correspond to the molecular weights of the PTKs lck and fyn, respectively (Lehuen et al., 1995). Transmembrane proteins in lipid rafts, such as CD45 (Edmonds and Ostergaard,

2002), could also bridge Thy-1 to PTKs. This would be consistent with the mechanism of signal initiation by the GPI-anchored protein CD73, which signals in a lck- and CD45-dependent manner (Resta and Thompson, 1997).

The MAPKs ERK1/2, p38 MAPK, and JNK have all been identified as points of signal integration between the TCR and CD28 (Chen et al., 1997; Su et al., 1994; Zhang et al., 1999). However, as elaborated previously, it is unclear how these MAPKs function with respect to TCR-1-induced CD25 expression, and nothing is known about their role in Thy-1-induced CD25 expression. I found that inhibition of ERK1/2 or JNK prevented Thy-1-induced CD25 expression, but inhibition of p38 MAPK had no effect. This result was unexpected, as p38 MAPK has previously been identified as an important mediator of Thy-1-induced T cell activation (Haeryfar and Hoskin, 2001) and, in my own studies, Thy-1/CD28-induced IL-2 production. However, I also found that p38 MAPK inhibition did not affect CD25 expression induced by Thy-1/CD28 stimulation. Because I previously observed that p38 MAPK modulates IL-2 expression and IL-2R signalling, the fact that Thy-1-induced CD25 is not affected by p38 MAPK inhibition points to the existence of multiple Thy-1 signalling pathways, or at least a principle Thy-1 pathway in T cells that diverges upstream of p38 MAPK.

Leyton et al. have provided evidence that Thy-1 stimulation enhances TCR signalling by promoting phosphorylation of the transmembrane protein LAT (Leyton et al., 1999). LAT is coupled to the Ras pathway (Sommers et al., 2004), which is likely how Thy-1 stimulation activates ERK1/2. LAT is also coupled to PLCγ (Wange, 2000). I found that inhibition of PLC with the inhibitor U73122

strongly impaired Thy-1-induced CD25 expression. At the highest concentration tested (0.5 μM), the inhibitor may have been toxic because actin expression was reduced. However, there were no alterations in actin expression to account for decreased CD25 expression at the other doses of U73122 that were tested. Since LAT is activated by ZAP-70 (Zhang et al., 1998), and lck physically associates with ZAP-70 (Duplay et al., 1994), Thy-1 might initiate signalling at the level of lck activation mediated by a transmembrane adaptor protein. Indeed, it would be beneficial to confirm activation of ZAP-70 following Thy-1 stimulation, or examine Thy-1-induced CD25 expression in primary T cells that were treated with small interfering RNA (siRNA) to specifically knock down expression of lck, ZAP-70, or LAT.

Haeryfar et al. were able to restore CD3-induced T cell activation, that was hindered by mAb neutralisation of Thy-1, with the PKC activator phorbol 12-myristate 13-acetate (Haeryfar et al., 2005). Moreover, PKC is activated downstream of PLC during TCR stimulation, which is analogous to Thy-1 stimulation (Haeryfar et al., 2003). This suggests that PKC might be a component of the Thy-1 signalling pathway in T cells. I observed that inhibition of PKC impaired the ability of Thy-1 to trigger CD25 expression, though not completely. DAG, a component of the GPI anchor that tethers Thy-1 to the cell surface, may contribute to PKC activation (Pont, 1987), and PKC has previously been shown to mediate CD25 expression in the YT NK cell line, likely through NFκB activation (Shirakawa and Mizel, 1989).

Stimulation of Thy-1 results in Ca^{2+} mobilisation (Kojima et al., 2000), and therefore would be expected to activate Ca^{2+}-dependent signalling molecules, such as CaMKII and the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin. Perviously, our lab has shown that Thy-1-induced T cell activation is very sensitive to inhibition of calcineurin with CsA (Haeryfar and Hoskin, 2001). However, this could be accounted for by impaired IL-2 expression. I found that Thy-1-induced CD25 expression was also sensitive to calcineurin inhibition by CsA, as well as blockade of CaMKII, though inhibition of CaMKII did not dramatically affect CD25 expression. Both CaMKII and calcineurin are activated by calmodulin; however, CaMKII is involved in NFκB activation while calcineurin activates NFAT (Crabtree, 1999; Haeseleer et al., 2002; Ishiguro et al., 2006). Taken together with the finding that PKC inhibition did not completely block CD25 expression, the minimal effect of CaMKII inhibition on Thy-1-induced CD25 expression may indicate that NFκB plays a relatively minor role in Thy-1 signalling. Conversely, the total reduction in CD25 expression caused by calcineurin inhibition suggests that NFAT is the major transcription factor responsible for driving CD25 expression in response to Thy-1 stimulation.

Because Thy-1 has been implicated in the provision of signal 1 and signal 2 (Haeryfar and Hoskin, 2004), which are both coupled to PI3K, I predicted that PI3K would be important in mediating Thy-1-induced CD25 expression. Indeed, the PI3K inhibitor wortmannin prevented CD25 expression. Although previous work from our lab also implicated PI3K in Thy-1 signalling (Haeryfar and Hoskin, 2001), it was not possible to distinguish between a role for PI3K in Thy-1

signalling and its association with costimulation. In contrast, my studies revealed a direct role for PI3K in Thy-1 signalling in T cells.

I observed that inhibition of caspases with the pan-caspase inhibitor z-VAD-fmk enhanced CD25 expression after stimulation of Thy-1. Caspases are major regulators of apoptosis (Salvesen, 2002), but are also involved in TCR signalling (Chun et al., 2002; Misra et al., 2005) and, at least in human T cells, CD25 expression (Falk et al., 2004). The signalling molecules affected by all the other inhibitors in this study are activated rapidly (within minutes) following T cell stimulation, while caspase activation follows much slower kinetics. Therefore, the observed increase in CD25 expression due to caspase inhibition was not likely due to a direct effect on the signalling pathway that mediates CD25 expression. Rather, it follows that Thy-1 signalling might result in T cell death by apoptosis and the enhancing effect of caspase inhibition on CD25 expression was simply due to the presence of more viable cells capable of expressing CD25 in response to Thy-1 stimulation when CD25 expression was asessed. This was suprising, because stimulation of Thy-1 triggers protein synthesis, which is evident both from my studies on Thy-1-induced CD25 expression, and from protein assay data generated during Western blot experiments (data not shown). Moreover, to my knowledge, Thy-1 has been shown to induce apoptosis in thymocytes, but not in primary T cells. However, at this point, I cannot rule out the possibility that Thy-1 signalling might upregulate T cell surface molecules, such as FasL, that promote activation-induced cell death (AICD). Alternatively, caspases may target substrates in the T cell that are involved in mediating CD25 expression that have

not yet been identified. If this were true, caspases might serve a negative regulatory role in the induction of CD25 expression.

A recent report demonstrates that neuronal Thy-1 signalling involves VASP, CREB, and PKA (Chen et al., 2007), which are also present in T cells and are known to be involved in T cell activation (Boonyaratanakornkit et al., 2005; Krause et al., 2000). I found that VASP and CREB were both rapidly phosphorylated following Thy-1 stimulation of T cells, and that inhibition of PKA blocked the induction of CD25 expression. In neurons, Thy-1-induced PKA activation precedes MEK activation, which is followed by CREB activation. Although MEK is clearly involved in Thy-1-induced CD25 expression, the interplay between these molecules in T cells remains to be determined in the context of Thy-1 signalling. Though consistent with its role in neuronal Thy-1 signalling, the finding that PKA mediates CD25 expression in T cells is somewhat puzzling. In my experiments, PKC and PKA both appear to promote Thy-1 signalling, since inhibition of either kinase impaired Thy-1-induced CD25 expression. Recently, it was demonstrated that PKC and PKA actually oppose each other in the context of T cell activation (Hermann-Kleiter et al., 2006). Specifically, inhibition of PKC blocks IL-2 production in T cells, but this efect can be reversed by inhibition of PKA. It would therefore be interesting to examine the effect of PKA inhibition on Thy-1/CD28-induced T cell activation, which involves IL-2 expression. PKA may play opposing roles in the signalling pathways that drive IL-2 production and CD25 expression in T cells. Alternatively, Thy-1 may trigger more than one pathway involving PKA. Nevertheless, I have

demonstrated that VASP, and CREB are activated in T cells as a result of Thy-1 stimulation, and have identified PKA as an important component of Thy-1 signalling in T cells. Collectively, my results in primary T cells demonstrate that, with the exception of p38 MAPK and caspases, all signalling molecules tested that are known to be important in TCR signalling are also involved in Thy-1-mediated CD25 expression (Table 6.1).

Interestingly, I also found that anti-Thy-1 mAb treatment of the 1H3.1 hybridoma T cell line resulted in both apoptosis (Figure A.3) and IL-2 expression (Figure A.4). Interestingly, inhibition of caspases with z-VAD-fmk did not dramatically enhance IL-2 production by these T cell hybridomas. 1H3.1 cells might be a useful tool for studying the mechanism of Thy-1-induced apoptosis and associated signalling. However, my preliminary observations with this cell line also emphasise the importance of studying signalling within the context of an appropriate model. The fact that caspase inhibition enhanced CD25 expression in primary T cells but had no effect on IL-2 expression in the T cell hydridomas, coupled with the fact that primary T cells do not even make IL-2 in response to Thy-1 stimulation, demonstrates that fundamental differences in Thy-1-associated signalling pathways exist between primary T cells and immortalised T cells. Great care should therefore be taken when comparing data acquired in different experimental systems.

Table 6.1. The Effect Of Pharmacological Inhibitors On Thy-1-Induced CD25 Expression. T cells were pretreated with inhibitors of protein tyrosine kinases (PTKs), MAPK/ERK 1 (MEK1), p38 MAPK, JNK, phospholipase C (PLC), protein kinase C (PKC), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), calcineurin, phosphatidylinositol-3 kinase (PI3K), caspases, or protein kinase A (PKA), stimulated with anti-Thy1- mAb-coated micro beads, and CD25 expression was analysed by Western blot. Results are summarised in the table below.

Inhibitor	**Signalling molecule targeted**	**Effect on Thy-1-induced CD25 expression**
herbimycin A	PTKs	Decreased
PD98059	MEK1	Decreased
SB203580	p38 MAPK	No change
SP600125	JNK	Decreased
U73122	PLC	Decreased
calphostin C	PKC	Decreased
KN-93	CaMKII	Decreased
CsA	calcineurin	Decreased
wortmannin	PI3K	Decreased
z-VAD-fmk	caspases	Increased
H-89	PKA	Decreased

6.3 Thy-1 Stimulation Induces Treg Cells

Control treatments in [^{3}H]TdR incorporation assays and IL-2 ELISA experiments included T cells that were stimulated with anti-Thy-1 mAb-coated beads, in the absence of costimulatory CD28 mAbs. Exposure to anti-Thy-1 mAbs alone never induced appreciable [^{3}H]TdR incorporation or IL-2 production in T cell cultures, while CD25 expression was consistently promoted by Thy-1 stimulation. The expression of CD25 following Thy-1 triggering, along with the absence of [^{3}H]TdR incorporation or IL-2 production is consistent with the phenotype of Treg cells (Hickman et al., 2006). Likewise, Thy-1 promotes LAT phosphorylation (Leyton et al., 1999), which is a critical step in the development of Treg cells (Koonpaew et al., 2006). However, Thy-1 is associated with $CD8^+$ T cell effector function (Haeryfar et al., 2005; Kojima et al., 2000) and is reportedly expressed in greater abundance on the surface of $CD8^+$ T cells in comparison to $CD4^+$ T cells (de Mello Coelho et al., 2004), suggesting the main role of Thy-1 might be to drive $CD8^+$ T cell responses, rather than those of $CD4^+$ T cells. I nonetheless found that Thy-1/CD28 stimulation induced a greater response in $CD4^+$ T cells than in $CD8^+$ T cells, indicating that Thy-1 signalling is equal in strength, if not more robust, in the $CD4^+$ T cells (Figure A.5).

I was able to induce CD25 expression in whole T cells with plate-bound anti-Thy-1. It was critical to develop a bead-free activation system for these experiments, as mAb-coated beads might have complicated subsequent functional assays. In fact, 5 µg/ml of plate-bound anti-Thy-1 stimulated greater CD25 expression on purified $CD4^+$ T cells than was present on the surface of

naturally arising $CD4^+CD25^+$ Treg cells. I also observed that $CD4^+$ T cells stimulated with plate-bound anti-Thy-1 mAbs did not produce appreciable amounts of IL-2 or incorporate [^{3}H]TdR, which was consistent with the hyporesponsive phenotype of natural Treg cells. Moreover, both natural Treg cells and Thy-1-induced $CD4^+CD25^+$ T cells did not incorporate [^{3}H]TdR in response to subsequent CD3/CD28 stimulation.

I found that Thy-1-induced $CD4^+CD25^+$ T cells consistently suppressed CD3/CD28-driven $CD4^+CD25^-$ T cell activation. In at least one experiment, the suppressive capacity of the Thy-1-induced $CD4^+CD25^+$ T cells was superior to that of the naturally arising Treg cells. However, in general the suppression induced by the two cell types was comparable. Naturally arising $CD4^+CD25^+$ Treg cells impair the ability of responder T cells to express IL-2 following TCR stimulation (Thornton and Shevach, 1998). To determine if this mechanism of suppression was also utilised by Thy-1-induced $CD4^+CD25^+$ T cell-mediated suppression, I assayed for IL-2 in the culture supernatants obtained from my suppression experiments. To my surprise, the Thy-1-induced $CD4^+CD25^+$ T cells failed to impair IL-2 production by $CD4^+CD25^-$ responder T cells after CD3/CD28 stimulation. Moreover, in response to CD3/CD28 stimulation, the Thy-1-induced $CD4^+CD25^+$ T cells produced a greater amount of IL-2 than the $CD4^+CD25^-$ responder T cells. This was not consistent with the established phenotype of $CD4^+CD25^+$ Treg cells in mice, which are not capable of transcribing the IL-2 gene (Su et al., 2004). Thy-1-stimulated T cells possess the ability to suppress despite expressing copious amounts of IL-2, and are therefore unlike any subset

of Treg cells previously described in the literature. To date, although not universal, the most widely accepted Treg cell marker is FoxP3 (Sakaguchi, 2004). This transcriptional regulator represses expression of the IL-2 gene (Schubert et al., 2001). Because Thy-1-induced $CD4^+CD25^+$ T cells retained the ability to express IL-2 in response to CD3/CD28 stimulation, I predicted that FoxP3 would not be expressed by these cells. Indeed, flow cytometry revealed that the Thy-1-stimulated cells were $FoxP3^-$.

Clearly a great deal of work remains to be completed in order to decipher the mechanism by which Thy-1-induced $CD4^+CD25^+FoxP3^-$ Treg cells mediate suppression. However, the work describing naturally arising $CD4^+CD25^+$ Treg cells contains several clues as to how Thy-1-induced $CD4^+CD25^+$ T cells may function.

When recovering $CD25^+$ T cells from the anti-Thy-1 mAb-coated plates, I noticed a significant variation in cell viability. I found that $CD25^+$ T cells ranged in viability from 99% to as low as 30%, and that the viability was not dependent on the density of the cells at the beginning of the assay (data not shown). Activated T cells upregulate expression of FasL, and undergo AICD through Fas/FasL interactions (Green et al., 2003). Since cell density was not a factor in cell death, Thy-1-induced FasL expression was unlikely to be modulating the reduced viability. This line of reasoning also ruled out a perforin/granzyme-mediated killing mechanism, since it also would require juxtaposition of the T cells in order to occur.

TGF-β is a cytokine that has been implicated in Treg cell suppressive function. Because TGF-β can also be used to induce Treg cell function (Chen et al., 2003), I added TGF-β to my anti-Thy-1 mAb-coated plates in an attempt to enhance the ability of Thy-1 signalling to induce Treg cells. However, all the T cells died in the presence of the TGF-β (data not shown). So if TGF-β were ultimately involved in the suppressive function of Thy-1-induced Treg cells, then induction of Treg cell function by Thy-1 stimulation would have to be coupled to resistance to TGF-β, for example by downregulating expression of TGF-β receptors.

The abundance of IL-2 produced by Thy-1-induced Treg cells in response to CD3/CD28 stimulation suggests that IL-2 is important either in the suppressive function of the Treg cells, or in their survival. IL-2 is also important in the development of naturally occurring Treg cells (Malek, 2003); however, minimal IL-2 production was evident during the induction of CD25 expression by Thy-1 signalling in Thy-1-induced Treg cells, which therefore must develop in an IL-2-independent manner. Interestingly, IL-$2^{-/-}$ and $CD25^{-/-}$ FoxP3-knock-in Treg cells were shown to be capable of suppressing immune responses, but did not survive as long as control Treg cells. Indeed, in the IL-2-deficient cells, numerous genes involved in cell cycle control and cell growth were not expressed as highly as in control cells (Fontenot et al., 2005a). In this regard, STAT5 has been identified as an important factor in IL-2-induced Treg cell homeostasis (Antov et al., 2003). Consistent with this finding, $CD4^{+}CD25^{+}$ Treg cells, which do not undergo autocrine IL-2 signalling (Long and Adler, 2006), are believed to rely on IL-2 from

normal responder T cells to induce their suppressor function (Thornton et al., 2004). Indeed, IL-2 might also be an important factor in the survival of Thy-1-induced Treg cells since reduced viability was observed during Thy-1 stimulation when IL-2 was not being produced.

The fact that neither the $CD4^+CD25^-$ responder T cells nor the Thy-1-induced $CD4^+CD25^+$ Treg cells exhibited enhanced [^{3}H]TdR incorporation in the presence of significantly elevated concentrations of IL-2 suggested that IL-2R signalling was impaired in these cells or that signals transmitted through the IL-2R were no longer translated into progression through the cell cycle. Duthoit et al. report that IL-2R signalling is perturbed downstream of STAT5 in naïve $CD4^+$ T cells in the presence of $CD4^+CD25^+$ Treg cells (Duthoit et al., 2005). Consistent with my findings, exogenous IL-2 failed to induce responder T cell activation in the presence of Treg cells in this study. Treg cell-induced ablation of CD25 expression on the responder cells could account for the inability to become activated in response to IL-2, though the complete absence of CD25 is unlikely. In fact, responder T cells have been shown to upregulate CD25 expression in spite of Treg cell activity (Duthoit et al., 2005). The same dissociation of the IL-2R signalling pathway from the induction of cell cycle may be at play both in the failure of Thy-1-induced Treg cells to incorporate [^{3}H]TdR in response to CD3/CD28 stimulation, and in their ability to suppress normal T cell activation.

Duthoit et al. have also described transiently non-responsive T cells that possess suppressor function (Duthoit et al., 2004). These T cells were activated and cultured for several days, during which they became refractory to

restimulation. Interestingly, during this time the T cells also acquired the ability to suppress T cell activation in a contact-dependent and IL-2-independent fashion. However, unlike Thy-1-induced Treg cells, these cells do not produce IL-2. The authors demonstrate that the naïve $CD4^+$ T cells failed to exit the G1 phase of the cell cycle (Duthoit et al., 2004). I have yet to perform cell cycle analysis on responder T cells activated in the presence of Thy-1-induced Treg cells, which would determine whether the cells were in fact unable to transit through the cell cycle, or were simply being killed by the Treg cells. In light of this new information on the function of murine Thy-1, it would be important to revisit human CD7, an ortholog of murine Thy-1, in similar functional studies. CD7 has already been shown to participate in T cell activation as a source of costimulation (Stillwell and Bierer, 2001), and CD7-directed antibody therapy has been shown to delay acute rejection of kidney allografts (Lazarovits et al., 1993). However, no other functional properties have yet been assigned to this surface molecule.

Collectively, my data contain several novel and important findings, which are summarised in Figure 6.2. First, I have described a novel IL-2-producing Treg cell. To my knowledge, this has never before been reported in either humans or mice. Second, the absence of IL-2 in supernants from T cells stimulated with Thy-1 mAbs suggests that, unlike normal Treg cells, the development of Thy-1-induced $CD4^+CD25^+$ T cells is IL-2-independent. I have also provided the first evidence that stimulation of a single cell surface protein can induce a regulatory phenotype in normal T cells. To my knowledge, all other studies on the induction of Treg cells rely on the stimulation of the TCR with mAbs or antigen, plus

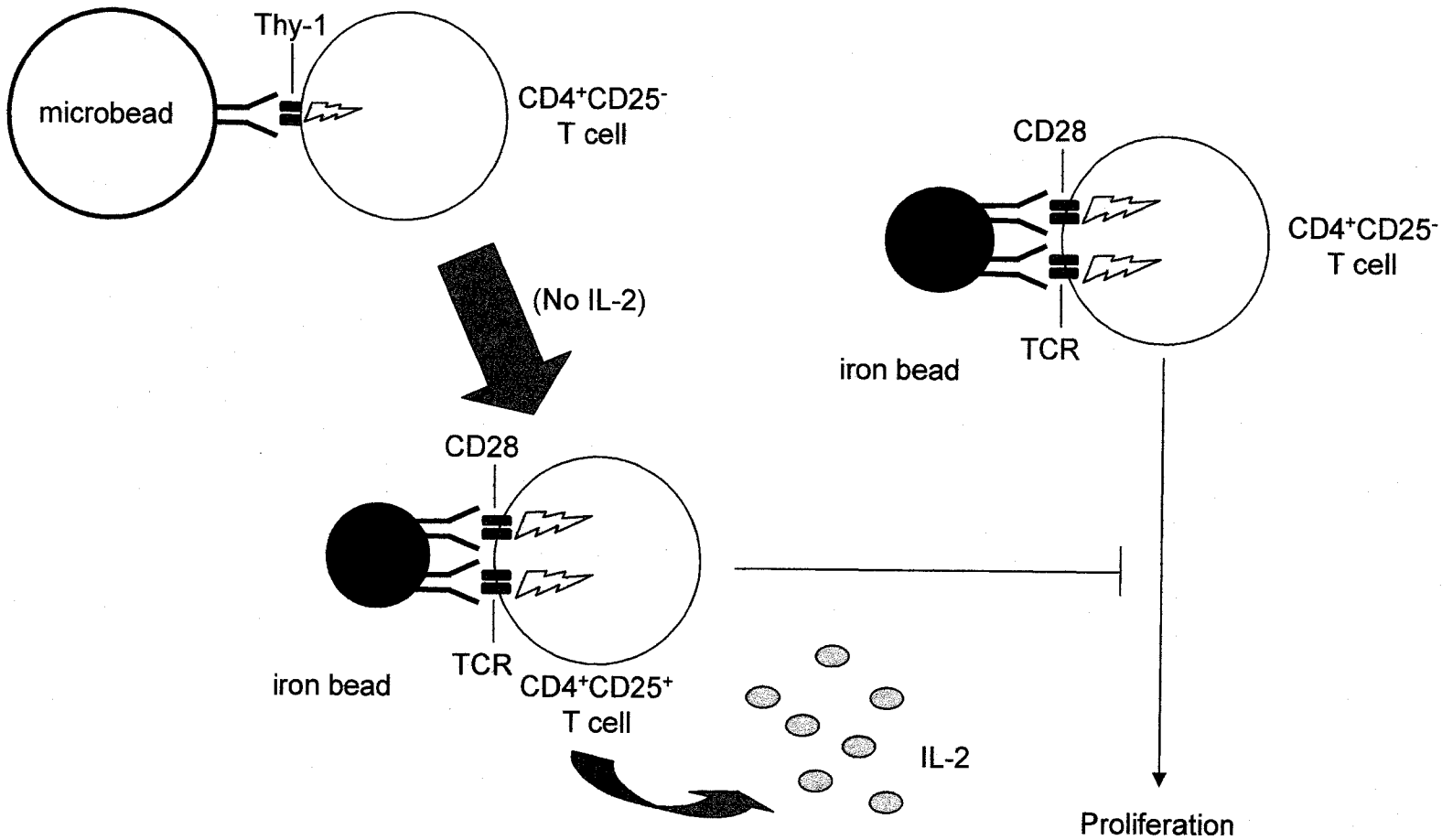


Figure 6.2. Thy-1 Stimulation Converts Naïve T Cells Into Treg Cells. Antibody-mediated triggering of Thy-1 signalling induces $CD4^+CD25^-$ T cells to express CD25, but not IL-2. Thy-1-induced $CD4^+CD25^+$ T cells produce substantial amounts of IL-2 in response to CD3/CD28 stimulation, and suppress CD3/CD28-induced proliferation of $CD4^+CD25^-$ responder T cells.

costimulation or other signals provided by cytokines. Lastly, and most importantly, I have described a new function for Thy-1 in T cell biology. Though preliminary and not yet complete, my study on Thy-1-induced Treg cell function has led to perhaps the most exciting discovery regarding Thy-1 since it was first described more than 40 years ago.

6.4 Strengths And Weaknesses Of This Study

The two major strengths of this thesis research are the use of primary T cells, and the minimisation of confounding signalling events. Studies on Thy-1 in T cell biology have been reported using primary T cells, as well as T cell clones and T cell lines. It would be naïve to think that T cell clones and T cell lines are equally representative of T primary cells. Also, most reports on Thy-1 are based on experiments where non-pure T cells are activated with antibodies in the presence of accessory cells, which could provide an indeterminate combination of costimulatory interactions that would contribute to any observed cellular responses. Mine is the first study that addresses Thy-1 signalling alone, or in the context of a defined source of costimulation.

One general weakness of these studies is context. I used antibodies to stimulate Thy-1 because, as yet, the immunological ligand of Thy-1 remains unidentified. It is assumed that these antibodies mimic that true Thy-1 ligand, but this is only conjecture. Antibodies can bind several different domains on Thy-1, not all of which are associated with stimulation. My work, and the work of many other Thy-1 researchers, is therefore based on the assumption that the Thy-1 ligand will bind the same domain on Thy-1 as these antibodies and have the

same stimulatory effect. Also, because the Thy-1 ligand is unknown, we do not know which costimulatory molecules are expressed in close enough proximity to costimulate Thy-1 signals. Therefore, is Thy-1/CD28-mediated T cell activation even relevant? Stimulation of a different costimulatory molecule may be more appropriate, or perhaps no surface molecules are expressed in the proper configuration to costimulate Thy-1 signalling at all. While this possibility questions the physiological relevance of my work, the fact remains that Thy-1 function may be generally conserved among other GPI-anchored proteins that can be properly costimulated. My work may therefore contribute to our understanding of GPI-anchored proteins whose physiological ligands are already known.

Another weakness of this study is the possible non-specific effects of the pharmacological inhibitors used. Genetically modified T cells, or T cells treated with siRNAs to knock down specific signalling molecules might be more appropriate tools for studying the signalling components of the Thy-1 activation pathway. Also, because impaired T cell activation or CD25 expression was only assessed at one time point, it is possible that these compounds simply delayed Thy-1-induced responses. In addition, as yet it is not known if any of these compounds affect Thy-1 expression on T cells. Indeed, human Thy-1 expression is regulated by PKC (Mason et al., 1996); still other molecules identified as key components of a Thy-1 signal transduction pathway in T cells might also modulate Thy-1 expression, which could account for the observed effects on Thy-1-induced CD25 expression.

Thy-1 is not internalised by T cells after anti-Thy-1 mAb-mediated stimulation (Haeryfar et al., 2003). Therefore, it is possible that anti-Thy-1 mAbs remaining on the surface of Thy-1-induced $CD4^+CD25^+$ T cells are involved in their suppressor function. Flow cytometry can be used to check for the presence of anti-Thy-1 antibodies on these cells. Alternatively, soluble anti-Thy-1 mAbs could be added to cultures containing responder T cells and anti-CD3/anti-CD28 mAb-coated beads to determine if the presence of the antibody alone can inhibit CD3/CD28-driven responses.

6.5 Future Directions And Concluding Remarks

My research has contributed significantly to the existing body of information on the possible immunological relevance of T cell-associated Thy-1. However, despite these new discoveries, many questions remain to be answered. The relatively short, and single, period of $[^3H]$TdR incorporation that results from Thy-1/CD28 stimulation suggests that the T cells were only going through, at most, one round of division. In fact, they may not even be completing mitosis. Recent studies in our lab have revealed that while T cells stimulated with anti-CD3 antibodies plus costimulation provided by DCs increase in number, T cells stimulated instead with anti-Thy-1 antibodies do not (S. Furlong, personal communication). Because Thy-1/CD28 stimulation triggers $[^3H]$TdR incorporation, the cells are clearly entering S-phase. Cell cycle analysis of Thy-1/CD28-stimulated T cells would identify at which stage the cells are blocked. This information might give clues as to deficits in Thy-1 signalling that result in impaired mitosis, as well as major points of divergence/difference between Thy-1

and TCR signals. Alternatively, the IL-2 made by the T cells in response to Thy-1/CD28 stimulation may be killing them. IL-2 is a cytokine that functions both as a growth factor and as a death factor. It can trigger the expression of apoptosis-inducing death receptors and their ligands by T cells (Zheng et al., 1998), and has been shown to inhibit the expression of Fas-associated death domain (FADD)-like IL-1β-converting enzyme (FLICE) inhibitory protein (FLIP), which normally prevents apoptosis. If Thy-1/CD28 stimulation cannot drive T cell proliferation, one must question the possible physiological function of Thy-1 in this context. In the thymus where there would be no shortage of costimulation, Thy-1 signalling might serve to simply trigger IL-2 production. Alternatively, Thy-1/CD28 stimulation may be important in priming T cells for activation. For example, if Thy-1 signalling were costimulated in the context of "danger", T cells would be more aptly prepared to become activated in response to antigenic stimulation. To my knowledge, no other studies have assessed Thy-1-induced T cell proliferation in any way other than [^{3}H]TdR incorporation. Before any complex cell cycle analyses are performed, Thy-1-induced T cell activation should be examined with simple carboxyfluoroscein succinimidyl ester (CFSE) dye labelling to confirm whether or not Thy-1/CD28-stimulated cells do in fact divide.

To date, my description of the Thy-1 signalling pathway in T cells is the most extensive. However, my results suggest that multiple pathways might be activated by Thy-1, or at least that the principal pathway diverges as some point. Elucidation of the mechanism of Thy-1 signalling in T cells remains to be

completed. The manner by which Thy-1 can initiate intracellular signalling events from the surface of the cell, in particular, is of great interest. I have traced Thy-1 signalling to PLC activation, and propose that Thy-1 signalling is mediated by a physical association with a transmembrane protein that interacts with lck. Immunoprecipitation studies on Thy-1 before and after stimulation would identify Thy-1-associated transmembrane proteins that might carry out this function.

The observation that Thy-1 signalling induces a Treg cell phenotype in normal T cells is hopefully only the beginning of what promises to be a very interesting story on a new function of Thy-1. However, the mechanism by which Thy-1-induced $CD4^+CD25^+$ T cells mediate suppression of T cell activation remains to be elucidated. If suppression is contact-dependent, it will be important to further phenotype the Thy-1-induced Treg cells to determine which cell surface molecules might be important to their function. Alternatively, if cell-to-cell contact is not involved, the production of soluble factors can be assessed en masse with a cytokine array. The fact that Thy-1-induced Treg cells express IL-2 in response to CD3/CD28 stimulation suggests that this cytokine may be a major factor in the suppression of T cell activation. The IL-2 may function in suppression by promoting apoptosis in the responder cell population. If this is true, it would be interesting to determine if Thy-1-induced Tregs are resistant to the death-inducing effects of IL-2, or if they too die in culture.

Recent work from our lab has revealed that T cells stimulated with Thy-1 antibodies, in the context of strong costimulation provided by DCs, express twice as much IL-17 as TCR-stimulated T cells (S. Furlong, personal communication).

This novel observation suggests yet another possible function for Thy-1, namely the induction of Th17 cells. However, it is unlikely that a definitive physiological function will be assigned to Thy-1 until its immunological ligand is identified. Thy-1 possesses an RLD integrin-binding sequence (Leyton et al., 2001), and, indeed, several integrins have been identified that can bind Thy-1, including astrocyte β_3 integrin (Leyton et al., 2001), leukocyte $\alpha_M\beta_2$ integrin (Wetzel et al., 2004), and monocyte $\alpha_X\beta_2$ integrin (Choi et al., 2005). As yet, none of these integrins have been studied in the context of T cell stimulation, so their involvement in triggering Thy-1-mediated T cell responses remains unknown.

I have obtained the Thy-1-Fc chimeric protein used by Leyton et al. to identify β_3 integrin as a Thy-1 ligand (Leyton et al., 2001), and assessed flow cytometry, immunohistochemistry, and immunoprecipitation as possible ways to identify new potential ligands for Thy-1. Flow cytometry with Thy-1-Fc-labelled C6 glioma cells, which should express a Thy-1 ligand, did not reveal positive staining (Figure A.6). However, the presence of a potential Thy-1 ligand in mouse brain tissue could be confirmed by immunohistochemistry (Figure A.7); $\alpha_V\beta_3$ is one such possible ligand (Leyton et al., 2001). Despite the RLD integrin-binding motif of Thy-1, other molecules may interact with and stimulate T cell-associated Thy-1. In this regard, I was able to immunoprecipitate multiple proteins from lymphoid tissue with the Thy-1-Fc protein (Figure A.8). However, preliminary mass spectrometry data were inconclusive, and none of these proteins have yet been identified. This technique will be continued in the lab to identify new potential Thy-1 binding partners.

In summary, my results clarify the role of MAPKs in Thy-1-induced T cell activation, and highlight a number of components of the TCR signalling pathway that are involved in Thy-1-induced gene expression in T cells. Lastly, and perhaps most interesting of all, I have provided the first experimental evidence that stimulation of a GPI-anchored protein can induce a regulatory phenotype in T cells. I expect that this new information will advance our understanding of Thy-1, and bring us closer to fully understanding its role in T cell biology.

Appendix – Supplementary Figures

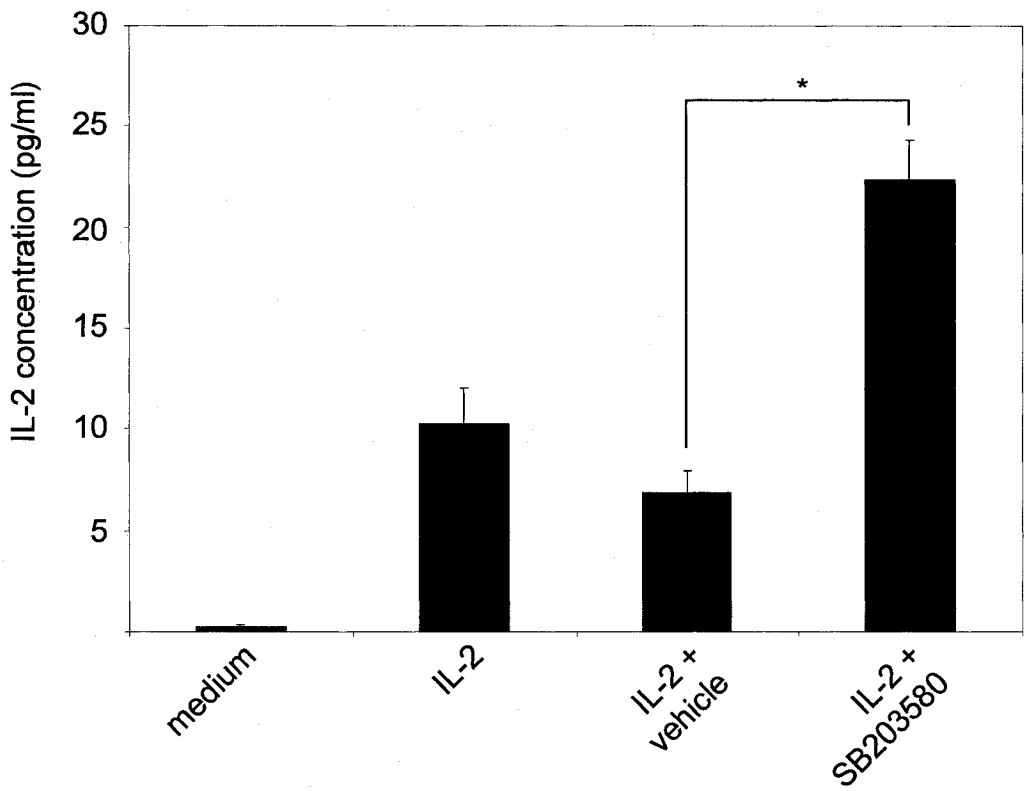


Figure A.1. Inhibition Of p38 MAPK Results In Elevated IL-2 Levels In CTLL-2 T Cell Supernatants. CTLL-2 IL-2-dependent T cells were pretreated with 10 μM SB203580 (p38 MAPK inhibitor), DMSO vehicle control, or medium, and then exposed to 50 U/ml IL-2 or medium alone. After 120 h, culture supernatants were collected and IL-2 levels were assessed in quadruplicate wells by sandwich ELISA. Data from one experiment are presented as mean IL-2 concentration (pg/ml) ± SD (n=3). * denotes $p < 0.001$.

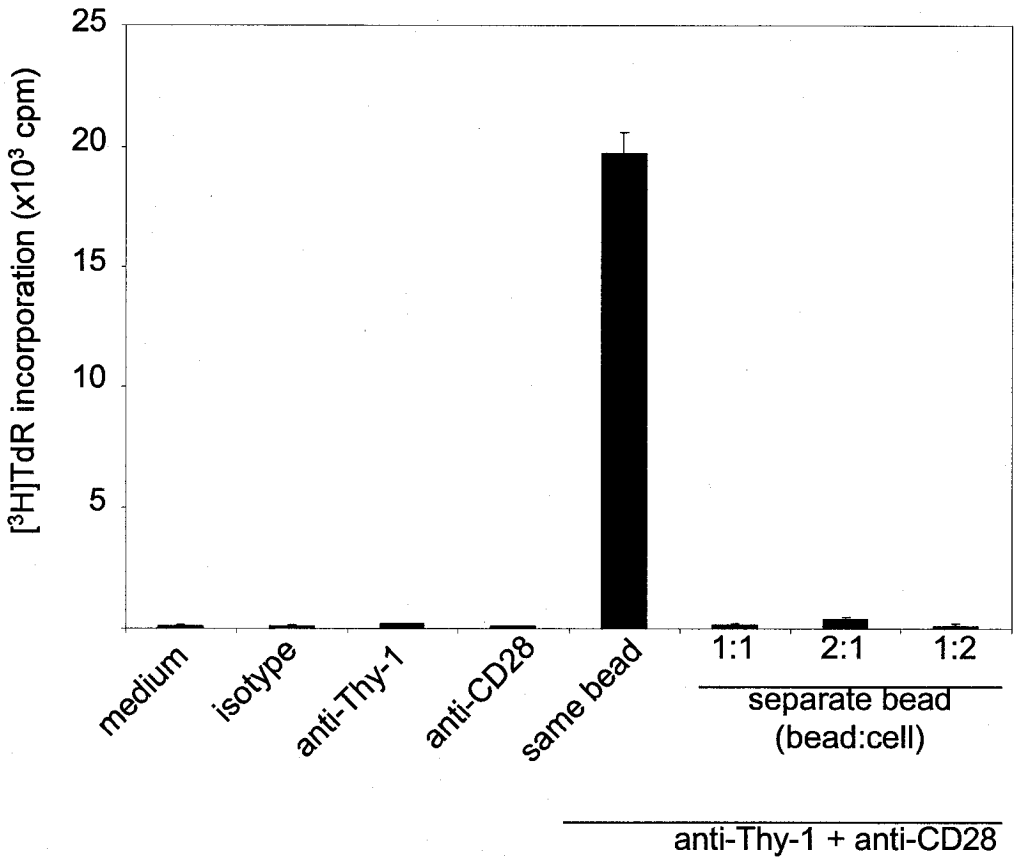


Figure A.2. Anti-Thy-1 mAb And Anti-CD28 mAb Coated Onto Separate Beads Do Not Activate T Cells. T cells were exposed to anti-Thy-1 mAb (10 µg/ml) and anti-CD28 mAb (5 µg/ml) either on the same bead (1 bead per cell) or separate beads at the indicated bead-to-cell ratios. Control treatments were isotype-matched antibody-coated beads, anti-Thy-1 mAb-coated beads alone, and anti-CD28 mAb-coated beads alone. [^{3}H]TdR incorporation was measured in quadruplicate wells after 48 h. Data from one experiment are presented as average cpm ± SD (n=2).

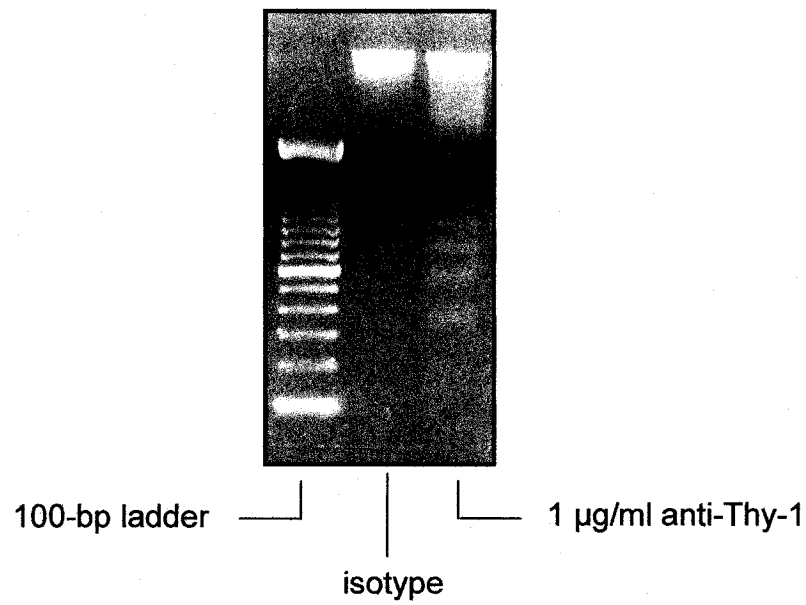


Figure A.3. Anti-Thy-1 Stimulation Induces Apoptosis In 1H3.1 T Cells. 1H3.1 T cells were treated with 1 µg/ml anti-Thy-1 mAb or isotype control for 24 h. DNA was harvested, stained with ethidium bromide in an agarose gel and visualised under UV light. The laddering pattern visible in the anti-Thy-1 mAb-treated lane is indicative of apoptosis. Results from one representative experiment are shown (n=3).

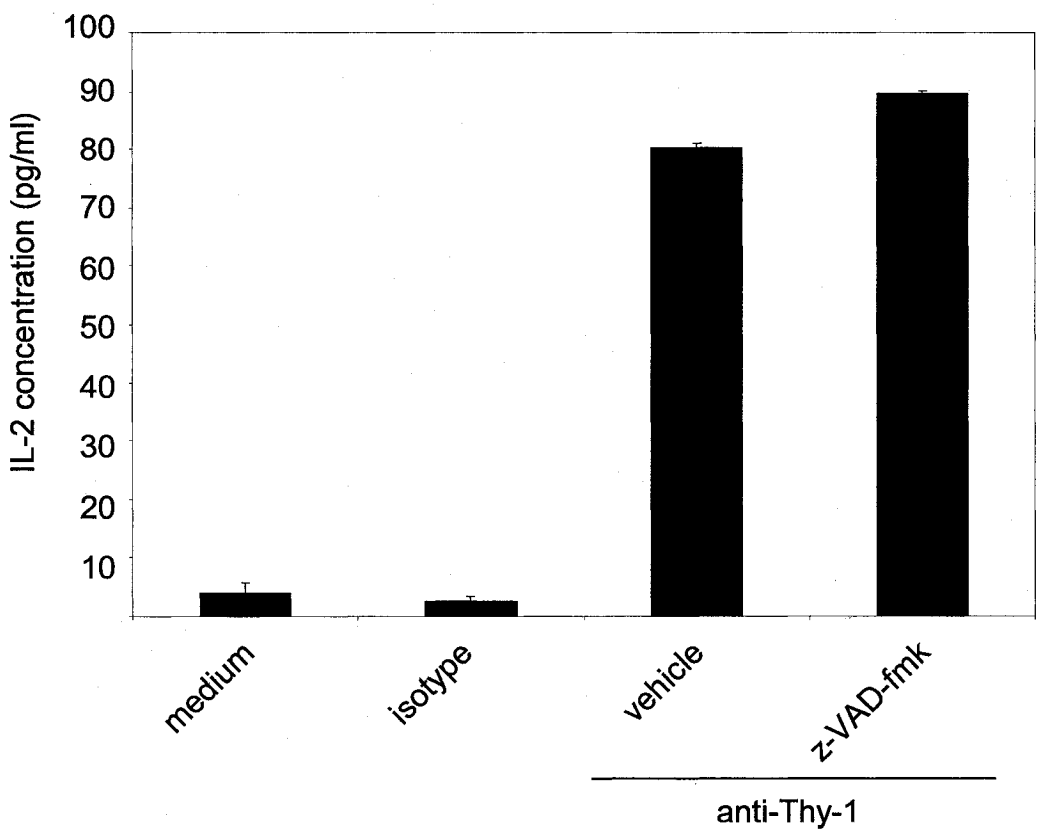


Figure A.4. Thy-1 Stimulation Induces IL-2 Production By 1H3.1 T Cells. 1H3.1 T cells were pretreated with 25 μM z-VAD-fmk (general caspase inhibitor), vehicle or medium for 30 minutes and exposed to 1 μg/ml anti-Thy-1 antibody, isotype control antibody or medium. IL-2 production was measured in quadruplicate wells after 24 h by sandwich ELISA. Data are presented as mean IL-2 concentration (pg/ml) ± SD. Results are from one experiment.

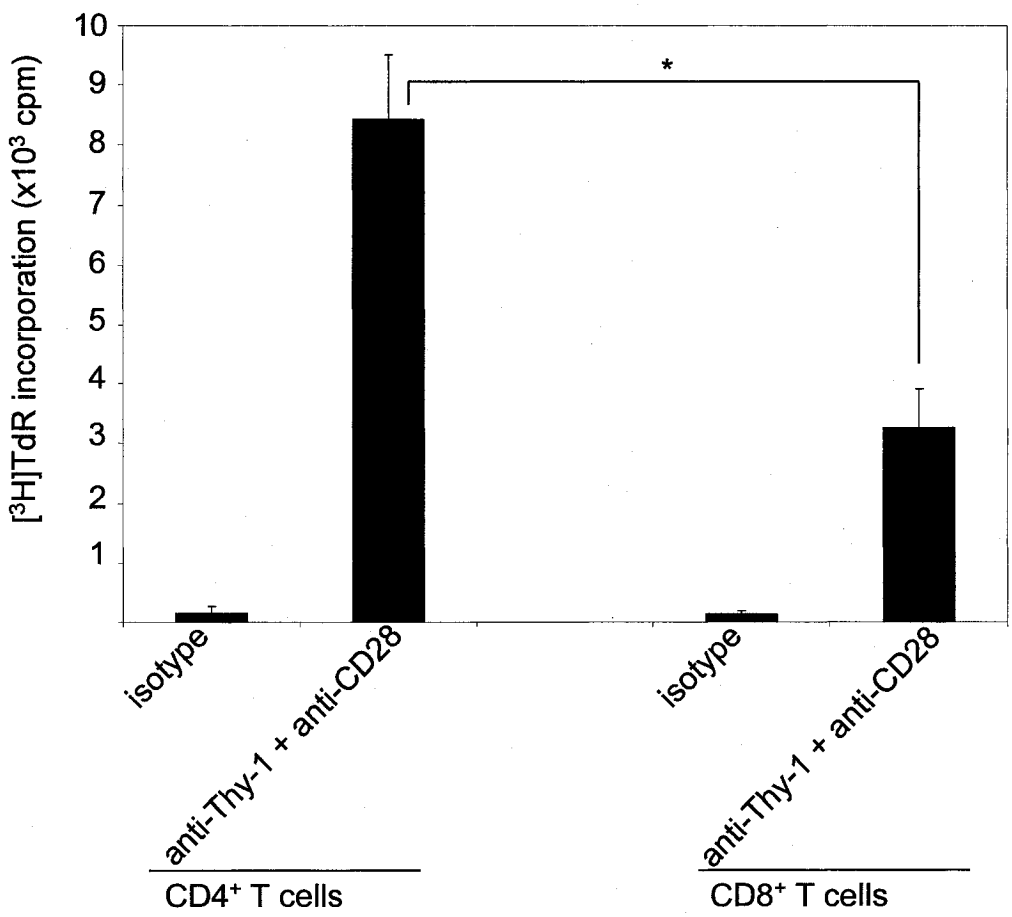


Figure A.5. $CD4^+$ T Cells Are More Responsive Than $CD8^+$ T Cells To Thy-1/CD28 Stimulation. $CD4^+$ and $CD8^+$ T cells were exposed to beads coated with anti-Thy-1 mAb (10 µg/ml) and anti-CD28 mAb (5 µg/ml) or isotype control antibodies at a 1:1 bead-to-cell ratio. [^{3}H]TdR incorporation was measured in quadruplicate wells after 48 h. Data from one experiment are presented as average cpm ± SD (n=2). * denotes $p < 0.001$.

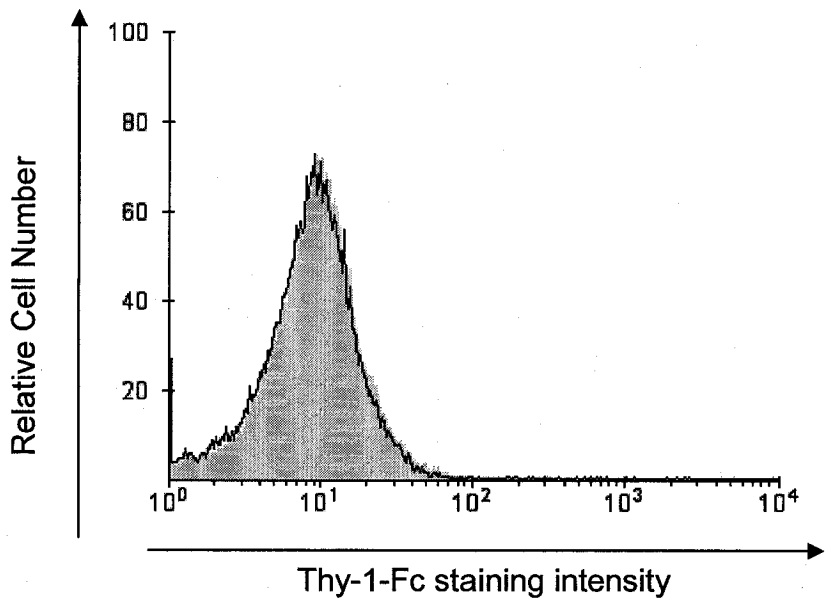
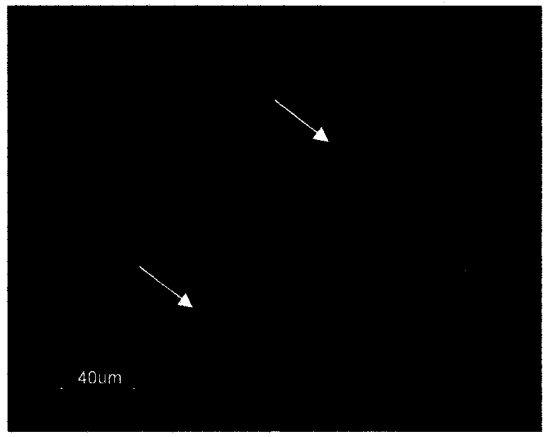


Figure A.6. Thy-1-Fc Binding To C6 Rat Glioma Cells Is Not Detectable By Flow Cytometry. C6 rat glioma cells were stained with 10 µg/ml human IgG_1 (filled peak) or Thy-1-Fc (open peak) and Alexa Fluor® 488-conjugated goat anti-human IgG. The cells were then analysed by flow cytometry to detect Thy-1-Fc chimera binding. Results from one experiment are shown.

Thy-1-Fc + Alexa488 anti-human IgG



Alexa488 anti-human IgG

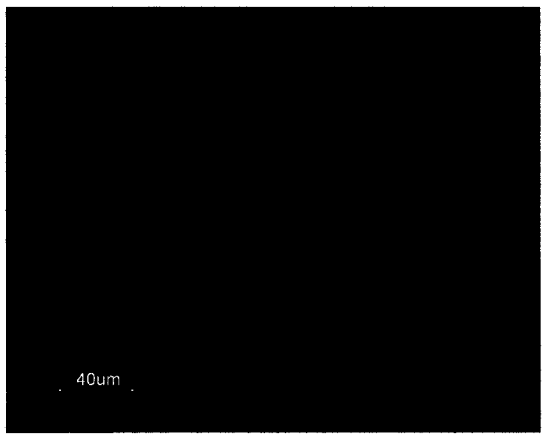


Figure A.7. Thy-1-Fc Binds To Cells In Mouse Brain Tissue. C57BL/6 mouse brain tissue was fixed in UMFix reagent, sectioned and stained with 10 µg/ml Thy-1-Fc and Alexa Fluor® 488-conjugated goat anti-human IgG, or Alexa Fluor® 488-conjugated goat anti-human IgG alone. Tissue sections were visualised on a fluorescence microscope. Arrows indicate positively stained cells.

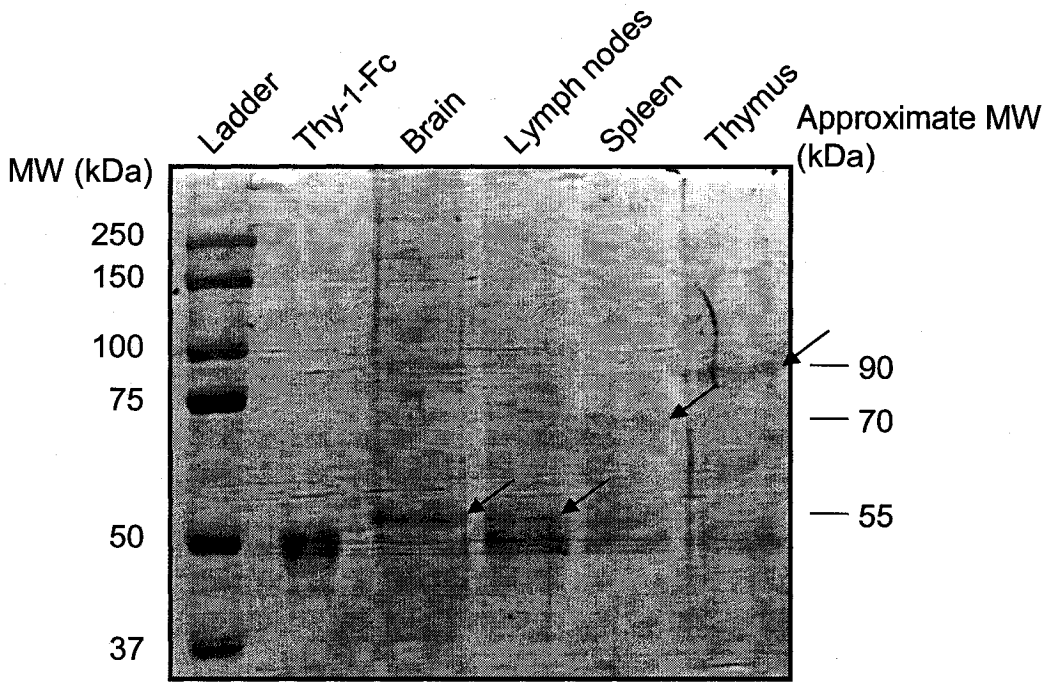


Figure A.8. Thy-1-Fc Interacts With Multiple Proteins In Lymphoid Tissues. Lymph node, spleen, and thymus tissue homogenates were immunoprecipitated with Thy-1-Fc. Samples were resolved by SDS-PAGE and stained with Brilliant Blue R protein dye. Results from one experiment are shown (n=2). Arrows indicate major interactions.

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