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Requirement for CD2 signaling in the costimulation and induction of murine cytotoxic T lymphocytes

by

Bruce L. Musgrave

Submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Dalhousie University

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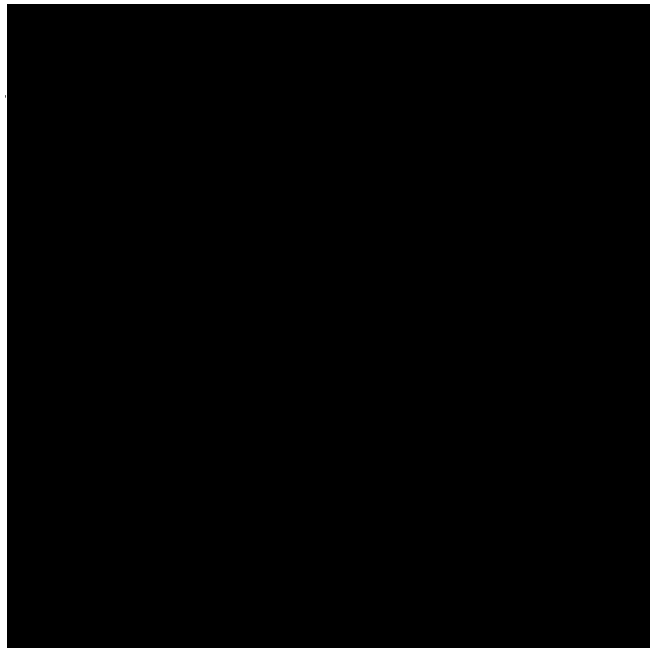

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TABLE OF CONTENTS

1.0 Introduction.....1

1.1 Induction of cytotoxic T lymphocytes.....1

(a) CTL activation.....1

(b) TCR/CD3 signal transduction.....3

1.2 Cytokines involved in CTL activation.....7

(a) IL-2 and IL-2R.....7

(b) IL-2-like cytokines: IL-15 and IL-21.....9

(c) IFN-γ.....10

1.3 Reorganization of T cell surface molecules during T cell activation.....12

(a) Involvement of the cytoskeleton in T cell activation.....12

(b) Unresolved issues with current models of T cell activation.....14

(c) The immunological synapse.....15

1.4 Cytolytic mechanisms.....17

(a) Apoptosis.....17

(b) Steps in cytolysis.....18

(c) Granule-mediated exocytosis.....18

(d) Fas – Fas ligand-mediated apoptosis.....20

(e) TRAIL-mediated apoptosis.....21

(f) TNF-mediated apoptosis.....22

1.4 Costimulation and the regulation of immune activation.....22

(a) Cytokine mRNA half-life.....22

(b) Regulation of anergy and apoptosis.....23

1.5 CD2-CD48 costimulation.....24

(a) CD2 structure.....24

(b) Ligands of CD2: CD48, CD58, CD59.....26

(c) Antigen-independent activation of T cells through CD2.....29

(d) Signal transduction through CD2.....30

(e) CD2 costimulation of T cells.....34

1.6 Objectives.....38

2.0 Methods and Materials.....40

2.1 Mice.....40

2.2 Medium and reagents.....40

2.3 T cell Isolation and activation by anti-CD3 mAb.....42

2.4 T cell proliferation assay.....42

2.5 ^{51}Chromium-release assay.....43

2.6 RNA isolation.....43

2.7 Semi-quantitative RT-PCR.....44

2.8 Enzyme-linked immunosorbent assay (ELISA).....46

2.9 Western blot analysis.....47

2.10 Flow cytometry analysis (FACS).....48

2.11 T cell-target cell conjugation assay.....49

2.12 Statistical analysis.....49

3.0 Results.....50

Section I: CD2 signaling is required for non-specific cytotoxic T lymphocyte induction by anti-CD3 antibody.....50

3.1 Blockade of CD2-CD48 interactions in anti-CD3-activated T cell cultures inhibits cell proliferation and the development of cytotoxic activity.....50

3.2 Inhibitory effects of anti-CD2 mAb on CD8+ AK-T cell proliferation and cytotoxicity57

3.3 AK-T cells exhibit altered phosphorylation of tyrosine residues on cytoplasmic proteins in the presence of anti-CD2 mAb.....57

3.4 Anti-CD2 mAb fail to inhibit T cell activation by combinations of PMA and ionomycin or PMA and anti-CD3 mAb.....63

3.5 CD2-CD48 interactions are required during the early stages of anti-CD3-mediated T cell activation.....63

3.6 CD2-CD48 blockade does not cause a shift in the kinetics of AK-T cell activation.....63

3.7 CD2 signaling promotes AK-T cell adhesion to P815 target cells.....69

3.8 IL-2 and IFN-γ production in AK-T cell cultures is downregulated in the presence of anti-CD2 or anti-CD48 mAb.....77

3.9 CD25 expression is downregulated but CD119 expression by AK-T cells is enhanced in the absence of CD2 signaling.....77

3.10 Granzyme B and perforin expression is downregulated in AK-T cell cultures treated with anti-CD2 mAb, but is unaffected by anti-CD48 mAb treatment.....81

3.11 Perforin mRNA expression is downregulated in unstimulated T cells treated with anti-CD2 mAb.....89

3.12 Anti-CD2 mAb do not inhibit CTLL-2 cell proliferation.....89

3.13 Exogenous IL-2 and IFN-γ restore normal function to AK-T cells induced in the absence of costimulation through CD2.....89

3.14 Exogenous IL-2 and IFN-γ restore normal function to AK-T cells induced in the presence of anti-CD48 mAb.....94

3.15 IL-2 is required for AK-T cells to gain the ability to conjugate to P815 target cells....94

3.16 Granzyme B and perforin expression is restored in anti-CD2 mAb-treated AK-T cells by the addition of exogenous IL-2 and IFN-γ.....101

3.17 2B4 mRNA is undetectable in AK-T cell cultures as assessed by RT-PCR.....101

Section II: Signaling through CD2 enhances IL-2 and IFN-γ mRNA stability in T lymphocytes: Evidence that CD2 meets criteria for a costimulatory molecule.....108

3.18 Blockade of CD2-CD48 interactions prevents proliferation in anti-CD3-activated T cell cultures.....108

3.19 IL-2, IFN-γ, and CD25 mRNA levels are decreased but CD119 mRNA levels are unaffected in T cells activated in the absence of CD2 signaling.....113

3.20 IL-2 and IFN-γ mRNA half-life is reduced in the absence of CD2 signaling.....113

3.21 CD2 signaling enhances T cell proliferation in response to sub-optimal stimulation with anti-CD3 mAb-coated microspheres.....118

3.22 IL-2 and IFN-γ expression are augmented by CD2 signaling.....121

3.23 IL-2 and IFN-γ mRNA half-life is enhanced by CD2 signaling.....121

3.24 Dilution analysis of IL-2, IFN-γ, CD25, CD119, and GAPDH confirms RT-PCR results of neat cDNA samples was under non-saturating conditions.....126

3.25 Determination of the appropriate number of PCR cycles for amplification of IL-2, IFN-γ, CD25, CD119, and GAPDH mRNA.....126

4.0 Discussion.....132

Section I: CD2 signaling is required for non-specific cytotoxic T lymphocyte induction by anti-CD3 antibody.....132

4.1 T cell activation and costimulation.....132

4.2 CD2 signaling in T cell activation.....134

4.3 Kinetics of $CD8^+$ T cell activation.....135

4.4 Early requirements for T cell activation.....136

4.5 Enhanced cytokine production by CD2 signaling.....140

4.6 CD2 signaling and the induction of T cell cytotoxicity.....141

4.7 Negative signaling through CD2.....143

4.8 T cell adhesion to target cells.....145

4.9 2B4 on T cells.....148

Section II: Signaling through CD2 enhances IL-2 and IFN-γ mRNA stability in T lymphocytes: Evidence that CD2 meets criteria for a costimulatory molecule...149

4.10 CD2 and CD28 in T cell costimulation.....149

4.11 Stabilization of cytokine mRNA via costimulation.....150

4.12 Semi-quantitative RT-PCR.....153

4.13 General conclusions.....155

5.0 References.....160

LIST OF FIGURES

Figure 1. Blockade of CD2-CD48 interactions in anti-CD3 mAb-activated T cell cultures inhibits cell proliferation.....51

Figure 2. Blockade of CD2-CD48 interactions in anti-CD3 mAb-activated T cell cultures inhibits the development of cytotoxic activity.....54

Figure 3. Inhibitory effects of anti-CD2 mAb on CD8+ AK-T cell proliferation and cytotoxicity.....58

Figure 4. AK-T cells exhibit altered phosphorylation of tyrosine residues on cytoplasmic proteins in the presence of anti-CD2 mAb.....61

Figure 5. Anti-CD2 mAb fail to inhibit T cell activation by PMA / ionophore- and PMA / anti-CD3 mAb.....64

Figure 6. CD2-CD48 interactions are required during the early stages of T cell activation..66

Figure 7. CD2-CD48 blockade does not alter the kinetics of AK-T cell activation.....70

Figure 8. CD2-CD48 interactions play a minor role in the adhesion of AK-T cells to P815 mastocytoma cells.....73

Figure 9. Effect of CD2 blockade on adhesion molecule expression.....75

Figure 10. IL-2 and IFN-γ mRNA expression in AK-T cell cultures is downregulated in the presence of anti-CD2 mAb.....79

Figure 11. CD25 expression is downregulated but CD119 expression by AK-T cells is enhanced in the absence of CD2 signaling.....82

Figure 12. Granzyme B and perforin expression is downregulated in AK-T cell cultures treated with anti-CD2 mAb, but is unaffected by anti-CD48 mAb treatment.....84

Figure 13. Granzyme B and perforin protein expression is downregulated in AK-T cell cultures treated with anti-CD2 mAb.....87

Figure 14. Perforin mRNA expression is downregulated in unstimulated T cells exposed to anti-CD2 (RM2.5) mAb for 6h.....90

Figure 15. Anti-CD2 mAb do not inhibit CTLL-2 cell proliferation.....92

Figure 16. Exogenous IL-2 and IFN-γ restore normal function to AK-T cells induced in the absence of costimulation through CD2.....95

Figure 17. Exogenous IL-2 and IFN-γ restore normal function to AK-T cells induced in the presence of anti-CD48 mAb.....98

Figure 18. Exogenous IL-2 restores conjugation to AK-T cells induced in the absence of costimulation through CD2.....102

Figure 19. Exogenous IL-2 restores conjugation to AK-T cells induced in the presence of anti-CD48 mAb.....104

Figure 20. AK-T cell expression of granzyme B and perforin is restored by the addition of exogenous IL-2 and IFN-γ.....106

Figure 21. 2B4 mRNA is undetectable in AK-T cell cultures.....109

Figure 22. CD2 and/or CD28 blockade inhibits anti-CD3 mAb-induced T cell proliferation.....111

Figure 23. Activated T cells express decreased levels of mRNA coding for cytokines and cytokine receptors in the absence of CD2 and/or CD28 signaling.....114

Figure 24. CD2 blockade decreases IL-2 and IFN-gamma mRNA stability.....116

Figure 25. CD2 signaling enhances T cell proliferation.....119

Figure 26. CD2 and CD28 signaling enhances expression of IL-2, IFN-gamma and CD25 mRNA by activated T cells.....122

Figure 27. IL-2 and IFN-gamma mRNA stability is enhanced by CD2 signaling.....124

Figure 28. Dilution analysis of IL-2, IFN-γ, CD25, CD119, and GAPDH confirms RT-PCR of neat cDNA samples was under non-saturating conditions.....127

Figure 29. Determination of the appropriate number of PCR cycles for amplification of IL-2, IFN-γ, CD25, CD119, and GAPDH mRNA.....130

LIST OF TABLES

Table 1. CD2 signaling promotes AK-T cell adhesion to P815 target cells.....72

Table 2. IL-2 and IFN-γ production in AK-T cell cultures is downregulated in the presence of anti-CD2 or anti-CD48 mAb.....78

Abstract

Although signal transduction through CD2 is known to be involved in T cell activation, the costimulatory role of CD2 in cytotoxic T lymphocyte development is not entirely clear. CD2 is a surface molecule found on most thymocytes and all mature T cells which is known to function in adhesion and the regulation of cytokine production and responsiveness. In this study, we show that the interaction of CD2 with its ligand CD48 is critical for the induction of cytotoxic effector function by T lymphocytes since anti-CD3-activated killer T (AK-T) cell induction is inhibited in the presence of anti-CD2 and anti-CD48 monoclonal antibodies (mAb). Inhibition is due to decreased conjugation of effector cells to allogeneic target cells and decreased cytolytic molecule expression. One likely explanation for the observed inhibitory effects is that CD2 blockade downregulates IL-2 and IFN-γ synthesis. Both IL-2 and IFN-γ are of critical importance for the development of T cell cytotoxicity. Evidence is provided that blockade of CD2-CD48 interactions resulted in decreased IL-2 and IFN-γ protein and mRNA expression in anti-CD3 activated T cell cultures. Furthermore, culture in the presence of anti-CD2 mAb downregulated perforin and granzyme B mRNA transcripts in AK-T cells. Granzyme B and perforin are two key molecules in the granule-mediated pathway of killing. Moreover, downregulation of baseline perforin expression in anti-CD2 mAb treated AK-T cell cultures, as well as decreased background perforin expression in resting T cells exposed to anti-CD2 mAb indicates that CD2 may have negative signaling properties. Restoration of granzyme B and perforin expression, as well as AK-T cell effector function upon addition of exogenous IL-2 and IFN-γ to anti-CD2 mAb treated AK-T cells at initiation of culture is consistent with the cytokine-dependent nature of the granzyme B/perforin cytolytic machinery. Although signaling through CD28 and LFA-1 has been implicated in enhancing the stability of mRNAs coding for IL-2 and IFN-γ, little is known about the role of CD2 signaling in cytokine mRNA stabilization. Here, we show that CD2 signaling enhances the stability of both IFN-γ and IL-2 mRNA. These results confirm the costimulatory role of CD2 in T cell induction and suggest that inhibition of T cell activation and consequent effector responses by CD2-CD48 blockade involves changes in IL-2 and IFN-γ mRNA stability. In conclusion, the results of this study indicate that costimulatory signaling through CD2 is critical for the development of cytotoxic T cell activity.

ABBREVIATIONS AND SYMBOLS

Act D	actinomycin D
AP-1	activating protein-1
APC	antigen presenting cell
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pair
BSA	bovine serum albumin
CHO	Chinese hamster ovary
^{51}Cr	^{51}chromium
cRPMI	complete RPMI 1640 medium
CTL	cytotoxic T lymphocyte
DAG	diacylyglycerol
DISC	death-inducing signaling complex
Dok	downstream of tyrosine kinase
ELISA	enzyme-linked immunosorbent assay
FADD	Fas-associated death domain
FasL	Fas Ligand
FcR	Fc receptor
FCS	fetal calf serum
FLIP	FLICE (caspase-8)-inhibitory proteins
GAPDH	glyceraldehyde phosphodehydrogenase
GPI	glycosylphosphatidylinositol
h	hour

HEPES	*N*-2-hydroxyethylpiperazine-*N*-2ethane sulfonic acid
[^{3}H]TdR	tritiated thymidine
ICAM	intercellular adhesion molecule
ICOS	inducible costimulatory molecule
IFN	interferon
IL	interleukin
IgSF	Immunoglobulin superfamily
IP_3	inositol 1,4,5 triphosphate
ITAM	immune receptor tyrosine-based activation motif
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
kDa	kilodalton
LAK	lymphokine-activated killer cell
LAT	Linker for activation of T cells
LCMV	lymphocytic choriomeningitis virus
LFA	lymphocyte function-associated antigen
LPS	lipopolysacharide
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
min	minute
MTOC	microtubule organization center
NBT	nitro blue tetrazolium

NFAT	nuclear factor of activated T cells
NK	natural killer
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PI 3-K	phosphatidylinositol 3-kinase
PIP_2	phosphatidylinositol 4,5 biphosphate
PIP_3	phosphatidylinositol 3,4,5 triphosphate
PKC	protein kinase C
PLCγ1	phospholipase C-γ1
PMA	phorbol 12-myristate 13-acetate
PTK	protein tyrosine kinase
RT	reverse transcription
SEA	staphylococcal enterotoxin A
sec	seconds
SDS	sodium dodecyl sulfate
SH	Src homology
SLP-76	SH2-domain containing leukocyte protein of 76 kDa
SMAC	supramolecular activation cluster
STAT	signal transducer and activator of transcription
TAE	Tris acetate EDTA buffer
TBS	Tris-buffered saline

TCR	T cell receptor
Th	T helper
TNF	Tumor necrosis factor
TRADD	TNF receptor-associated death domain
TRAIL	TNF-related apoptosis-inducing ligand
ZAP-70	zeta-associated protein of 70kDa

1.0 Introduction

1.1 Induction of cytotoxic T lymphocytes

(a) CTL activation

Cytotoxic T lymphocytes (CTL) are important effector cells in host immune surveillance against neoplasia and infection by many intracellular pathogens (Kagi *et al.* 1996). CTL protect the host by inducing apoptosis in transformed or infected cells. $CD8^+$ T cell expansion and differentiation into functional effector cells is linked to signal transduction through specific cell surface receptors which provide at least two distinct signals to induce optimal activation and proliferation (Liu and Linsley 1992; Mescher 1995). The primary signal is the result of T cell receptor (TCR) engagement by antigenic peptides presented in the context of self MHC molecules, and is facilitated by adhesion molecules such as LFA-1 which mediate formation of T cell-antigen presenting cell (APC) conjugates (Bachmann *et al.* 1997). TCR triggering leads to the activation of receptor-associated protein tyrosine kinases such as ZAP-70, $p56^{lck}$, $p59^{fyn}$, phospholipase C-γ1, and phosphoinositide 3-kinase which ultimately result in transcription factor translocation to the nucleus (Hutchcroft *et al.* 1998).

A secondary signal resulting from the interactions of costimulatory proteins and their ligands is also required for T cell activation. The secondary signal results from the interaction of costimulatory molecule / ligand pairs such as CD28/B7, CD40/CD40L, CD2/CD48 or CD58 on the surface of the T cell and APC, respectively (Watts and Debenedette 1999; Liu and Linsley 1992). The combination of TCR and costimulatory signaling stimulates the optimal activation of T cells and induces T cell production of

cytokines such as interleukin (IL)-2 and interferon (IFN)-γ that promote further T cell differentiation (Liu and Linsley 1992). Blocking these costimulatory signals inhibits T cell activation (Lenschow *et al.* 1992). Interestingly, IL-12 can substitute for CD28 costimulation during CTL induction (Makrigiannis *et al.* 2001). Ligation of CD28 and CD2 results in enhanced production of IL-2 by T cells (Gimmi *et al.* 1991; Rosenthal-Allieri *et al.* 1995). Correspondingly, signaling through CD2 and CD28 has been shown to induce nuclear translocation of the transcription factor NFAT (Ghosh *et al.* 1996; Tanaka *et al.* 1997), which has been implicated in the expression of IL-2 and other molecules important for T cell activation (Rooney *et al.* 1995). Additionally, costimulation of T lymphocytes via CD28 slows degradation of IL-2 and IFNγ mRNA (Collins *et al.*1994; June *et al.* 1990). Traditionally, it was believed that $CD8^+$ killer T cells required IL-2 produced by $CD4^+$ Th cells in order to fully differentiate into effector T cells. However, $CD8^+$ cytotoxic T cells are, in fact, capable of developing into functional CTL in the absence of $CD4^+$ T helper cells (Buller *et al.* 1987; Rahemtulla *et al.* 1991; Zhan *et al.* 2000).

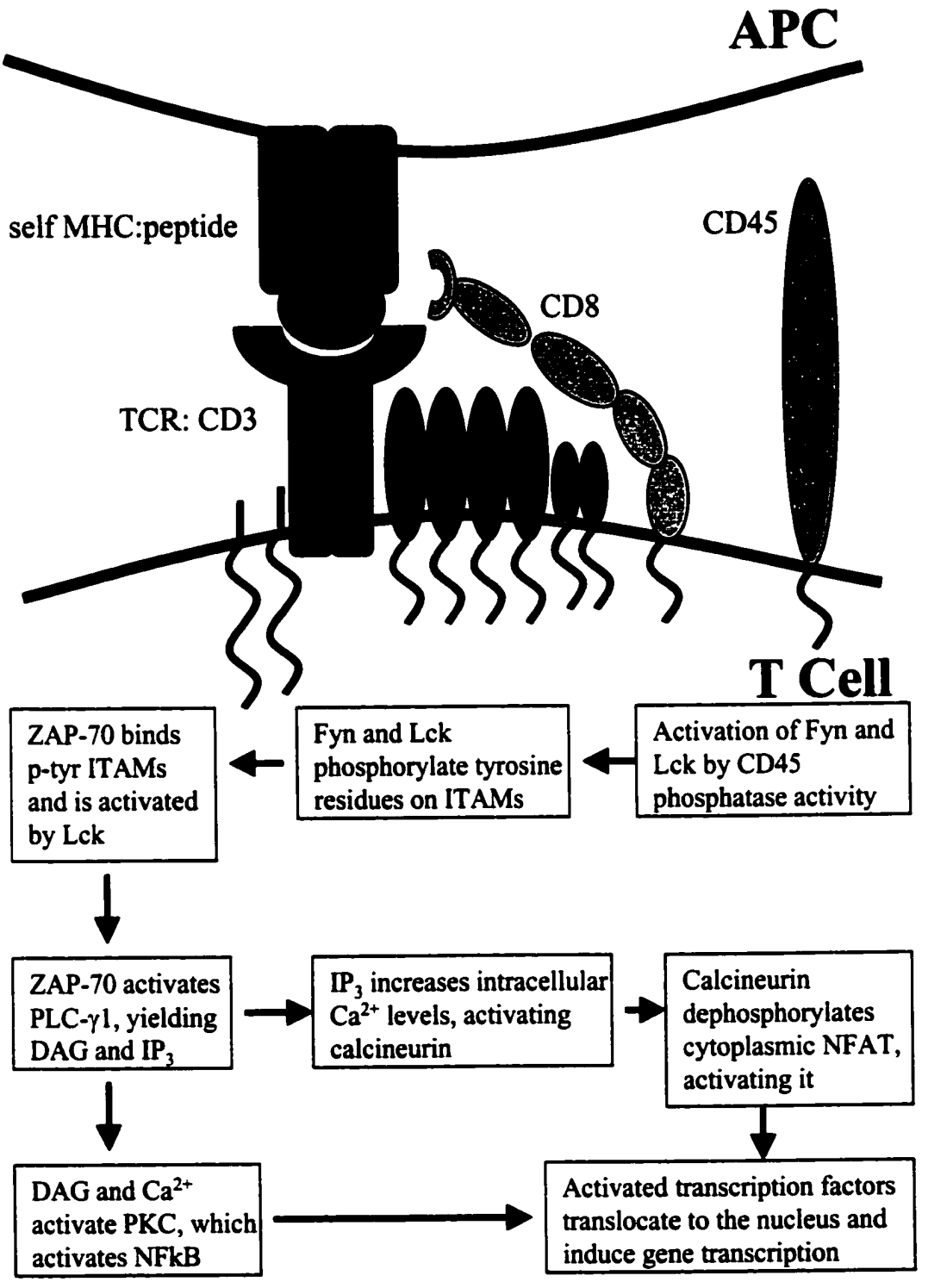
Anti-CD3ε mAb are able to activate T cells in the absence of specific antigen, resulting in the use of these mAbs to study T cell activation through TCR stimulation (Leo *et al.* 1987). FcR^+ accessory cells such as B cells or macrophages must be present to cross-link the anti-CD3 mAb or, alternatively, cross-linking anti-isotype antibody is required for T cell activation to occur (Schwab *et al.* 1985). Thus, anti-CD3 mAb F(ab')2 fragments are unable to induce T cell activation (Hirsch *et al.* 1989). T cells can also be activated through immobilized anti-CD3 mAb in combination with exogenous IL-2 or anti-CD28 mAb (Vine *et al.* 1989; June *et al.* 1987; Blair *et al.* 1998). Anti-CD3 mAb

induce T cell transcription of important cytokine genes such as IL-2 and IFN-γ (Thompson *et al.* 1989). T cell activation by anti-CD3 mAb leads to a proliferative response and MHC-unrestricted cytotoxicity (Stankova *et al.* 1989). As such, anti-CD3 mAb provide a useful tool to investigate T cell activation and its consequences.

(b) TCR/CD3 signal transduction

T cell activation is initiated following the engagement of the TCR/CD3 complex by a specific antigenic peptide-MHC molecule on an APC. The TCR consists of αβ or γδ heterodimers involved in binding MHC-peptide complexes and CD3 γ, δ, and ε chains and ζζ homodimers involved in signal transduction (Caplan and Baniyash 2000). The initial T cell activation event is the phosphorylation of CD3 immunoreceptor tyrosine-based activation motifs (ITAMs) tyrosine residues on γ, δ, ε, and ζ chains by a src-family PTK such as Lck or Fyn (Wange and Samelson 1996) (Diagram 1). Lck is associated with the cytoplasmic tail of CD4 and CD8. TCR ligation brings CD4 or CD8 into close proximity of CD3 ITAMs and the ζ chain, allowing Lck to phosphorylate the tyrosine residues. Once phosphorylated, ITAMs associated with the ζ chain serve as docking sites for proteins such as ZAP-70 which possess SH2 domains. ZAP-70 is recruited via its pair of SH2 domains to phosphorylated ζ-chain and is activated by phosphorylation by Lck (Weiss and Littman 1994). An alternative model of CD4 and CD8 involvement in TCR-mediated signaling suggests that these coreceptors may not shuttle Lck to ITAMs. Rather, high affinity binding of Lck to ITAM-bound phosphorylated ZAP-70 may be the means by which the coreceptors "find" MHC-peptide-TCR complex (Thome *et al.* 1995).

Diagram 1.


APC
self MHC:peptide
CD45
CD8
TCR: CD3
T Cell
Activation of Fyn and Lck by CD45 phosphatase activity
Fyn and Lck phosphorylate tyrosine residues on ITAMs
ZAP-70 binds p-tyr ITAMs and is activated by Lck
ZAP-70 activates PLC-γ1, yielding DAG and IP3
IP3 increases intracellular Ca2+ levels, activating calcineurin
Calcineurin dephosphorylates cytoplasmic NFAT, activating it
DAG and Ca2+ activate PKC, which activates NFkB
Activated transcription factors translocate to the nucleus and induce gene transcription

CD45 is a phosphatase which may initially dephosphorylate two inhibitory tyrosine residues on Lck or Fyn (reviewed in Trowbridge and Thomas 1994). Dephosphorylation of the C-terminus tyrosine residue by CD45 exposes the SH2 domain, facilitating interactions with other signaling molecules and increasing Lck and Fyn activity (Ostergaard *et al.* 1989). Several researchers have demonstrated that CD45 substrates include Lck, Fyn, CD3 ζ-chain, and ZAP-70 (Mustelin *et al.* 1989; Mustelin *et al.* 1992; Mustelin *et al.* 1995; Furukawa *et al.* 1994).

Following activation, ZAP-70 phosphorylates two adapter proteins termed Linker for activation of T cells (LAT) and SH2-domain containing leukocyte protein of 76 kDa (SLP76) (Zhang *et al.* 1998a; Qian *et al.* 1996; Mege *et al.* 1996). LAT is a membrane-associated protein which functions to recruit phospholipase C γ1 (PLC-γ1) to the plasma membrane (Zhang *et al.* 1998a). The precise functions of LAT and SLP76 are not yet known, but LAT also appears to function as a platform for the assembly of the PLCγ1 signaling complex, while SLP76 binds to LAT and may possibly regulate the entire process (Asada *et al.* 1999; Liu *et al.* 1999; Pivniouk and Geha 2000; Zhang *et al.* 1998a; Zhang *et al.* 1998b). ZAP-70 kinase activity results in the phosphorylation of LAT. Tyrosine-phosphorylated LAT is subsequently confined to plasma membrane rafts (Montixi *et al.* 1998; Xavier *et al.* 1998). It is here that LAT recruits necessary signaling molecules such as Grb2, PLCγ1, PI 3-kinase p85 subunit, SLP-76, and Vav via SH2 domain interactions (Zhang *et al.* 1998a). PLCγ1 is subsequently phosphorylated and activated, most likely by ZAP-70. However, SLP-76 can bind to the SH3 domain of Lck through its proline-rich region (Sazenbacher *et al.* 1999). After the tyrosine phosphorylation of SLP-76 on tyrosine residues, presumably by ZAP-70, SLP-76 could

interact with SH2-domain containing proteins and form a signaling complex (Su *et al.* 1999). Itk has been shown to bind to phosphorylated SLP-76, which may also bind PLC-γ1 since PLC-γ1 is a confirmed Itk target (Su *et al.* 1999).

Once activated, PLC-γ1 associates with the plasma membrane where its substrates are located. PLC-γ1 is responsible for regulating the hydrolysis of phosphatidylinositol (4,5) biphosphate (PIP_2) and the production of diacylyglycerol (DAG) and inositol 1,4,5 triphosphate (IP_3). LAT may also play a role in the activation of phosphatidylinositol 3-kinase (PI 3-K) by recruiting the inactive p85 subunit to the plasma membrane where it interacts with the p110 subunit. Active PI 3-K phosphorylates PIP_2 to generate phosphatidylinositol (3,4,5) triphosphate (PIP_3) (Zhang 1998a; Ward *et al.* 1996). PI 3-K is required for T cell activation since inhibitors of this kinase prevent mouse CTL development (Phu *et al.* 2001). PLC-γ1 transfers the terminal phosphate of ATP to the D-3 position of the inositol head groups of phosphoinositide lipids and generates three products in the process, the key product being inositol (1,4,5)-trisphosphate (IP_3) (Kapeller and Cantley 1994). IP_3 stimulates the release of membrane sequestered intracellular Ca^{2+} stores and opens plasma membrane Ca^{2+} channels. Grb2 recruits signaling molecules such as Vav which results in actin reorganization, and Ras which leads to MAPK activation. As such, this signaling pathway is critical for the elevation of intracellular Ca^{2+} levels and activation of protein kinase C (PKC) family members. Ca^{2+} binds calmodulin, forming a complex which activates a serine/threonine phosphatase called calcineurin. Dephosphorylation of cytoplasmic NFAT by calcineurin allows cytoplasmic NFAT to translocate to the cell nucleus. DAG in combination with Ca^{2+} activates PKC that phosphorylates serine/threonine residues on various substrates,

eventually activating transcription factors such as NFκB by releasing inhibitory IκB through its phosphorylation (Reviewed in Ilangumaran *et al.* 2000; Bauch *et al.* 2000; Cantrell *et al.* 1996). The end result is transcriptional activation of the IL-2 gene and commitment of the T cell to cell cycle and a round of division (Finco *et al.* 1998).

1.2 Cytokines involved in CTL activation

(a) IL-2 and IL-2R

In 1965, interleukin (IL)-2 was first described as an unknown growth factor in the supernatants of mitogen-stimulated T cell cultures (Kasakura and Lowenstein 1965). This small (~15 kDa) molecule is critical for the full induction of T cell proliferation and the development of cytotoxic activity (Smith 1988; Smyth *et al.* 1990). In support of this is the observation that IL-2-deficient mouse T cells are much less able to respond to mitogenic stimuli than are wild-type T cells (Schorle *et al.* 1991). IL-2 promotes the differentiation of CD8+ T cells into functional CTL (Selvan *et al.* 1990) through upregulating the expression of granzymes and perforin genes (Smyth *et al.* 1990; Liu *et al.* 1989). IL-2 is also capable of upregulating the expression of anti-apoptotic Bcl-2 (Miyazaki *et al.* 1995). In addition to promoting T cell growth, IL-2 has been shown to stimulate B cell proliferation and increased antibody production (Ceuppens and Stevens 1986). IFN-γ production by natural killer (NK) cells is upregulated in response to IL-2 (Ortaldo *et al.* 1984) and NK cell differentiation to lymphokine-activated killer cells is induced by IL-2 (Grimm *et al.* 1983).

IL-2 binds to a trimeric receptor that consists of a combination of three subunits designated the α, β, and γ chains (Ohashi *et al.* 1989; Nakarai *et al.* 1994). Although a

combination of the β and γ subunits will bind IL-2 and transduce an activation signal, the αβγ receptor binds IL-2 with much higher affinity (Taniguchi and Minami 1993). T cells only express the high affinity α subunit (CD25) following activation whereas the β subunit is expressed constitutively on $CD8^+$ T cells and is upregulated on $CD4^+$ T cells following activation (Ohashi *et al.* 1989). The γ subunit is also expressed constitutively on T cells (Nakarai *et al.* 1994). Interestingly, the γ subunit is also a component of receptors for other cytokines including IL-4, IL-7, IL-9, and IL-15 (Kishimoto *et al.* 1994; Giri *et al.* 1994).

The IL-2R delivers intracellular signals through associated Janus kinase (JAK) and signal transducers and activator of transcription (STAT) molecules (Gaffen *et al.* 1995; Beadling *et al.* 1994). IL-2R-associated JAK1 and JAK3 rapidly become activated and tyrosine phosphorylated following receptor interaction with IL-2 (Liu *et al.* 1997). The importance of JAK3 in IL-2R signaling is underscored by the observation that JAK3-deficient mice are defective in T cell development (Park *et al.* 1995). JAK phosphorylation is followed by recruitment and subsequent JAK-mediated tyrosine phosphorylation of STAT5 monomers (Gaffen *et al.* 1995). Although STAT5 has been linked to IL-2R signaling, a recent study examining STAT5 knockout mice suggests that STAT5 is not required for IL-2 and IL-2R expression (Moriggl *et al.* 1999). Phosphorylation of STAT5 molecules leads to their dimerization or oligomerization via reciprocal phosphotyrosine-SH2 domain interactions (Shuai *et al.* 1994). The activated STAT transcription factors subsequently translocate to the nucleus where they activate gene transcription by binding to response elements. The JAK/STAT signaling pathway is rapidly activated within 1-15 minutes of receptor stimulation (Silvennoinen *et al.* 1993),

and is also rapidly downregulated, usually within 2 hours (Lee *et al.* 1997). Ultimately, IL-2R signaling culminates in the activation of transcription factors, which include c-fos, c-jun, and c-myc (Shibuya *et al.* 1992; Hatakeyama *et al.* 1991).

(b) IL-2-like cytokines: IL-15 and IL-21

Like IL-2, IL-15 is a 14-15 kDa members of the four alpha-helix bundle family of cytokines that stimulate T cell proliferation. However, IL-15 is expressed in a wider variety of cell types than IL-2. Although IL-15 binds the β and γ chains of the IL-2 receptor complex, IL-15 also binds a specific high-affinity receptor chain, IL-15Rα (reviewed in Perera 2000). Receptors for IL-2 and IL-15 share signaling molecules, including JAK-1, JAK-3, STAT-3, and STAT-5 in T cells (Waldmann *et al.* 1998). Since receptors for IL-2 and IL-15 utilize common receptor subunits and signaling molecules, it is not surprising that IL-15 can compensate for a lack of IL-2 in lymphocyte activation (Ye *et al.* 1996). However, IL-2 and IL-15 are not completely identical in function. A recent study showed that IL-15 is able to reverse tumor-derived TGF-β blockade of IL-2-induced proliferation and STAT3 and STAT5 phosphorylation in T cells (Campbell *et al.* 2001). Recently, another cytokine closely related to IL-2 and Il-15 was discovered (Parrish-Novak *et al.* 2000). This cytokine has been designated IL-21, and its receptor, IL-21R. Although the role of IL-21 in T cell activation is unclear at present, *in vitro* assays indicate that this cytokine binds the γ chain of the IL-2R complex and has a role in stimulating T cell proliferation (Asao *et al.* 2001; Vosshenrich and DiSanto 2001).

(c) IFN-γ

Another cytokine that is important for the optimal activation of T cells is IFN-γ. The structure of IFN-γ consists of two identical polypeptide chains that associate noncovalently to form a 50 kDa molecule (Gray *et al.* 1982; Gray *et al.* 1983). This cytokine has been shown to upregulate MHC class I and II expression on professional APCs, as well as on epithelial and endothelial cells (Wong *et al.* 1983; Skoskiewicz *et al.* 1985; Momburg *et al.* 1986). IFN-γ has also been shown to increase expression of IL-2Rα (CD25) on T cells (Giovarelli *et al.* 1988). IFN-γ promotes a Th1-type immune response by inhibiting Th2 cell proliferation (Gajewski *et al.* 1988). Mice which are deficient in JNK2 fail to generate Th1 effector cells, most likely due to a lack of IFN-γ production (Yang *et al.* 1998a; Sabapathy *et al.* 1999). IFN-γ induces granzyme B expression by CTL (Fitzpatrick *et al.* 1996) and renders tumor cells more susceptible to killing by CTL due to increased MHC class I expression (Feinman *et al.* 1986). In light of studies that demonstrate increased surface expression of apoptosis-promoting TRAIL as a result of IFN-γ stimulation, it is likely that at least some of the observed anti-tumor effects of IFN-γ are due to enhanced TRAIL-mediated killing of tumor cells by cytotoxic effector cells (Kayagaki *et al.* 1999).

The IFN-γR is a heterodimeric complex consisting of two subunits designated IFN-γR1 and IFN-γR2 (Aguet *et al.* 1988; Soh *et al.* 1994). The IFN-γR1 subunit or α chain, responsible primarily for IFN-γ-binding, is expressed constitutively at moderate levels on the surface of both lymphoid and nonlymphoid cell types whereas the IFN-γR2 subunit or β chain, responsible primarily for signal transduction, is also expressed constitutively, but with a more restricted expression pattern and at much lower levels

(Farrar and Schreiber 1993; Valente *et al.* 1992; Pernis *et al.* 1995). The IFN-γR complex forms following binding of an IFN-γ dimer to two IFN-γR1 chains, followed by the association of two IFN-γR2 chains to the complex. It is also thought that the β chain stabilizes the complex formed between IFN-γ and the α chain since IFN-γ binding is increased 4-fold when the β chain is present (Marsters *et al.* 1995). Formation of this complex is essential for effective signal transduction induced by IFN-γ (Reviewed in Pestka *et al.* 1997). Treatment of T cells with phorbol ester or anti-CD3 antibodies has been shown to effect the induction of β chain expression mRNA (Sakatsume and Finbloom 1996), suggesting that activation enhances T cell responsiveness to IFN-γ. Following binding of IFN-γ to the IFN-γR, the resulting complex is internalized by the T cell. Within an acidified compartment, the complex dissociates and IFN-γ and the IFN-γR2 chain are degraded. The free IFN-γR1 chain is then recycled to the cell surface (Reviewed in Bach *et al.* 1997).

The IFN-γR2 is responsible primarily for signal transduction, and its cytoplasmic tail forms docking sites for JAK1 and JAK2 (Pestka *et al.* 1997; Darnell *et al.* 1994), which, by close association, activate each other. IFN-γ induced tyrosine-phosphorylation of IFN-γR2 leads to formation of a docking site for STAT1 (Greenlund *et al.* 1994). Activated JAK1 and JAK2 subsequently activate STAT1. Following phosphorylation and dimerization of STAT1, STAT1 1translocates to the nucleus where it binds to sites on DNA designated IFN-γ-activating sequences (GAS), ultimately inducing transcription of several genes important for T cell activation (Reviewed in Decker *et al.* 1997).

1.3 Reorganization of T cell surface molecules during T cell activation

(a) Involvement of the cytoskeleton in T cell activation

A cellular cytoskeleton was observed more than twenty years ago in the form of a Triton X-100 (nonionic detergent) insoluble complex that retained a similar shape and size as untreated cells (Ben-Ze'ev *et al.* 1979). Since that time, research into the involvement of the cytoskeleton in cellular processes has advanced a great deal. The cytoskeleton acts as an important cellular framework that spans the entire cell. It is involved in nearly every cellular process including cell migration, protein trafficking, and signal transduction (Caplan and Baniyash 2000). Two reports describing the reorientation of the microtubule organizing center (MTOC) and the actin-based cytoskeleton in both CTL and T helper (Th) cell function provided early indications that the cytoskeleton plays a significant role in T cell activation (Geiger *et al.* 1982; Kupfer *et al.* 1987). The cytoskeleton provides the essential structural support necessary for the activation of T cells (Cerottini and Brunner 1972; Serrador *et al.* 1999). This support must be maintained during the entire activation process for a T cell to fully differentiate into an effector cell (Valitutti *et al.* 1995). Furthermore, involvement of the cytoskeleton in T cell activation events offers plausible explanations for several unresolved issues in the traditional model of T cell activation.

Current literature suggests two roles for the cytoskeleton in T cell activation. The primary function of the cytoskeleton is the regulation of T cell form and movement, as well as stabilization of T cell-APC contact formation (i.e. supramolecular activation cluster (SMAC) or immunological synapse) (Dustin and Cooper 2000; Acuto and Cantrell 2000). However, the cytoskeleton is also vital as a scaffold for signaling

components (Dustin and Cooper 2000). By functioning as a scaffold for signaling components, the cytoskeleton may facilitate the interaction of molecules that would otherwise be unlikely to interact. Association with the cytoskeleton allows these signaling molecules to either assemble in the correct configuration or maintain a configuration that is critical for their function. In accordance, the ability of chemical inducers of dimerization and synthetic ligands to activate numerous signaling molecules by facilitating their interactions has been documented by several researchers, providing further evidence that association of appropriate signaling molecules alone is able to induce their activation (Holsinger *et al.* 1995; Klemm *et al.* 1997; Spencer *et al.* 1996).

TCR engagement is an absolute requirement for stabilization of the contact zone between a T cell and APC (Negulescu *et al.* 1996; Malissen 1999). Without TCR engagement, a T cell will break contact with a particular APC and seek contact with another (Negulescu *et al.* 1996; Donnadieu *et al.* 1994). It is interesting to note that stable T cell-APC conjugates will not form following cytochalasin D treatment of T cells. Cytochalasin D is a drug that prevents actin polymerization by binding the ends of actin filaments (Delon *et al.* 1998). This study indicates the requirement for cytoskeletal involvement in the formation of a T cell: APC conjugate and suggests that in the absence of the cytoskeleton, TCR engagement will not occur.

A rapid reorganization of T cell surface receptors is required for a T cell to successfully interact with an APC since TCR molecules are found at locations other than the leading edge of a cell and are present in relatively low abundance (Demotz *et al.* 1990; Dustin and Cooper 2000). Changes to the cytoskeletal architecture are responsible for this reorganization of TCR (Shaw and Dustin 1997). Changes in the architecture of

the cytoskeleton are detectable in T cells almost immediately following the initial interaction with APCs prior to antigen-recognition (Delon *et al.* 1998). Since these changes take place prior to engagement of the TCR, their regulation is likely TCR-independent and is presumably mediated by costimulatory molecules such as CD2, CD28, and LFA-1 (Acuto and Cantrell 2000).

(b) Unresolved issues with current models of T cell activation

The conventional model of T cell activation proposes that T cell activation occurs when a TCR binds a specific peptide presented in the context of a self MHC molecule on an APC. The ligand binding subunits ($\alpha\beta$ chains) of the TCR do not transduce signals. Rather, the cytoplasmic tails of the other subunits (γ, δ, ε, and ζ chains) signal through the activity of associated molecules such as PTKs. However, this model of T cell activation possesses several notable flaws. The TCR binds specific peptide-MHC complexes with relatively low affinity (Alam *et al.* 1996). Furthermore, the number of specific peptide-MHC complexes on an APC for a particular TCR is very small (Demotz *et al.* 1990). This is not consistent with the fact that a large number of MHC-peptide complexes and considerable time would be required for the TCR to coordinate the initial adhesion of a T cell to an APC (Dustin *et al.* 1996a). Furthermore, large adhesion molecules present on the surface of T cells such as CD43 and CD45 (~45nm) make an initial interaction between the much smaller TCR and MHC-peptide complex (~15nm) exceedingly unlikely (Shaw and Dustin 1997). Formation of an immunological synapse between a T cell and APC (rather than simple random receptor-ligand interactions)

during their interaction serves to explain many of these unresolved issues surrounding traditional models of T cell activation.

(c) The immunological synapse

It is generally accepted that initiation of TCR signaling does not require a stabilized contact zone between the T cell and APC. However, prolonged contact is necessary in order to achieve optimal activation of T cells. Shaw (Shaw and Dustin 1997) proposed that the arrangement of membrane proteins in the area of contact (immunological synapse) between a T cell and APC, rather than simply engagement of the TCR, is the cause of T cell activation. The immunological synapse is a product of cytoskeletal reorganization and has the effect of allowing key surface proteins and associated protein kinases to associate with their substrates. Furthermore, highly glycosylated molecules like CD45 are excluded from the immunological synapse, thus reducing the anti-adhesive effect of their large size and strong negative charge (Shaw and Dustin 1997). Also, since CD45 functions as a potentially inhibitory phosphatase, its exclusion from the immunological synapse also allows for the phosphorylation of both Lck at both tyrosine residues and Lck substrates (Ostergaard *et al.* 1989; Shaw and Dustin 1997). It is likely that close association of molecules such as CD2 and CD28 with the TCR stabilize the interaction of similarly sized TCRs with appropriate ligands and also promote the exclusion of large molecules such as CD45 and LFA-1 from the immunological synapse. Viola and colleagues (Viola *et al.* 1999) have shown that detergent-insoluble glycolipid domains (rafts) congregate at the site of CD28 ligation.

This finding is noteworthy, considering that lipid rafts are rich in protein kinases (Zhang *et al.* 1998b).

Hence the immunological synapse consists of two subregions: an internal zone and an external zone. The internal zone is the location of smaller molecules (~15nm) including TCR, CD4 or CD8, CD2, CD28, and associated PTKs such as Lck and Fyn, as well as adapter molecules such as LAT and SLP76 (Monks *et al.* 1998; Grakoui *et al.* 1999; Zhang *et al.* 1998b). Ligation of costimulatory molecules generates increased filamentous actin (F-actin) at the site of ligation (Acuto and Cantrell 2000) and a concomitant accumulation of lipid rafts at the immunological synapse (Viola *et al.* 1999). Engagement of CD28 with B7-family members stimulates activity of the Rho GTPase Vav1 that controls changes to the structure of the actin cytoskeleton, resulting in the accumulation of lipid rafts in the inner zone of the immunological synapse (Henning and Cantrell 1998; Acuto and Cantrell 2000). These events ultimately organize the signaling molecules within the inner zone of the immunological synapse and promote signal transduction through the TCR. Inhibitors of actin polymerization, such as cytochalasin D, prevent the sustained increase in intracellular calcium levels, which normally occurs following effective TCR signaling (Delon *et al.* 1998).

The external zone of the immunological synapse houses larger molecules (~45nm) such as CD45, LFA-1, and Talin, and serves to maintain a broad peripheral ring of high affinity adhesion interactions (Shaw and Dustin 1997; Grakoui *et al.* 1999). Talin is a large actin- and integrin-binding protein that has been proposed as a regulator of the high affinity form of LFA-1 in resting T cells (Dustin and Cooper 2000). Talin is thought to maintain LFA-1 in the low affinity form via interactions with the actin cytoskeleton

through vinculin, thus preventing lateral movement in the membrane (Sampath *et al.* 1998). T cell activation signals abrogate this inhibitory effect and allow for lateral movement of LFA-1 (Kucik *et al.* 1996). Talin is also believed to participate in the cytoskeleton-mediated recruitment of LFA-1 to the immunological synapse (Kupfer and Singer 1989). Integrin-dependent signals are believed to supplement TCR stimulation primarily through the induction of structural changes to the cytoskeleton. However, reorientation of the MTOC toward the immunological synapse is integrin-independent, requiring only TCR-mediated signaling (Sedwick *et al.* 1999). Thus, the functional role of the cytoskeleton is unclear in this respect. It is most definitely a structural support network for signaling molecules and plays a major role in assembling the functional immunological synapse, but may also play a more subtle role in the orchestration of actual signaling events by PTKs and other cytoskeleton-associated signaling molecules.

1.4 Cytolytic mechanisms

(a) Apoptosis

The ability for its individual cells to undergo apoptosis is critical for an organism to be able to regulate immune activation and prevent tumor growth (reviewed in O'Connor *et al* 2000). Apoptotic cells have a number of characteristic features. For example, chromatin condenses in an apoptotic cell. The cell membrane blebs and the DNA of the apoptotic cell becomes fragmented. The cell subsequently shrinks and fragments into sealed apoptotic bodies (reviewed in Berke 1994).

Apoptosis is induced by a variety of stimuli, including those provided by CTL, but the end result is activation of caspase-8 or caspase-9 (initiator caspases). The initiator

caspases then activate downstream effector caspases including caspases-3, -6, and –7 (reviewed in Zheng and Flavell 2000). It is generally believed that cell death results from the cleavage of substrates such as actin or inhibitors of endonucleases. Apoptosis is also induced via increased mitochondrial permeability, resulting in the release of cytochrome c into the cytosol where it binds Apaf-1 (apoptotic protease activating factor). Activated Apaf-1 proceeds to activate caspase-9, which then activates the effector caspase-3 (Shi 2001; Ranger *et al.* 2001). Apoptosis is the end result of these signaling events.

(b) Steps in cytolysis

Cytolysis of target cells by CTL involves a series of events beginning with the formation of a stable conjugate between the effector CTL and the target cell. Following conjugate formation, polarization of golgi and cytolytic granules within the cytoplasm of the CTL to the contact patch near the target cell is observed. Apoptosis is induced primarily through an extracellular Ca^{2+}-dependent granule exocytosis pathway and/or a Ca^{2+}-independent Fas-/TRAIL-receptor ligation pathway. The CTL then dissociates from the target cell, which undergoes apoptosis within 15 min to 4h. During this time, the CTL searches for another potential target cell (reviewed in Berke 1994).

(c) Granule-mediated exocytosis

The Ca^{2+}-dependent mechanism by which CTL induce apoptosis in target cells involves the exocytosis of the apoptosis-inducing granzymes, which are packaged along with perforin in cytoplasmic granules of CTL. Perforin and granzyme expression is dependent on T cell activating cytokines such as IL-2 and IFN-γ (Liu *et al.* 1989;

Fitzpatrick *et al.* 1996). Upon recognition of an appropriate target cell by TCR ligation, CTL exocytose the contents of their cytoplasmic granules into the localized contact patch between the two cells, and these cytotoxic molecules enter the target cell cytoplasm and nucleus (Yanelli *et al.* 1986). The rudimentary model of granule exocytosis suggested that granzymes (most notably granzyme B) enter the target cell through pores formed in the plasma membrane of the target cell by perforin polymers and induce apoptosis. However, recent data supports an alternative role for perforin. This molecule does, in fact, form pores in the target cell, but pore formation (and thus membrane damage) is thought to stimulate the endocytic uptake of granule constituents. Endosomal disruption allowing granzymes to escape endosomes into the cytoplasm of the target cell rather than simply formation of large transmembrane pores appears to be the true function of perforin (Reviewed in Smyth *et al.* 2001). Accordingly, the perforin-mediated uptake of granzymes is specific and selective since smaller proteins other than the contents of cytotoxic granules are not delivered to the cytoplasm of target cells via perforin (Browne *et al.* 1999). Interestingly, there is evidence to support the existence of a perforin receptor (Berthou *et al.* 2000), though this data has yet to be substantiated and the functional relevance of a perforin-specific receptor is not yet known. Based on the observation that granzyme B can enter target cells in the absence of perforin but remains in the endosomal compartment (Froelich *et al.* 1996), it was suggested that a receptor for granzyme B might also exist. A mannose 6-phosphate death receptor that allows granzyme B to enter endosomes during CTL-mediated apoptosis was subsequently identified (Motyka *et al.* 2000), though as is the case with the perforin receptor, the functional significance of this receptor has not yet been established.

Although the precise mechanism by which apoptosis is induced by granule mediated exocytosis is not yet known, caspase-3 and caspase-8 are activated by granzyme B (Yang 1998b; Medema *et al.* 1997). It is worth noting that many viruses are able to inhibit caspase-8 activity and prevent CTL-induced apoptosis of the host cell (Meinl *et al.* 1998), but granzyme B is able to induce apoptosis via caspase-3 activity independently of caspase-8 since caspase-3 can directly activate Bid and thus induce apoptosis (Barry *et al.* 2000). These observations underscore the fact that CTL are especially capable of inducing apoptosis in potential target cells in spite of protective mechanisms employed as a result of viral infection or transformation.

(d) Fas - Fas ligand-mediated apoptosis

Fas (CD95) is a widely expressed 45-52 kDa cell surface molecule member of the TNF family. Fas ligand (FasL/CD95L) exhibits a more restricted expression pattern than Fas, being expressed primarily on killer cells which utilize this molecule to kill Fas-expressing target cells in a calcium-independent fashion (Anel *et al.* 1995). Oligomerization of Fas is required for the induction of an apoptotic signal, since Fas monomers and dimers do not induce apoptosis (Dhein *et al.* 1992). Surprisingly, although the granule exocytosis mechanism of cytolysis was discovered prior to FasL-mediated apoptosis, more is known of the elements of the Fas signaling pathway. Ligation of FasL to Fas results in oligomerization of Fas and recruitment of FADD (Fas-associated protein death domain) and pro-caspase-8 via their death domains (DD). These molecules comprise a complex known as the DISC (death-inducing signaling complex) which is the site of pro-caspase-8 cleavage to activated caspase-8 (Peter and Krammer 1998; Scaffidi

et al. 1999). FasL-mediated apoptosis is regulated through expression of pro- and anti-apoptotic molecules, including the Bcl-2 family of proteins and FLICE (caspase-8)-inhibitory proteins (FLIP) (reviewed in Scaffidi *et al.* 1999).

(e) TRAIL-mediated apoptosis

TRAIL (APO-2L), the TNF-related apoptosis-inducing ligand, was identified through analysis of sequence homology to other members of the TNF family (Wiley *et al.* 1995). TRAIL occurs as a 33-34 kDa surface-bound or 19-20 kDa secreted molecule expressed primarily by activated T cells and B cells, NK cells, dendritic cells, and monocytes (Mariani and Krammer 1998a; Fanger *et al.* 1999; Zamai *et al.* 1998; Griffith *et al.* 1999; Mariani and Krammer 1998b). TRAIL has been shown to induce apoptosis in many tumor cell lines while sparing non-transformed cells (Mariani and Krammer 1998a; Wiley *et al.* 1995). In T cells, expression of TRAIL is induced through TCR-mediated signaling (Musgrave *et al.* 1999). TRAIL binds TRAIL-R1 (DR4) and TRAIL-R2 (DR5) receptors that induce apoptosis. Two additional receptors designated TRAIL-R3 (DcR1) and –R4 (DcR2) fail to transmit an apoptotic signal and may, therefore, serve as decoy proteins (reviewed in Degli-Esposti 1999). Although other apoptosis-inducing members of the TNF family cause significant toxicity upon systemic administration (Fiers 1991), recombinant soluble TRAIL is able to function without any overt toxicity when administered to mice (Walczak *et al.* 1999). However, human hepatocytes and rodent primary neuron and neuron-like cells are sensitive to TRAIL-mediated apoptosis *in vitro,* which suggests that caution is warranted in any potential use of TRAIL to treat human disease (Ozoren *et al.* 2000; Martin-Villalba *et al.* 1999). Nevertheless, the ability of

TRAIL receptors to synergize with chemotherapeutic drugs to eliminate neoplastic cells suggests a potential application in cancer therapy (Ashkenazi *et al.* 1999). This synergistic activity can be attributed to activation of distinct apoptotic pathways since TRAIL-mediated apoptosis is mitochondria-independent while chemotherapeutic agents trigger a mitochondria-dependent pathway of apoptosis. Thus far, it appears that TRAIL requires caspase-8 for the induction of apoptosis (Mariani *et al.* 1997). This observation is further supported by evidence that inhibition of caspase-8, but not caspase-3 or caspase-9, abrogates TRAIL-induced apoptosis (Walczak and Krammer 2000). Along with caspase-8, FADD is also required for apoptosis induced by TRAIL and these two proteins are important components of the DISC (Sprick *et al.* 2000).

(f) TNF-mediated apoptosis

CTL are capable of inducing apoptosis via surface-bound tumor necrosis factor (TNF) (Kriegler *et al.* 1988). TNF induces cell death through a mechanism that involves adapter proteins similar to those used in FasL-mediated apoptosis. Briefly, TNF binds to the TNF-R and induces trimerization of this receptor, thus assisting in its interaction with the DD of TRADD (TNF receptor-associated death domain). TRADD is recruited to the membrane complex and subsequently recruits FADD to the complex. Signaling events downstream from this point on are similar in both the FasL and TNF pathways of apoptosis (reviewed in Screaton and Xu 2000).

1.4 Costimulation and the regulation of immune activation

(a) Cytokine mRNA half-life

mRNAs which encode transcription factors and cytokines are characterized by an exceptionally short half-life (<30 min) (Caput *et al.* 1986). One important mechanism of immune regulation is the control of transcription factor and cytokine mRNA half-life. Enhanced cytokine mRNA stability is achieved through costimulation provided by T cell interactions with APCs. Costimulation of T lymphocytes via CD28 slows degradation of IL-2 and IFNγ mRNA, indicating that CD28 signaling stabilizes mRNA (Collins *et al.* 1994; June *et al.* 1990). Costimulatory signaling may influence cytoplasmic proteins binding AUUUA motifs present in the 3'-untranslated region of cytokine mRNA, which have been demonstrated to facilitate degradation (Caput *et al.* 1986). To date, CD2 signaling has only been shown to play a role in the stabilization of CD40L mRNA (Murakami *et al.* 1999). In this study, the authors showed that CD2 signaling increases CD40L protein expression on newly activated CD4+ T cells through increased mRNA stabilization. CD28 signaling has also been shown to stabilize CD40L mRNA (Klaus *et al.* 1994). Signaling through CD2 and CD28 has also been shown to induce nuclear translocation of the transcription factor NFAT (Ghosh *et al.* 1996; Tanaka *et al.* 1997.), which is required for expression of IL-2 and other molecules important for T cell activation (Rooney *et al.* 1995).

(b) Regulation of anergy and apoptosis

Another means by which costimulatory signaling regulates immune activation is by controlling the induction of anergy and apoptosis. Although CD2 ligation has been reported to induce an apoptotic signal in T cells (Fournel *et al.* 1998; Dumont *et al.* 1998), other investigators have shown that CD2 ligation is also capable of reversing T

cell anergy (Boussiotis *et al.* 1994). Differential signaling gives CD2 a unique role among costimulatory molecules since signaling from other costimulatory molecules is able to prevent anergy but not reverse an established anergic state. The mechanism of differential signaling by CD2 is not yet known.

1.5 CD2-CD48 costimulation

(a) CD2 structure

CD2 is a 50-55 kDa cell surface glycoprotein with signal transducing properties that is expressed on murine T and B cells, and on human T and NK cells (Moingeon *et al.* 1989). The primary ligands of CD2 are CD48 in rodents and CD58 (LFA-3) in humans (Selvaraj *et al.* 1987; Kato *et al.* 1992). Through interactions with its counter-receptor, CD2 promotes the initial stages of T cell contact with either APCs or target cells (Springer 1990). However, CD2-mediated adhesion is distinct from integrin-mediated adhesion since CD2-mediated adhesion does not require TCR stimulation (Moingeon *et al.* 1991; Dustin and Springer 1989). CD2 is especially well suited to facilitate TCR-peptide-MHC interactions and immune recognition since the intercellular distance spanned by this interaction (~140 A°) is approximately that of TCR-peptide-MHC. Additionally, T cell activation is further enhanced since large molecules such as CD45 phosphatase, which will reduce the half-life of phosphorylated tyrosine residues, are excluded from the contact area (Wang *et al.* 1999).

The CD2 protein consists of an extracellular portion with overall dimensions of 25 x 20 x 75 A° that consists of four sections: a N-terminal Immunoglobulin superfamily (IgSF) domain (domain 1), a C-terminal IgSF domain (domain 2), a linker region, and a

stalk (Jones *et al.* 1992; Bodian *et al.* 1994). CD2 also contains an α-helix transmembrane region and a 117 amino acid cytoplasmic tail that contains 4 proline-rich regions that resemble binding sites for SH3 domains but no tyrosine residues (Davis and van der Merwe 1996; Rosenthal-Allieri *et al.* 1995). Nuclear magnetic resonance (NMR) analysis has shown that domain 1 of CD2 consists of an anti-parallel β-barrel formed by two β-sheets (Driscoll *et al.* 1991).

The stalk and linker regions of CD2 are highly conserved between species (Jones *et al.* 1992; Tavernor *et al.* 1994). The linker region is particularly important for CD2 function since it facilitates binding to CD48 / CD58. Binding is enhanced because the linker region provides up to 20 degrees of flexibility between domain 1 and 2. The linker region also extends the length of the extracellular domain and thus, orientates domain 1 so that the binding face of CD2 forms a plane nearly parallel with the cell surface (Jones *et al.* 1992). Increased flexibility attributed to the linker region is likely to be important during CD2 ligand binding. However, the observation that certain combinations of anti-CD2 antibodies are able induce antigen-independent activation or apoptosis of T cells suggests that function-related conformational changes in CD2 very likely depend on linker region flexibility (Rouleau *et al.* 1993; Rosenthal-Allieri *et al.* 1995). An alternate explanation is that specific combinations of anti-CD2 antibodies activate T cells by inducing formation of the large aggregates of surface molecules that are required for signaling (Davis and van der Merwe 1996).

Like the stalk and linker region, the 117 amino acid cytoplasmic tail of CD2 is also highly conserved between species (71-84% at the nucleotide level between four sequenced homologues) (Williams *et al.* 1987; Tavernor *et al.* 1994). The most

conserved regions are 4 proline-rich regions that resemble binding sites for SH3 domains (such as $p56^{lck}$, $p59^{fyn}$, and PI-3 kinase) but contain no tyrosine residues (Davis and van der Merwe 1996; Rosenthal-Allieri *et al.* 1995; Cohen *et al.* 1995). Transmembrane structures including TCR/CD3, CD4, CD5, CD8, CD45 (Beyers *et al.* 1992; Schraven *et al.* 1990) and cytoplasmic molecules such as Fyn, Lck, PI 3-kinase (Beyers *et al.* 1992; Carmo *et al.* 1993; Shimizu *et al.* 1995) have been reported to associate with the cytoplasmic tail of CD2. The cytoplasmic tail of CD2 is critical for intracellular signaling since it is required for T cell activation via specific combinations of anti-CD2 antibodies (Bierer *et al.* 1988a; He *et al.* 1988).

(b) Ligands of CD2: CD48, CD58, CD59

Known ligands of CD2 consist of CD48, CD58, and CD59. CD2 (Sewell *et al.* 1986), CD48 (Killeen *et al.* 1988), and CD58 (Seed 1987; Wallner *et al.* 1987) are very similar in structure and constitute a distinct subfamily within the Immunoglobulin Superfamily (IgSF) (Killeen *et al.* 1988). CD2, CD48, and CD58 display similar patterns of conserved disulfide binds and consist of N-terminal and C-terminal IgSF domains. CD58 (LFA-3) was the first ligand of CD2 to be described (Hunig 1985; Selvaraj *et al.* 1987) and is expressed on a range of hematopoeitic and non-hematopoeitic human tissues (Smith *et al.* 1990). Although rodent CD2 is structurally homologous to human CD2, CD58 has not been detected in rodents and the only known rodent ligand is the structurally similar CD48 (Kato *et al.* 1992; van der Merwe *et al.* 1993a). Whereas CD58 is widely expressed in humans, CD48 displays a more restricted pattern of expression in rodents, being found only on lymphocytes, macrophages, and dendritic cells (Thorley-

Lawson *et al.* 1982; Arvieux *et al.* 1986; Reiser 1990). Anti-CD48 antibodies completely block CD2-mediated adhesion of rodent T cells to APC, suggesting that CD48 is the principal ligand of CD2 (Kato *et al.* 1992; Kato *et al.* 1993; Brown *et al.* 1995). Interestingly, CD48 is also expressed in human tissues, although its role as a CD2 ligand is minor in comparison to CD58.

CD2 and CD58 genes are located on human chromosome 1p13 (Sewell *et al.* 1988; Kingsmore *et al.* 1989) whereas the gene encoding CD48 is found on chromosome 1q21-23 (Staunton *et al.* 1989). Genetic analysis suggests that duplication of one region of chromosome 1 led to the creation of these similar genes (Wong *et al.* 1990). Chromosomes 3 and 1 are the locations of genes encoding rodent CD2 and CD48, respectively (Wong *et al.* 1990). From an evolutionary perspective, it would appear that CD48 gene duplication occurred following the separation of humans and rodents, leading to the expression of both CD48 and CD58 in humans, but only CD48 in rodents. CD2 interacts with both CD48 and CD58 in humans but has a much higher affinity for CD58. Interestingly, rodent CD2 displays a much higher affinity for rodent CD48 than human CD2 displays for human CD48, suggesting that selective pressure led to an increase in the affinity of rodent CD2 for CD48 because CD48 is the lone ligand for CD2 in rodent species (Davis *et al.* 1998). It is interesting that the expression of CD58 is more widespread among human tissues than the expression of CD48 in rodents, suggesting that CD2 may function differently to some extent in rodents and humans. However, to date there is little concrete evidence in support of this idea.

The binding interactions between rodent CD2/human CD2 and rodent CD48/human CD58 are of relatively low affinity (K_d ~60-90μM or 10-20 μM,

respectively) with rapid dissociation rates on the order of K_{off}=4s^{-1} (Davis *et al.* 1998; Dustin *et al.* 1997; van der Merwe *et al.* 1994; van der Merwe *et al.* 1993b). Examination of the crystal structure of purified CD2 and CD58 has revealed a right-angled irregular interaction of major B-sheets with significant charge complementarity between the molecules Ig-like domains. Charge complementarity results from amino side chains forming hydrogen bonds at the faces of the molecules (Wang *et al.* 1999). Studies in rodents have revealed similar interactions (van der Merwe 1993b; McAlister *et al.* 1996; van der Merwe *et al.* 1995). Based on the high overall expression of CD2 and its ligands which are typically greater than 100 molecules μm^{-2}, the nature of these interactions suggest that cell-cell interactions result from weak and thus dynamic contacts between cells (Dustin *et al.* 1996b). Since ligation of cell surface molecules is limited by the mobility around the membrane of the interacting cells (Bell 1978), it is notable that CD48 (Killeen *et al.* 1988) and an isoform of CD58 (Seed 1987; Dustin *et al.* 1987a) are glycosylphosphatidylinositol- (GPI) anchored molecules. Compared to transmembrane-anchored molecules, GPI-anchored proteins are nearly 10-fold more mobile (Ferguson and Williams 1988). This increased mobility along with transient CD2-ligand interactions prevents CD2-ligand interactions from blocking the movement of TCR-peptide-MHC complexes freely through the cell-cell contact area, and likely facilitate the accumulation of TCR molecules at the contact patch and enhance recognition of cognate peptide on APCs. The finding that T cells recognize specific peptide-MHC complexes on APCs 50- to 100-fold more effectively in the presence of CD2-ligand interactions supports this model (Koyasu *et al.* 1990).

Along with CD48 and CD58, CD59 has also been found to bind human CD2. However, no rodent homologue of CD59 has been discovered (Hahn *et al.* 1992a; Deckert *et al.* 1992). CD59 is structurally dissimilar to CD2, CD48, and CD58, and is normally involved in protection against lysis by complement. As such, it is perhaps surprising that CD59 binds CD2. In fact, no direct interaction between purified CD2 and CD59 has been observed, suggesting that interactions between CD2 and CD59 may have no real physiological significance. In all likelihood, the role of CD59 in CD2-mediated immune activation is minor or nonexistent.

(c) Antigen-independent activation of T cells through CD2

An early report indicates that antigen-independent activation of T lymphocytes can be achieved by stimulation with specific pairs of anti-CD2 mAb (Hunig *et al.* 1987). Crosslinking of CD2 by certain combinations of mAb triggers proliferation of highly purified T cells, as well as IL-2 and IFN-γ secretion in the absence of an antigen-specific signal through the TCR (Meuer *et al.* 1984; Gonsky *et al.* 2000). This CD2-induced proliferative response was IL-2 dependent since stimulation could be abrogated by addition of anti-IL-2 antibody. It is unlikely that this phenomenon is simply the result of anti-CD2 antibodies aggregating CD2-associated structures such as TCR since the cytoplasmic domain of CD2 is required for activation of T cells via certain combinations of CD2 antibodies (Bierer *et al.* 1988a; He *et al.* 1988).

(d) Signal transduction through CD2

The most highly conserved portion of CD2 between species is its 117 amino acid cytoplasmic tail which is required for intracellular signaling through CD2 (Tavernor *et al.* 1994). This cytoplasmic tail contains 4 proline-rich regions that serve as binding sites for molecules with SH3 domains (such as $p56^{lck}$, $p59^{fyn}$, and PI-3 kinase) but has no tyrosine residues able to function as potential docking sites for SH2 domains (Davis and van der Merwe 1996; Rosenthal-Allieri *et al.* 1995; Pawson and Gish 1992). Through mutational analyses, it has been shown that these proline-rich regions are important, if not essential, for CD2 signaling (Bell *et al.* 1996; Hahn *et al.* 1992b). Although the signaling pathways activated through CD2 are not completely understood, it is clear that the CD2 signal transduction pathway shares many signaling molecules with the TCR and CD28 pathways but also possesses its own unique signaling molecules. Important signaling molecules that have been found in CD2 immunoprecipitates include CD3-ε and -ζ chains, Lck, Fyn, the p85 subunit of PI-3 Kinase, and CD45 (Schraven *et al.* 1993; Schraven *et al.* 1994; Beyers *et al.* 1992; Schraven *et al.* 1990; Offringa and Bierer 1993; Carmo *et al.* 1993; Shimizu *et al.* 1995; Bell *et al.* 1992). Similar to signaling through the CD3 complex, stimulation of CD2 induces PLC-γ1 activation (Kanner *et al.* 1992), which subsequently increases cytosolic Ca^{2+} levels (Pantaleo *et al.* 1987). Also, the tyrosine phosphorylation pattern is similar in activated T cells through CD3 complex- and CD2-associated signaling pathways (Ley *et al.* 1991).

There are conflicting reports as to whether the CD3 ζ-chain is required for CD2-mediated T cell activation. Although CD2-induced T cell signaling requires expression the CD3 ζ chain, TCR-CD3 complex triggering is not essential for T cell activation

through CD2 ligation (Howard *et al.* 1992). In TCR-mediated T cell activation, the ζ-chain of the CD3 complex interacts with Lck and Fyn and subsequently becomes phosphorylated (Weiss and Littman 1994). Phosphorylated ζ-chain ITAMs are then able to recruit ZAP-70 to the TCR/CD3 signaling complex. CD2 signaling also induces ζ-chain tyrosine phosphorylation (Samelson *et al.* 1990) and Lck activation (Danielian *et al.* 1992). Moreover, CD2-mediated signaling is functional in TCR-deficient T cells as long as the ζ-chain is expressed on the surface of the cell (Howard *et al.* 1992). Studies have shown that ZAP-70 is not recruited to the ζ-chain and is only weakly phosphorylated following CD2 stimulation, whereas CD3 stimulation leads to recruitment of ZAP-70 to the ζ-chain along with obvious ZAP-70 phosphorylation (Weissman *et al.* 1988; Jin *et al.* 1990; Hubert *et al.* 1996). In another study, ZAP-70-deficient T cells failed to proliferate or produce cytokines following CD2 stimulation (Ueno *et al.* 2000). Interestingly, CD2-mediated activation has been reported to occur in T cells expressing ζ-chains which lack functional ITAMs (Steeg *et al.* 1997). This finding suggests that CD2 signaling does not require ζ-chain signaling and also implies that ZAP-70 (which is a ζ-associated protein) is not required for CD2 signaling. This is consistent with studies that show that CD2 signaling is able to activate phospholipase C (PLC)-γ1 and mitogen-activated protein kinases (MAPK) independently of ZAP-70 activity (Hubert *et al.* 1996; Ueno *et al.* 2000). In contrast, a recent report demonstrates that ZAP-70 is absolutely required for CD2-mediated Ca^{2+} influx, ζ-chain phosphorylation, and stimulation of T cell proliferation and cytokine production (Meinl *et al.* 2000). ZAP-70 is also required for CD2-mediated activation of NFAT (Lin *et al.* 2001). A possible explanation for these apparently conflicting reports comes from a study

in which phosphorylation of several proteins was detected in ZAP-70 deficient T cells following CD2 stimulation (Ueno *et al.* 2000). Taken together, the data suggest that CD2 signaling utilizes both ZAP-70-dependent and -independent pathways. Transcription factors such as NFAT are involved in CD2 stimulation of cytokine production by T cells (Ueno *et al.* 2000).

Another important transcription factor implicated in CD2 signaling in T cells is AP-1, a heterodimer composed c-Fos and c-Jun (Karin 1995; Hughes and Pober 1996). Activation of AP-1, along with NFκB and NFAT is required for induction of IL-2 gene transcription (Karin 1995; Hughes and Pober 1996; Rao *et al.* 1997; Kalli *et al.* 1998). AP-1 activation is regulated by the ERK pathway (Cobb and Goldsmith 1995; Nishida and Gotoh 1993) and by the c-Jun N-terminal kinase (JNK) pathway (Hughes and Pober 1996; Su *et al.* 1994). Activated ERK and JNK phosphorylate transcription factors which upregulate c-Fos and c-Jun, respectively. It is thus likely that stimulation through CD2 activates the ERK and JNK signaling cascades.

As previously mentioned, CD2 signaling activates src-family PTKs $p56^{lck}$ and $p59^{fyn}$ (Danielian *et al.* 1991; Carmo *et al.* 1993). In addition, CD45 is associated with the cytoplasmic tail of CD2 (Schraven *et al.* 1990) where this phosphatase is thought to regulate the activity of Lck and Fyn (Tonks *et al.* 1988). At present, it is unclear whether CD2 signaling depends on Lck or Fyn or both. A recent study demonstrated that Fyn rather than Lck is primarily associated with CD2 (Gassmann *et al.* 1994). This is in line with the finding that CD2-mediated signaling in Jurkat T cells is Lck-independent (van Oers *et al.* 1996; Iwashima *et al.* 1994; Sunder-Plassmann and Reinherz 1998). In fact, Lck-deficient T cells have been shown to produce IL-2 following CD2 stimulation

(Sunder-Plassmann and Reinherz 1998). In is interesting to note that unlike CD2-mediated stimulation, TCR-mediated stimulation of IL-2 production is Lck-dependent. (Straus *et al.* 1992). In contrast, Fyn is indispensable for CD2-mediated induction of T cell proliferation but TCR signaling was independent of Fyn (Fukai *et al.* 2000). Based on the available evidence, it would seem that Fyn is the principal src-family PTK in CD2 signaling whereas Lck is required for TCR signaling.

The phosphorylation of $p62^{dok}$ has been shown to occur following CD2 stimulation (Nemorin and Duplay 2000), and may be involved in the regulation of TCR-mediated T cell activation (Nemorin *et al.* 2001). Activation of T cells through CD2, but not through the TCR, leads to phosphorylation of $p56^{dok}$ and $p62^{dok}$, and CD2-mediated phosphorylation of these molecules is dependent on Lck (Nemorin and Duplay 2000). This finding is somewhat surprising considering the overall requirement for Fyn in CD2 signaling, and suggests that CD2 signaling is not entirely independent of Lck. Interestingly, although Lck is required for phosphorylation of dok (Downstream Of tyrosine Kinase) proteins via CD2 signaling, and TCR-mediated signaling relies heavily on Lck activity, activation via CD2 but not TCR induces phosphorylation of $p56^{dok}$ and $p62^{dok}$ (Nemorin and Duplay 2000). Also, although TCR stimulation does not induce phosphorylation of $p62^{dok}$, TCR stimulation in combination with CD2 stimulation induces a dramatic increase in the phosphorylation of $p62^{dok}$ compared to CD2 stimulation alone (Harriague *et al.* 2000). The function of $p62^{dok}$ is unclear, but due to the presence of proline-rich regions, $p62^{dok}$ may serve to interact with other signaling molecules with SH3 domains (such as PLC-γ1). However, if $p62^{dok}$ is over-expressed in CD2-stimulated T cells, a decrease in PLC-γ1 phosphorylation, ERK activation, and Ca^{2+}

mobilization is observed (Nemorin *et al.* 2001), implying that $p62^{dok}$ may also regulate CD2 signaling in T cells.

(e) CD2 costimulation of T cells

Ligation of CD2 results in enhanced production of IL-2 by T cells (Rosenthal-Allieri *et al.* 1995). Additionally, signaling through CD2 has been shown to induce nuclear translocation of the transcription factor NFAT (Tanaka *et al.* 1997), which has been implicated in the expression of IL-2 and other molecules important for T cell activation (Rooney *et al.* 1995). An elegant study using CD48-expressing CHO cells as APCs found that CD48-expressing CHO cells stimulated more potent activation (i.e. increased proliferation and IL-2 production) of OVA-specific T cells than control CHO cells which lacked CD48 (Latchman and Reiser 1998). Interestingly, the authors noted that CD2 had costimulatory activity similar to CD28, further supporting an important role for CD2 in T cell induction. Another study showed that increasing LFA-3 expression on melanoma cells strongly enhances cytokine secretion by melanoma-specific CTL (Le Guiner *et al.* 1998). These studies indicate that CD2 costimulation augments specific immune reactions and thus facilitates T cell activation.

CD2 is especially well suited to facilitating TCR signaling since both CD2 and the TCR are similar dimensions, each spanning a distance of approximately 140 A° when bound to CD48 or CD58 and peptide-MHC, respectively (Shaw and Dustin 1997; van der Merwe *et al.* 1995). Both CD2 and the TCR are approximately 6-fold smaller than the large intercellular adhesion molecules CD43 and CD45. Also, on resting T cells, the distribution of CD2 and TCRs would be expected to be randomly scattered over the cell

surface. Following initial T cell-APC interactions (likely integrin-mediated adhesion), CD2 adhesion will result in optimal positioning of TCRs for peptide-MHC interactions based on their similar dimensions. CD2 ligation would also be predicted to increase the density of TCR molecules in the T cell-APC contact area. Because CD2 ligands are GPI-anchored, they are inherently mobile throughout the cell membrane (Dustin *et al.* 1996b), and along with the low binding affinity of CD2, the resulting T cell-APC contact zone is likely very dynamic. This feature is particularly important since it allows many peptide-MHC molecules to diffuse through the contact zone, increasing the likelihood that a particular peptide will be encountered in spite of the low frequency of occurrence of any given peptide. Also, as a result of its smaller size, CD2-mediated adhesion will exclude the larger adhesion molecules from the immunological synapse (Shaw and Dustin 1997; Malissen 1999). TCR-triggering induces its recruitment to rafts (Montixi *et al.* 1998; Xavier *et al.* 1998). Since TCR recruitment to rafts facilitates TCR-mediated signaling, it is possible that costimulation through CD2 may serve to enhance TCR-mediated T cell activation by inducing raft formation at the site of TCR triggering, as has been shown for CD28 (Viola *et al.* 1999). It is also possible that CD2 blockade inhibits raft formation, and thus T cell activation. Although the precise sequence of signaling events required for the optimal activation of T cells has not yet been elucidated, the importance of PTK activity and tyrosine phosphorylation is well established (Weiss and Littman 1994; Mustelin 1994). Correspondingly, decreased phosphatase activity (primarily CD45) is important for increasing the half-life of tyrosine phosphorylated residues, thus enhancing T cell activation. Since it appears that CD2 ligation excludes the larger CD45 from the T cell-target cell contact area, CD2 likely facilitates T cell activation by allowing signaling

to occur for an increased period of time. This model is consistent with the concept that CD2 is not essential for T cell activation, but serves to optimize and facilitate this process (Killeen *et al.* 1992; Bachmann *et al.* 1999). If the affinity of the TCR for peptide was sufficiently high, activation could occur in the absence of CD2.

Although CD28 and CD2 share many associated signaling molecules, a recent paper by Yashiro and co-workers (Yashiro *et al.* 1998) showed that there is a fundamental difference in the costimulatory capacity of both molecules. Although both CD28 and CD2 costimulation induced proliferation in naïve T cells, CD28-costimulated T cells displayed substantial IL-2 production whereas apoptosis was observed in CD2-costimulated T cells. An earlier study by Parra and colleagues (Parra *et al.* 1997) showed that B7-1 costimulation of SEA-triggered CD8+ T cells results in long lasting T cell proliferation and production of large amounts of IL-2, but little IFN-γ. In contrast, CD58 binding to CD2 induced strong IFN-γ production but little IL-2 synthesis in $CD8^+$ T cells (Parra *et al.* 1997). It has also been demonstrated that CD28 signaling can prevent the induction of anergy in T cells (Harding *et al.* 1992) while CD2 signaling is able to reverse T cell anergy (Boussiotis *et al.* 1994). Thus, it appears that CD28 and CD2 signaling interact to regulate both activation and the induction of anergy in T cells. However, in addition to CD2 and CD28, a homologue of CD28 was recently detected in a screen for activation antigens on T cells (Hutloff *et al.* 1999). ICOS (inducible costimulatory molecule) is expressed on activated T cells (Hutloff *et al.* 1999; Yoshinga *et al.* 1999) and binds B7RP-1 on professional APCs. ICOS signaling enhances T cell proliferation and cytokine production, and is thought to be responsible for providing costimulation to previously activated T cells (Wallin *et al.* 2001). Although the role of

ICOS in the regulation of anergy in T cells has not been examined, based on its perceived function, it seems likely that ICOS is also involved in regulating T cell activation in combination with CD2 and CD28.

The finding that mice deficient in CD2 or CD28 demonstrate only slight defects in T cell activation and appear to have relatively normal activation parameters and function was initially surprising (Green *et al.* 1994; Shahinian *et al.* 1993; Killeen *et al.* 1992). The results of these studies suggested that neither CD2 nor CD28 are absolutely required for T cell function. In fact, due to their inherent similarities, it is more likely that CD28 or CD2 can compensate for each other in mice which lack one or the other of the two costimulatory molecules. Correspondingly, mice lacking both CD28 and CD2 are profoundly deficient in T cell activation (Green *et al.* 2000), suggesting that the combination of CD28 and CD2 signaling are required for optimal T cell activation. This study is also consistent with the idea that CD2 and CD28 are at least somewhat redundant in function.

Because T cells are primarily responsible for graft rejection and blockade of costimulatory signaling results in a greatly diminished T cell response, one possible means of enhancing graft survival is through blockade of T cell costimulation. Several early studies demonstrated the importance of CD2-CD48 interactions for optimal T cell activation (Bromberg *et al.* 1991; Guckel *et al.* 1991; Qin *et al.* 1994). These studies clearly showed that blockade of CD2-CD48 interactions results in suppression of T cell-mediated immunity with prolonged murine allograft and xenograft survival. Prolongation of graft survival was seen following administration of either anti-CD2 or anti-CD48 antibodies. Studies in rats have suggested that CD2 plays a key role in initiating allograft

rejection responses (Dengler *et al.* 1998). Other studies have shown that blockade of either CD2 (Chavin *et al.* 1992) or CD28 (Baliga *et al.* 1994; Turka *et al.* 1992) molecules and associated signaling pathways prolongs allograft survival. Although graft tolerance is not achieved when only CD2 or CD28 is blocked, combined blockade of CD2 and CD28 induces T cell hyporesponsiveness and tolerance to cardiac allografts in mice (Woodward *et al.* 1996). Mean survival times were 12.2+/-0.5 days for untreated controls, 24.8+/-1.0 days for anti-CD2 mAb alone, 55.0+/-2.0 days for CTLA4Ig alone, and >120 days with anti-CD2 mAb and CTLA4Ig combined. The observed effects were not due to clonal deletion or anergy since *in vitro* stimulation of lymphocytes from tolerant recipients with donor-specific alloantigen resulted in normal CTL and MLR responses. A more recent study found that survival of grafts secreting either CTLA4Ig or LFA-3Ig was enhanced compared to normal control grafts, with an additive enhancement of graft survival observed when both CTLA4Ig and LFA-3Ig were used in combination (Brady *et al.* 2001). Costimulation through CD2 is thus important for naïve T cells to become activated and differentiate into effector cells involved in graft rejection.

1.6 Objectives

The primary objective of this study was to determine the role of CD2 in the costimulation of CTL activation. Although the function of CD2 as an adhesion molecule is well established, its role as a costimulatory molecule is somewhat more controversial. An important role for CD2 in T cell activation is suggested by the fact that mitogenic stimulation of T cells has been achieved through combinations of anti-CD2 mAbs and blockade of CD2 interactions *in vivo* leads to prolonged allograft survival (Hunig *et al.*

1987; Chavin *et al.* 1992). CD2 has also been proposed to set the threshold for T lymphocyte activation (Bachmann *et al.* 1999). Moreover, a recent report that T cells from mice deficient in both CD2 and CD28 are profoundly impaired in terms of their ability to become activated and differentiate into effector cells (Green *et al.* 2000) suggests that CD2 may share costimulatory properties with CD28. However, the role of CD2 signaling during CTL development has not yet been investigated. I therefore sought to test the hypothesis that CD2 costimulates T cell activation and CTL development by examining the effect of blocking CD2 signaling with either anti-CD2 and/or anti-CD48 mAb on the induction of cytotoxic activity in cultures of mouse T cells activated *in vitro* with anti-CD3 mAb. I hypothesized that the role of CD2 in T cell activation involves enhancement of TCR-mediated signaling in addition to TCR-independent effects. Our findings indicate that CD2 is required to induce optimal expression of IL-2 and IFN-γ, which in turn promote granule-mediated cytotoxicity by CTL. Blockade of CD2 signaling results in decreased IL-2 and IFN-γ mRNA expression and stability within T cells and underscores the costimulatory capacity of CD2. In addition, our data suggest a possible negative regulatory signaling role for CD2 that is separate and distinct from its costimulatory functions.

2.0 METHODS AND MATERIALS

2.1 Mice

Female C57BL/6 mice (6-8 weeks old) (Charles River Canada, QC) were fed standard laboratory chow and water supplied *ad libitum* in the Carleton Animal Care Facility located in the Sir Charles Tupper Medical Building, Halifax, NS.

2.2 Medium and reagents

RPMI 1640 medium (ICN Biomedicals Canada Ltd., Mississauga, ON), was supplemented with 100 μg/ml streptomycin, 100 U/ml penicillin, and 10 mM L-glutamine (ICN Biomedicals), 5 μM HEPES buffer (Sigma Chemical Co., St. Louis, MO; pH 7.4) and 5% heat-inactivated (at 56°C for 30 min) fetal calf serum (FCS) (Life Technologies, Burlington, ON). Hereafter this is referred to as complete RPMI 1640 medium (cRPMI). cRPMI was filter-sterilized with Sterivex GS 0.22 μm filters (Millipore Corp., Bedford, MA) and stored in 250 ml sterile polystyrene tissue culture flasks (Sarstedt, St. Laurent, PQ) at 4°C.

Mouse rIFN-γ was from Genzyme Corp. (Cambridge, MA). Human rIL-2 was obtained from Collaborative Biomedical Products (Bedford, MA). Cytokine stocks were stored at –70°C or at -20°C following each use. Anti-asialoGM1 rabbit polyclonal antiserum was obtained from Wako Chemicals (Richmond, VA). Low-tox rabbit complement, rat IgG_{2a}, goat anti-hamster IgG antibody, FITC-conjugated rat anti-mouse CD25 mAb, FITC-conjugated rat anti-mouse CD8a mAb, FITC-conjugated rat anti-mouse CD117 mAb, FITC-conjugated goat anti-hamster IgG antibody, hamster anti-mouse CD28 mAb, purified hamster IgG isotypic control antibody, and anti-CD3ε mAb

(ascites) were purchased from Cedarlane Laboratories (Hornby, ON). Rat anti-mouse CD2 mAb (RM2.5), hamster anti-mouse CD48 mAb (BCM-1), and rat anti-mouse CD119 mAb (IFN-γ receptor α-chain) were acquired from Pharmingen Canada (Mississauga, ON). Rat anti-mouse CD2 (RM2.1) mAb was generously donated by Dr. K. Okumura (Juntendo University, Tokyo, Japan). Purified rat IgG isotypic control antibody was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Hamster IgG and FITC-conjugated mouse anti-rat IgG antibody were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Actinomycin D (Act D)phorbol-myristate-acetate (PMA), and ionomycin were purchased from Sigma Chemical Co. (St. Louis, MO). Act D and PMA were dissolved in ethanol, and ionomycin was dissolved in DMSO. Act D, PMA, and ionomycin stock solutions were stored at -20°C.

The murine P815 mastocytoma cell line and the hybridoma that produces rat anti-mouse LFA-1 (clone FD441.8) were obtained from ATCC (Manassas, VA). Dr. J. Bluestone (Univ. of Chicago, Chicago, IL) kindly supplied the hybridoma that produces hamster anti-mouse CD3 mAb (clone 145-2C11). Hybridoma clone 16-10A1, which produces hamster anti-mouse B7-1 mAb was purchased from ATCC. Hybridoma clone GL1, which produces rat anti-mouse B7-2 mAb was a kind gift from Dr. K. Hathcock (NCI, Bethesda, MD). All cell lines were maintained in cRPMI at 37°C and 5% CO_2 in a humidified incubator.

2.3 T cell isolation and activation by anti-CD3 mAb

Mice were sacrificed by cervical dislocation, spleens were aseptically removed, and homogenized in phosphate buffered saline (PBS; pH 7.4). Erythrocytes were removed by osmotic shock and spleen cells were passaged through nylon wool columns to deplete B cells and macrophages (Julius *et al.* 1973). Nylon wool non-adherent splenocytes were treated with anti-asialoGM1 antiserum and complement to deplete natural killer cells. The resulting T cells were resuspended in cRPMI and cultured at 8 x 10^6 cells per well in 24-well flat bottom tissue culture plates (Sarstedt, St. Laurent, PQ) for 48h at 37°C and 5% CO_2 in a humidified incubator. T cells were activated by the addition of hamster anti-mouse CD3 mAb (hybridoma supernatant from clone 145-2C11) at a final dilution of 1:20. Unless otherwise noted, all treatments (i.e., antibodies, cytokines, etc.) were performed prior to the addition of the anti-CD3 mAb. Alternatively, T cells were activated by the addition of antibody-coated 10 μm microspheres (Polysciences Inc., Warrington, PA) (coated with anti-CD3 ascites [0.01 μg/ml – 10 μg/ml] and anti-CD2 RM2.1 [5 μg/ml], anti-CD28 [5 μg/ml], or isotype control antibody) at a ratio of 2 T cells: 1 microsphere for 6h-48h at 37°C and 5% CO_2 in a humidified incubator.

2.4 T cell proliferation assay

Anti-CD3 activated T-cells in a 200 μl volume were transferred to quadruplicate wells of a 96 well round bottom microtitre plate (Sarstedt). The cultures were pulsed with 0.5 μCi of tritiated thymidine ([^{3}H]TdR; sp. Act. 65 Ci/mmol; ICN Biomedicals) per well and incubated at 37°C and 5% CO_2 for an additional 6h. Cultures were harvested onto glass fibre mats using a Titertek multiple sample harvester. [^{3}H]TdR incorporation was

measured in a Wallac 1410 liquid scintillation counter. Data are expressed as mean cpm +/- SD.

2.5 ^{51}Chromium-release assay

P815 mastocytoma cells were radiolabeled with in 100 μCi $Na_2{}^{51}CrO_4$ (ICN Biomedicals), washed 3 times in cRPMI, adjusted to $5x10^4$ cells/ml, and 100μl aliquots were combined with anti-CD3-activated T cells to yield the desired effector: target cell ratio. After 4h incubation at 37°C and 5% CO_2, the microtitre plate was centrifuged at 400 x *g* for 5 min and 100μl aliquots of cell-free supernatant were removed for analysis with a Beckman Gamma 8000 gamma counter. The maximum ^{51}Cr release was determined by lysing target cells with 10% SDS. The spontaneous ^{51}Cr release was determined by incubating the target cells with cRPMI alone. To quantitate cytotoxicity, the following equation was used:

[experimental release – spontaneous release] / [maximum release – spontaneous release] x 100 = % lysis. Data are expressed as mean % lysis +/- SD.

2.6 RNA Isolation

Total RNA was isolated from T-cells using TRIzol (Gibco BRL) as per the manufacturer's instructions. Briefly, 8 x 10^6 T cells were pelleted by centrifugation for 5 min at 400 x *g* and lysed in 1ml TRIzol in 1.5 ml eppendorf tubes. A 200 μl volume of chloroform was added and eppendorf tubes were vigorously shaken for 15 seconds followed by incubation at room temperature for 3 min. Following centrifugation for 15 min at 14,000 x *g* at 5°C, 450 μl of the aqueous phase was transferred to a new eppendorf

tube, and 500 µl of ice-cold isopropyl alcohol was added. Samples were incubated at room temperature for 10 min, then centrifuged at 14,000 x *g* for 10 min. The supernatant was carefully removed and 1 ml of 75% ethanol/ 25% RNAse free water was added to the RNA pellet. The samples were vortexed for 15 seconds, then centrifuged at 14,000 x *g* for 5 min. The supernatant was carefully removed and the RNA pellet was vacuum dried for 5 min, after which the RNA pellet was dissolved in 50 µl of pyrogen-free water.

2.7 Semi-quantitative RT-PCR

Reverse transcription of mRNA was carried out in a 20 µl volume containing 200 U Moloney murine leukemia virus reverse transcriptase, 1 µg random hexanucleotide primers, and 0.5 mM dNTPs . The reaction mixtures were incubated at 37°C for 1h, then 95°C for 10 minutes. The final volume was adjusted to 200 µl with pyrogen free water. cDNA in a total volume of 50 µl, along with 2.5 µl *Taq* DNA polymerase, 0.2 mM dNTPs, and 50 mM of each primer were used for subsequent PCR. The PCR reaction mixture was covered with 100µl of mineral oil to prevent evaporation. The following primers were used for PCR:

GAPDH (product: 247bp)	(F): 5'-ACTCACGGCAAATTCAACGGC-3'
	(R): 5'-ATCACAAACATGGGGGCATCG-3'
Perforin (product: 252bp)	(F): 5'-TCAATAACGACTGGCGTGTGG-3'
	(R): 5'-GTGGAGCTGTTAAAGTTGCGG-3'
Granzyme B (product:889bp)	(F): 5'-GCCCACAACATCAAAGAACAG-3'
	(R): 5'-GAGAACACATCAGCAACTTGGG-3'
FasL (product: 362bp)	(F): 5'-ATGGTTCTGGTGGCTCTGGT-3'

(R): 5'-GTTTAGGGGCTGGTTGTTGC-3'

IL-2 (product: 170bp) (F): 5'-TGATGGACCTACAGGAGCTCCTGAG-3'

(R): 5'-GAGTCAAATCCAGAACATGCCGCAG-3'

TRAIL (product: 513bp) (F): 5'-TCACCAACGAGATGAAGCAGC-3'

(R): 5'-CTCACCTTGTCCTTTGAGACC -3'

IFN-γ (product: 257bp) (F): 5'-CGCTACACACTGCATCTTGG-3'

(R): 3'-GGCTGGATTCCGGCAACA-3'

CD25 (product:523bp) (F): 5'-TACTGCAGAGAATTTCATCC-3'

(R): 5'-GTAGAGCCTTGTATCCCG-3'

CD119 (product:189bp)(F): 5'-AGTGGGGAGATCCTACATACGAAACATACGG-3'

(R): 5'-TTTCTGTCATCATGGAAAGGAGGGATACAG-3'

2B4 (product 190bp) (F): 5'-TTG TCC TGC TTG GTG ACC AAG-3'

(R): 5'-TGT TGC TAA CGT TGC AGG TGT-3'

PCR amplification was achieved using the following protocols:

GAPDH: 28 cycles, denaturation 94°C 30s, annealing 57°C 30s, synthesis 72°C 60s

Perforin: 32 cycles, denaturation 94°C 30s, annealing 57°C 30s, synthesis 72°C 60s

Granzyme B: 28 cycles, denaturation 94°C 30s, annealing 57°C 30s, synthesis 72°C 120s

FasL: 28 cycles, denaturation 94°C 30s, annealing 57°C 30s, synthesis 72°C 90s

IL-2: 28 cycles, denaturation 94°C 30s, annealing 57°C 30s, synthesis 72°C 60s

TRAIL: 28 cycles, denaturation 94°C 30s, annealing 57°C 30s, synthesis 72°C 120s

IFN-γ: 28 cycles, denaturation 94°C 30s, annealing 57°C 30s, synthesis 72°C 60s

CD25: 28 cycles, denaturation 94°C 30s, annealing 57°C 30s, synthesis 72°C 90s

CD119: 28 cycles, denaturation 94°C 30s, annealing 57°C 30s, synthesis 72°C 60s

2B4: 28 cycles, denaturation 94°C 30s, annealing 60°C 30s, synthesis 72°C 60s

PCR products were visualized by electrophoresis on ethidium bromide stained 1.8% agarose gels. Relative abundance of PCR products was determined by densitometric analysis of gel scans. The number of PCR cycles selected for amplification of cDNA was previously determined based on the generation of a PCR product during the exponential phase. For example, in order to determine the appropriate number of PCR cycles for determination of relative abundance of mRNA coding for IL-2, IFN-γ, CD25, CD119, and GAPDH, we determined the amplification curve of each PCR product by examining a range of cycles from 20 to 38. The appropriate number of cycles was determined based on the number of cycles required to amplify a product during the exponential phase without reaching a plateau of amplification. The appropriate number of cycles to choose for IL-2, IFN-γ, CD25, CD119, and GAPDH was 28. RT-PCR performed under these conditions allows for semi-quantitative analysis of mRNA levels if the difference is twofold or greater (Singer-Sam *et al.* 1990).

2.8 Enzyme-linked immunosorbent assay (ELISA)

Sandwich ELISA was used to measure cytokine levels in supernatants from 24 or 48h cultures of anti-CD3 activated T cells using paired mAb, recombinant cytokines and protocols supplied by Pharmingen Canada. Briefly, 96 well flat bottom plates (Sarstedt, St. Laurent, PQ) were coated with capture antibody (1 μg/ml anti-IL-2; 4 μg/ml IFN-γ) and incubated at 4 °C overnight. Plates were washed three times with TBS. Plates were coated with 2 mg/mL BSA in TBS, 200 μL per well for 2h at room temperature to block non-specific binding of antigen. Plates were washed three times with 200 μL TTBS

(0.05% Tween 20). Experimental samples were incubated at 4°C overnight and the plates were washed three times with 200 μL TTBS. Biotinylated secondary antibody was added (0.5 μg/ml IL-2; 0.5 μg/ml IFN-γ) at room temperature for 1h. Plates were washed 6 times with TTBS. Extravadin-peroxidase (1/1000 in 1.0 mg/mL BSA in TTBS) was added to each well and the plates were incubated at room temperature for 30 min. Plates were washed with TTBS 6 times and TMB substrate solution (Kirkegarde-Perry Laboratories) was added. The reaction terminated with 100 μL of NH_3PO_4. Plates were read to determine absorbence at a wavelength of 450 nm and based on comparison to a standard curve of known cytokine concentrations, the concentrations of each cytokine were determined.

2.9 Western blot analysis

Aliquots of 8 x 10^6 purified T cells were pelleted by centrifugation and resuspended in 0.05 ml ice-cold lysis buffer (10 mM Tris-EDTA, pH 7.4, 1% Nonidet P-40, 10 μg/ml aprotinin, leupeptin/pepstatin, 1mM phenylmethanesulfonyl fluoride, and 0.1 mM Na_3VO_4), placed on ice and vortexed every 3 min for 30 min. Cell lysates were cleared by centrifugation at 14000 x *g* for 10 min. Samples were diluted in an equal volume of Laemmli sample buffer and boiled for 5 min. Samples were separated on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes (Amersham Pharmacia). The blots were blocked with 5% non-fat milk in PBS-T (0.1M phosphate buffer containing 200mM NaCl and 0.05% Tween-20), and then probed with goat anti-mouse granzyme B (Santa Cruz Biotech., Santa Cruz, CA), rat anti-mouse perforin (Kamiya Biomedical Co., Tukwila, WA), or recombinant anti-phosphotyrosine:HRPO

antibody (BD Pharmingen, Mississauga, ON) at 1:2500 (perforin), 1:400 (granzyme B), and 1:3000 (phosphotyrosine) for 60 min at room temperature. An anti-goat AP (granzyme B, perforin) was used as the secondary at a dilution of 1:2000 (Santa Cruz Biotech., Santa Cruz, CA). BCIP-NBT colorimetric detection (granzyme B, perforin) and enhanced chemilumenescence (ECL) detection (anti-phosphotyrosine) were performed and recorded on Hyperfilm-ECL film (BioRad). Equal protein loading was confirmed by amido black staining of a duplicate nitrocellulose membrane.

2.10 Flow cytometry analysis (FACS)

Flow cytometric analysis was used to determine the percentage of CD2-, CD4-, CD8-, CD28-, CD48-, CD25-, CD11a-, CD54, and CD119-positive cells in 24 or 48h cultures of anti-CD3 activated T cells using a standard protocol. Briefly, T cells were pelleted at 400 x *g* for 5 min at 5 °C. Cells were washed in 1 mL PBS and resuspended in 1 mL FACS buffer (1% BSA [Sigma, Oakville, ON], 0.2% NaN_3 [Fisher Scientific, Nepean, ON], in PBS). Cells were subsequently incubated with goat anti-hamster IgG (Cedarlane, Hornby, ON) antibody at 1 μg/mL for 30 min on ice to prevent hamster IgG-specific secondary antibodies from binding cell bound anti-CD3 antibody (activating mAb). A 500 μl volume of FACS buffer was added and the cells were pelleted by centrifugation at 400 x g for 5 min at 5°C. Cells were incubated with appropriate primary antibody (1 μg/ml) for 30 min on ice, and then washed with 500 μl FACS buffer. Cells were subsequently treated with FITC-labeled secondary antibody (1 μg/ml) for 30 min on ice. Cells were washed with 500 μl FACS buffer three times and then cell pellets were resuspended in 500 μl 1% paraformaldehyde in PBS (v/v) as a fixative. The percentage of

fluorescent cells was determined via analysis with a fluorescence activated cell scanner (FACScan, Becton Dickinson, Franklin Lakes, NJ).

2.11 T cell-target cell conjugation assay

Powdered neutral red dye was dissolved in cRPMI at a concentration of 1 mg/ml. The solution was centrifuged for 10 min at 750 x *g* and the supernatant was filter sterilized and diluted 1:2 with cRPMI. The target P815 mastocytoma cells were incubated in the dye solution for 30 min at 37°C and 5% CO_2 in a humidified incubator, centrifuged at 400 x *g* for 10 min, then resuspended at 5 x 10^6 cells/ml in cRPMI. T cells were resuspended at an equivalent concentration in cRPMI. Equal 100 µl volumes of T cell and labeled P815 target cell suspensions were combined in Falcon 2054 round-bottom polystyrene tubes (Becton Dickinson and Co., Lincoln Park, NJ) and the resulting cell solutions were centrifuged for 3 min at 100 x *g* and incubated for 30 min at 37°C and 5%CO_2 in a humidified incubator. Following incubation, cell pellets were resuspended by gentle aspiration. The number of conjugated T cells bound to labeled P815 cells and unconjugated T cells was determined by visualization with a light microscope and the data expressed as % conjugation +/- SD.

2.12 Statistical analysis

Statistical analysis of data was performed via ANOVA and Student's t-test using the GraphPad Instat V2.04a program and MS Excel. P values less than 0.05 were considered to be statistically significant. Data are representative of at least 3 individual experiments.

3.0 RESULTS

Section I: CD2 signaling is required for non-specific cytotoxic T lymphocyte induction by anti-CD3 antibody

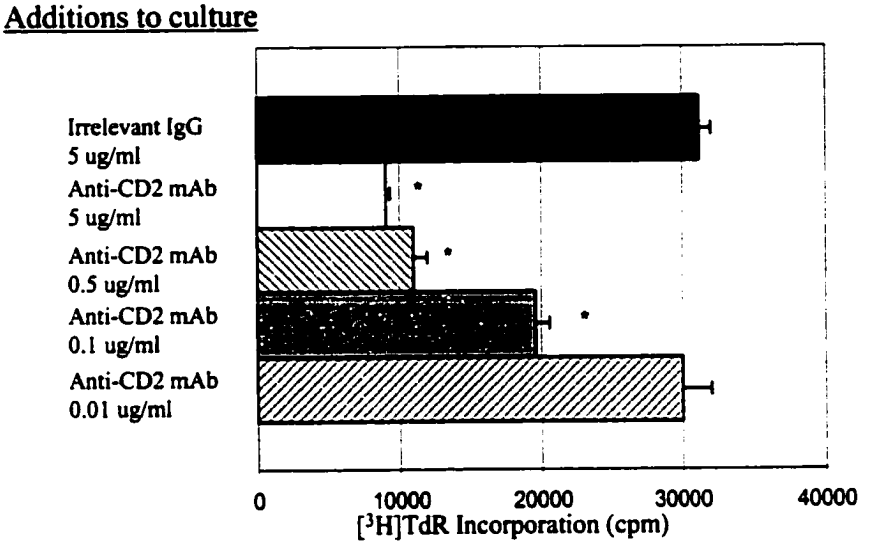
3.1 Blockade of CD2-CD48 interactions in anti-CD3-activated T cell cultures inhibits cell proliferation and the development of cytotoxic activity

We first determined the effect of various concentrations of anti-CD2 and anti-CD48 mAb on the development of proliferative and cytotoxic activity by anti-CD3 activated T cells. AK-T cell proliferation was assessed by tritiated thymidine incorporation at 48h of culture since it was previously established that peak AK-T cell proliferation occurs at this time point (Stankova *et al.* 1989). AK-T cell proliferation was increasingly decreased in cultures containing increasing concentrations of anti-CD2 mAb up to 0.5 μg/ml (**Figure 1A**) and anti-CD48 mAb up to 1.0 μg/ml (**Figure 1B**). Cytotoxicity of AK-T cells against allogeneic P815 mastocytoma cells was measured in a standard ^{51}Cr-release assay at 48h of culture since this is the time at which peak cytotoxic activity develops (Kaiser *et al.* 1993). The inhibitory effects of anti-CD2 mAb (**Figure 2A**) and anti-CD48 mAb (**Figure 2B**) on AK-T cell cytotoxicity paralleled the inhibitory effect of the mAbs on AK-T cell proliferation. Subsequent experiments, therefore, employed anti-CD2 and anti-CD48 mAb at 0.5 μg/ml and 1 μg/ml, respectively.

Figure 1. **Blockade of CD2-CD48 interactions in anti-CD3 mAb-activated T cell cultures inhibits cell proliferation**

T cell cultures were stimulated for 48h with anti-CD3 mAb in combination with anti-CD2 (**A**) or anti-CD48 (**B**) mAb or isotype control antibody at the indicated concentrations. Proliferation was assessed via [^{3}H]TdR incorporation. Data are shown as mean cpm of quadruplicate cultures +/- standard deviation (SD). Statistical significance was determined by one-way analysis of variance (ANOVA) and Bonferroni multiple comparison tests. Data are representative of three independent experiments.

A


Additions to culture
Irrelevant IgG
5 ug/ml
Anti-CD2 mAb
5 ug/ml
Anti-CD2 mAb
0.5 ug/ml
Anti-CD2 mAb
0.1 ug/ml
Anti-CD2 mAb
0.01 ug/ml
0
10000
20000
30000
40000
[3H]TdR Incorporation (cpm)

B

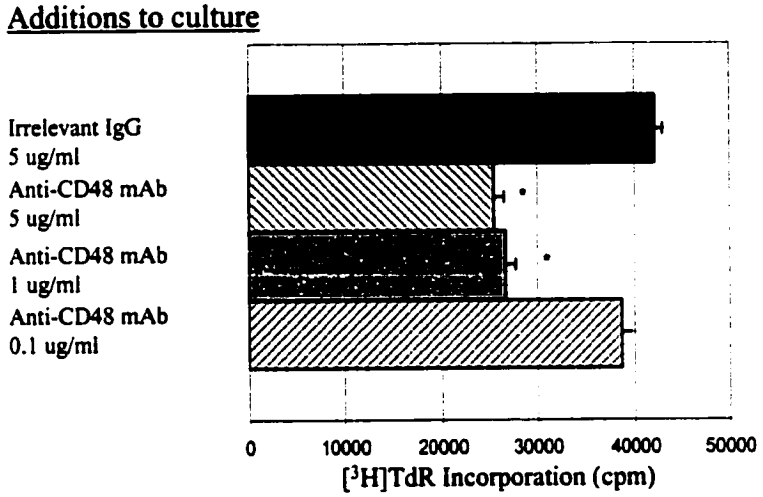
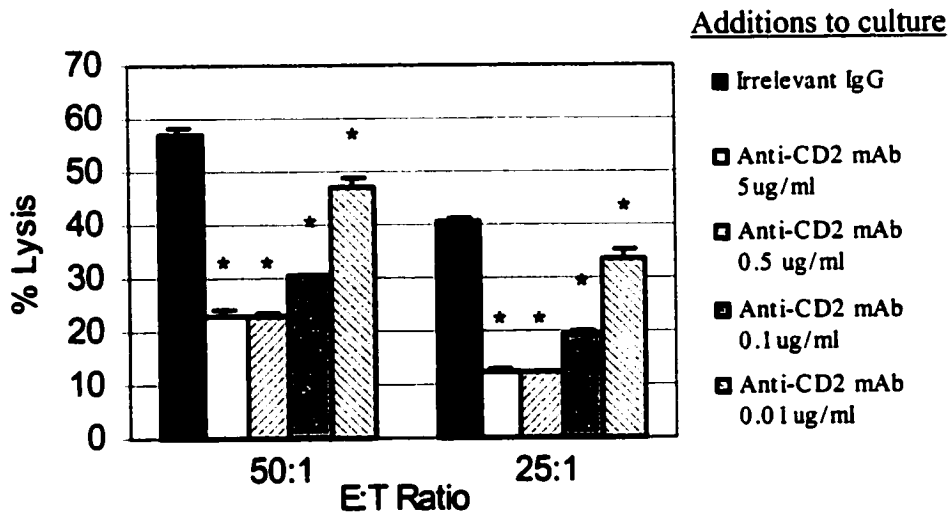

Additions to culture
Irrelevant IgG
5 ug/ml
Anti-CD48 mAb
5 ug/ml
Anti-CD48 mAb
1 ug/ml
Anti-CD48 mAb
0.1 ug/ml
0
10000
20000
30000
40000
50000
[3H]TdR Incorporation (cpm)

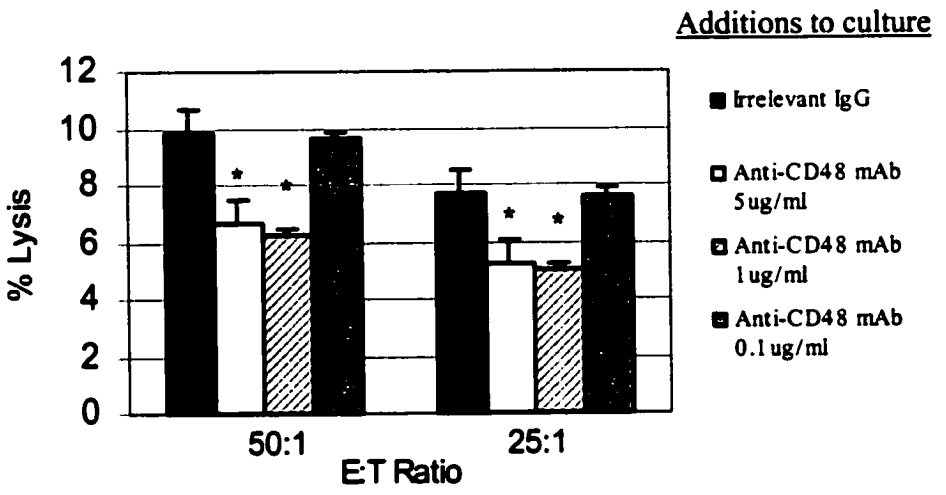
Figure 2. **Blockade of CD2-CD48 interactions in anti-CD3 mAb-activated T cell cultures inhibits the development of cytotoxic activity**

T cell cultures were stimulated for 48h with anti-CD3 mAb in combination with anti-CD2 (**A**) or anti-CD48 (**B**) mAb or isotype control antibody at the indicated concentrations. P815 tumor target cells were labeled with $Na_2\ ^{51}CrO_4$ as described in *Methods*. Data are shown as mean % specific lysis +/- SD for triplicate wells. Statistical significance was determined by one-way analysis of variance (ANOVA) and Bonferroni multiple comparison tests for 50:1 and the 25:1 effector: target cell ratios. Data are representative of three independent experiments.

A


Additions to culture
Irrelevant IgG
Anti-CD2 mAb 5ug/ml
Anti-CD2 mAb 0.5 ug/ml
Anti-CD2 mAb 0.1ug/ml
Anti-CD2 mAb 0.01ug/ml
% Lysis
70
60
50
40
30
20
10
0
50:1
25:1
E:T Ratio

B


Additions to culture
Irrelevant IgG
Anti-CD48 mAb 5ug/ml
Anti-CD48 mAb 1ug/ml
Anti-CD48 mAb 0.1ug/ml
% Lysis
12
10
8
6
4
2
0
50:1
25:1
E:T Ratio

3.2 Inhibitory effects of anti-CD2 mAb on CD8+ AK-T cell proliferation and cytotoxicity

Since several recent reports have demonstrated that $CD4^+$ T cells and $CD8^+$ T cells respond differently to certain stimuli (Elloso and Scott 2001; Phu *et al.* 2001), we next determined whether CD2 blockade differentially affects $CD8^+$ T cell cultures by examining the proliferative and cytotoxic response of CD4-depleted and unfractionated anti-CD3 activated T cell cultures performed in the presence of anti-CD2 mAb. The inhibitory effect of anti-CD2 mAb on T cell proliferation was similar in both CD4-depleted and unfractionated T cell cultures, as assessed by tritiated thymidine incorporation at 48h of culture (**Figure 3A**). AK-T cell cytotoxicity against P815 mastocytoma cells was also similarly inhibited by anti-CD2 mAb in CD4-depleted and unfractionated T cell cultures, as measured at 48h of culture using a standard ^{51}Cr-release assay (**Figure 3B**).

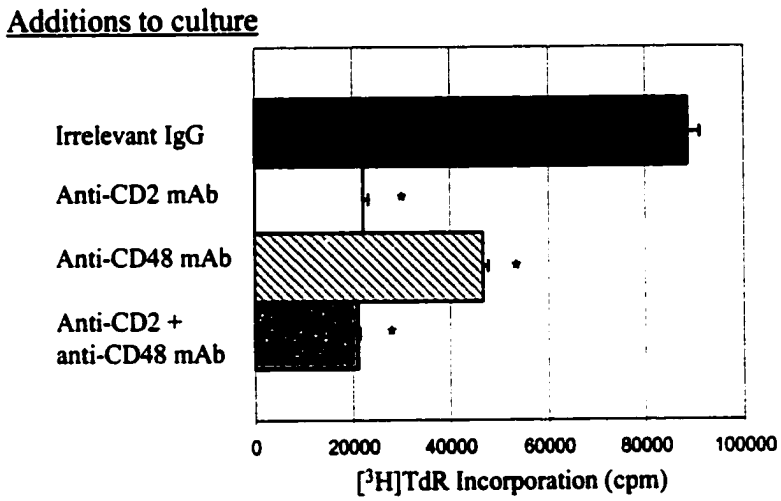
3.3 AK-T cells exhibit altered phosphorylation of tyrosine residues on cytoplasmic proteins in the presence of anti-CD2 mAb

To determine whether blockade of CD2 signaling in murine T cells influences PTK activity during T cell activation, we examined the phosphorylation of tyrosine residues on cytoplasmic proteins of AK-T cells following short-term stimulation with anti-CD3 mAb in the presence of anti-CD2 blocking antibodies. **Figure 4** indicates that several low molecular weight (33-40kDa) proteins exhibit decreased phosphorylation of tyrosine residues in AK-T cells at both 30 min and 60 min following activation in the presence of anti-CD2 mAb.

Figure 3. **Inhibitory effects of anti-CD2 mAb on CD8+ AK-T cell proliferation and cytotoxicity**

Purified $CD8^+$ T cell cultures were stimulated for 48h with anti-CD3 mAb in the presence of the indicated mAbs. (**A**) AK-T cell proliferation was assessed by $[^3H]$TdR incorporation and shown as the mean cpm of quadruplicate cultures +/- SD. Statistical significance was determined by Student's t-test. (**B**) P815 tumor target cells were labeled with $Na_2\ ^{51}CrO_4$ as described in *Methods*. Data are shown as mean % specific lysis +/- SD for triplicate wells. Statistical significance was determined by Student's t-test for 50:1 and the 25:1 effector: target cell ratios. Data are representative of three independent experiments.

A


Additions to culture
Irrelevant IgG
Anti-CD2 mAb
Anti-CD48 mAb
Anti-CD2 +
anti-CD48 mAb
0
20000
40000
60000
80000
100000
[3H]TdR Incorporation (cpm)

B

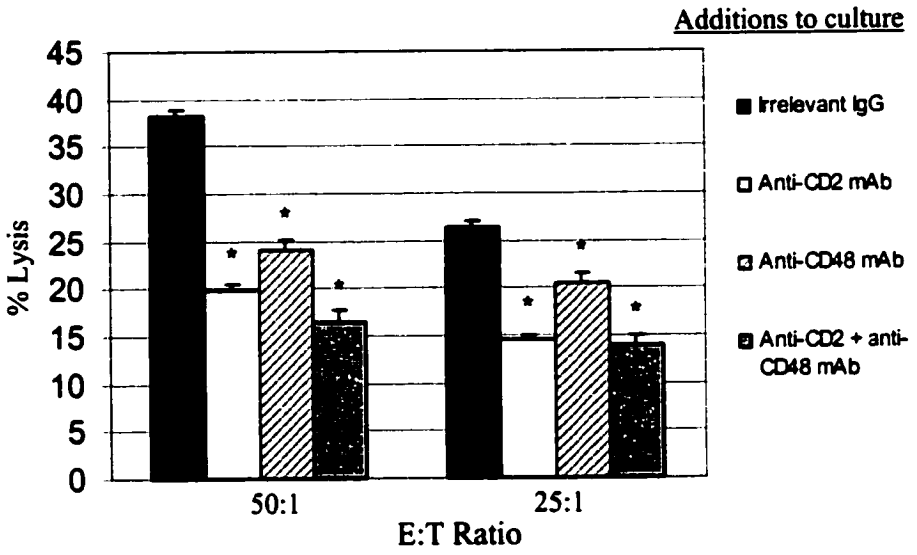
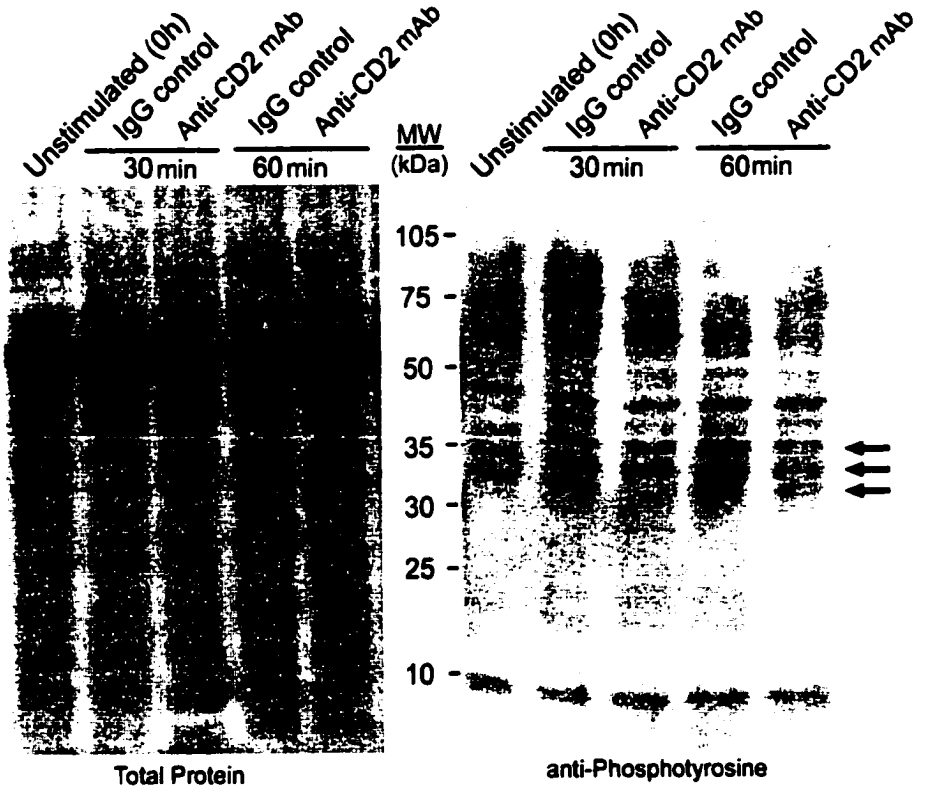

Additions to culture
45
40
35
30
25
20
15
10
5
0
% Lysis
50:1
25:1
E:T Ratio
Irrelevant IgG
Anti-CD2 mAb
Anti-CD48 mAb
Anti-CD2 + anti-CD48 mAb

Figure 4. **AK-T cells exhibit altered phosphorylation of tyrosine residues on cytoplasmic proteins in the presence of anti-CD2 mAb**
Cell lysates were prepared from unstimulated T cells and from T cells stimulated with anti-CD3 mAb in the presence of indicated antibodies at 30 min and 60 min of culture. The lysates were subjected to immunoblotting with a recombinant anti-phosphotyrosine mAb as described in *Methods*. Arrows indicate marked differences in the phosphorylation of tyrosine residues on AK-T cell proteins. Data are representative of three independent experiments.


Unstimulated (0h)
IgG control
Anti-CD2 mAb
IgG control
Anti-CD2 mAb
30 min
60 min
MW
(kDa)
105
75
50
35
30
25
10
Total Protein
anti-Phosphotyrosine

3.4 Anti-CD2 mAb fail to inhibit T cell activation by combinations of PMA and ionomycin or PMA and anti-CD3 mAb

In order to elucidate the effect of CD2 blockade on T cell signal transduction events which are distal to the T cell receptor, combinations of PMA and ionomycin or PMA and anti-CD3 mAb were added to T cell cultures in the presence or absence of anti-CD2 mAb. After 48h, proliferation was assessed by [^{3}H] TdR incorporation. **Figure 5** shows that anti-CD2 mAb had no effect on T cell activation by PMA and ionomycin or PMA and anti-CD3 mAb, indicating that inhibition due to CD2 blockade involves signaling events upstream of PKC activation and calcium mobilization.

3.5 CD2-CD48 interactions are required during the early stages of anti-CD3-mediated T cell activation

To determine the time at which CD2 signaling is required for optimal AK-T cell induction, purified T lymphocytes were cultured in the presence of anti-CD3 mAb for 48h and anti-CD2 mAb or an isotype control antibody were added at various time points up to 36 h after initiation of culture. AK-T cell cultures were assayed for cytotoxic activity and proliferative responses at 48h of culture. **Figures 6A** and **6B** clearly show that CD2 signaling is required during the initial 12h of culture for optimal AK-T cell induction.

3.6 CD2-CD48 blockade does not cause a shift in the kinetics of AK-T cell activation

To determine whether blockade of CD2-CD48 interactions alters the kinetics of AK-T cell activation, proliferation of AK-T cells induced in the presence of anti-CD2 mAb was

Figure 5. **Anti-CD2 mAb fail to inhibit T cell activation by PMA / ionophore- and PMA / anti-CD3 mAb**

AK-T cell proliferation at 48h in response to the indicated stimuli was assessed by [^{3}H]TdR incorporation. Anti-CD2 mAb was used at 0.5 μg/ml, PMA at 15 ng/ml, and ionomycin at 500 ng/ml. Data are shown as mean cpm of quadruplicate cultures +/- SD. Unstimulated T cells alone, with PMA, or with PMA + anti-CD2 mAb measured $127^{\pm}10$, $118^{\pm}2$, and $162^{\pm}85$ cpm, respectively. Statistical significance was determined by Student's t-test. (*) denotes a statistically significant difference in comparison to controls. Data are representative of three independent experiments.

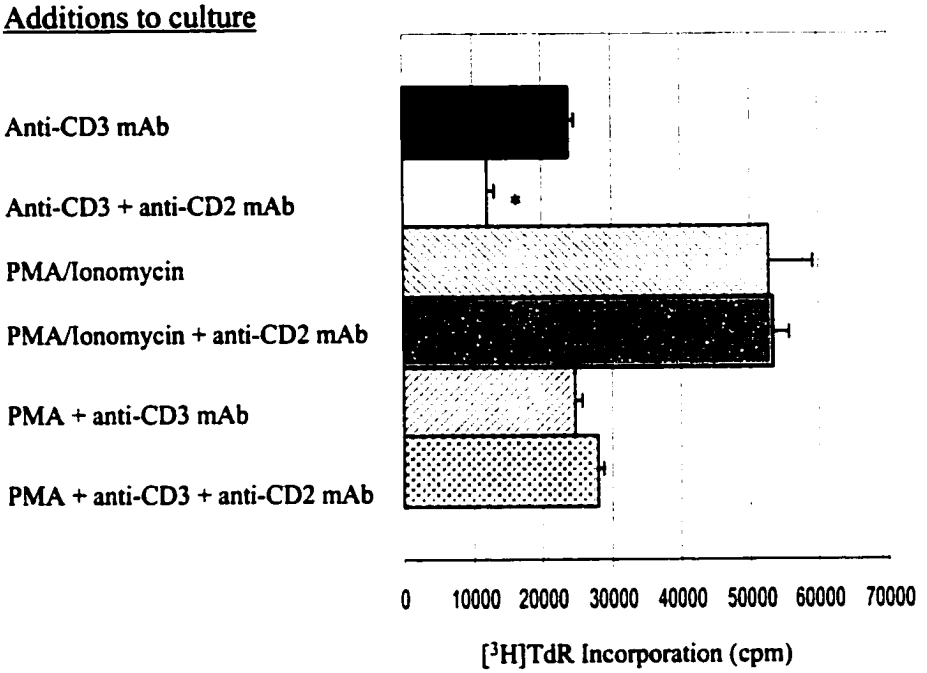
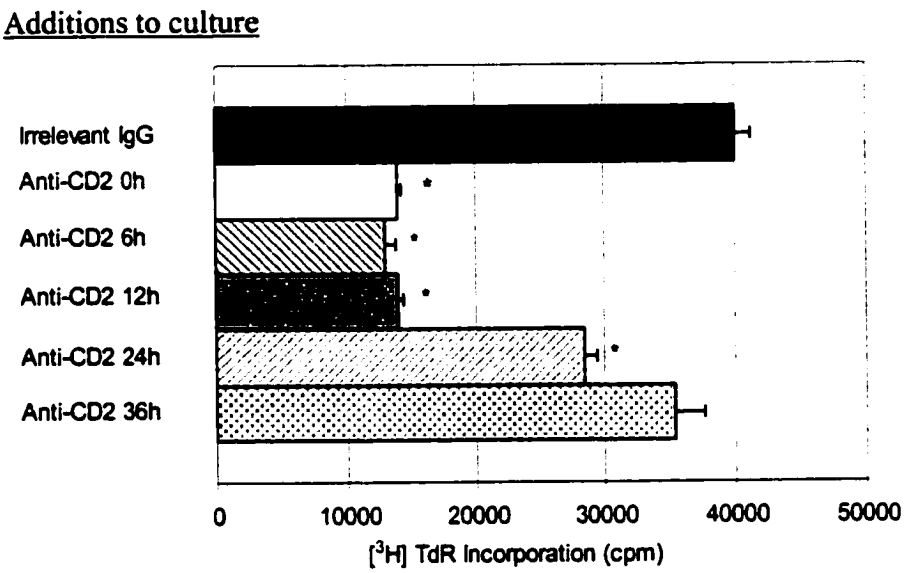

Additions to culture
Anti-CD3 mAb
Anti-CD3 + anti-CD2 mAb
PMA/Ionomycin
PMA/Ionomycin + anti-CD2 mAb
PMA + anti-CD3 mAb
PMA + anti-CD3 + anti-CD2 mAb
*
0
10000
20000
30000
40000
50000
60000
70000
[3H]TdR Incorporation (cpm)

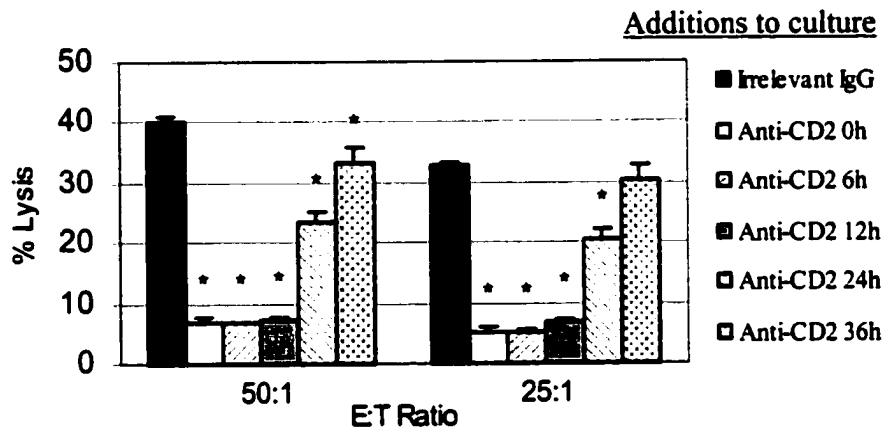
Figure 6. **CD2-CD48 interactions are required during the early stages of T cell activation**

T cell cultures were stimulated for 48h with anti-CD3 mAb. Anti-CD2 mAb were added up to 36h after addition of anti-CD3 mAb. (**A**) AK-T cell proliferation was assessed by [^{3}H]TdR incorporation and shown as mean cpm of quadruplicate cultures +/- SD. Statistical significance was determined by ANOVA and Bonferroni multiple comparison tests. (**B**) CTL cytotoxicity against P815 tumor target cells labeled with $Na_2\ ^{51}CrO_4$ was assessed and data are shown as mean % specific lysis +/- SD for triplicate wells. Statistical significance was determined by ANOVA and Bonferroni multiple comparison tests for 50:1 and the 25:1 effector: target cell ratios. Data are representative of three independent experiments.

A


Additions to culture
Irrelevant IgG
Anti-CD2 0h
Anti-CD2 6h
Anti-CD2 12h
Anti-CD2 24h
Anti-CD2 36h
0
10000
20000
30000
40000
50000
[3H] TdR Incorporation (cpm)

B


Additions to culture
Irrelevant IgG
Anti-CD2 0h
Anti-CD2 6h
Anti-CD2 12h
Anti-CD2 24h
Anti-CD2 36h
% Lysis
50
40
30
20
10
0
50:1
25:1
E:T Ratio

measured at various time-points up to 72h of culture. Peak proliferation was observed at 48h of culture in anti-CD2 mAb-treated cultures as well as in control cultures (**Figure 7**), indicating that CD2-CD48 blockade does not alter the kinetics of AK-T cell activation.

3.7 CD2 signaling promotes AK-T cell adhesion to P815 target cells

We next examined the ability of AK-T cells induced in the presence of anti-CD2 or anti-CD48 mAb to conjugate to P815 target cells to determine whether CD2 signaling promotes recognition/adhesion of tumor targets by AK-T cells. As shown in **Table 1**, CD2-CD48 blockade reduced the ability of AK-T cells to conjugate to P815 target cells compared to AK-T cells induced in the presence of an isotype control antibody. The inhibitory effect of blockade with anti-CD2 mAb on AK-T cell binding to target cells was greater than that of anti-CD48 mAb. However, residual AK-T cell bound anti-CD2 mAb is unlikely to play a major role in inhibiting AK-T cell conjugation to target cells since only a weak inhibitory effect was observed when saturating concentrations of ani-CD2 mAb were added directly to AK-T cells and P815 target cells in a 4h ^{51}Cr-release cytotoxicity assay (**Figure 8**). A comparable weak inhibitory effect was observed with anti-CD48 mAb while an additive effect was obtained when both mAb were used in combination. In comparison, strong inhibition was observed in the presence of anti-LFA-1 mAb.

Because adhesion molecules such as CD11a (LFA-1) and CD54 (ICAM-1) are critical in mediating AK-T cell adhesion to P815 target cells (Hoskin *et al.* 1998), we next examined surface expression of these molecules by AK-T cells induced in the presence of anti-CD2 mAb. Surprisingly, CD11a expression was only slightly decreased and CD54 expression was unaffected by anti-CD2 mAb treatment (**Figure 9**). AK-T cell surface

Figure 7. **CD2-CD48 blockade does not alter the kinetics of AK-T cell activation** T cell cultures stimulated by anti-CD3 mAb in the presence of the indicated antibodies were assayed for proliferation via [^{3}H]TdR incorporation at time points up to 72h of culture. Data is shown as mean cpm of quadruplicate cultures +/- SD. Data are representative of three independent experiments.

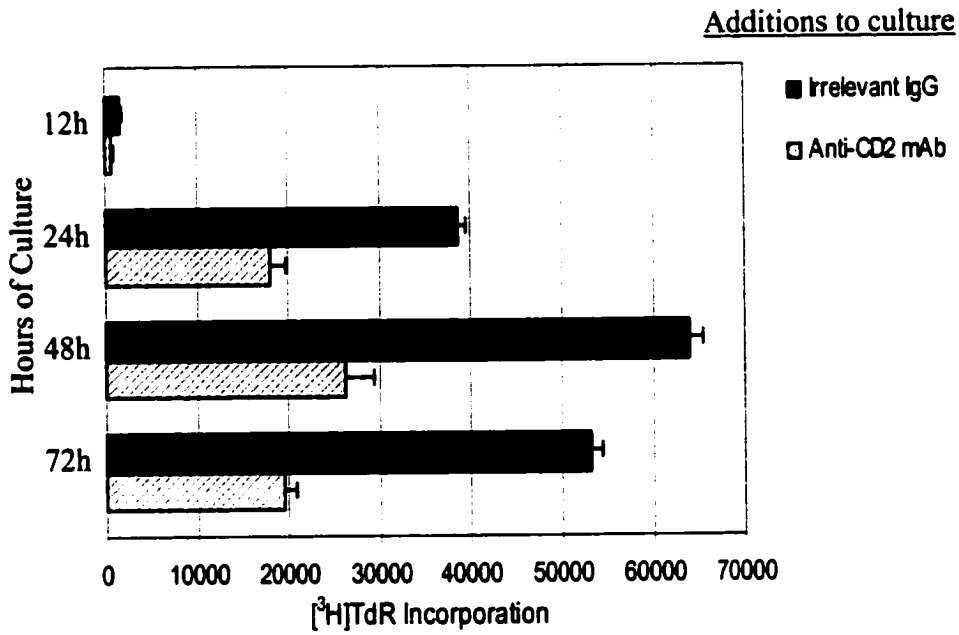

Additions to culture
Irrelevant IgG
Anti-CD2 mAb
Hours of Culture
12h
24h
48h
72h
0
10000
20000
30000
40000
50000
60000
70000
[3H]TdR Incorporation

Table 1. CD2 signaling promotes AK-T cell adhesion to P815 target cells

Treatment[a]	% Conjugation	% Inhibition	Significance (P[b])
Irrelevant IgG	30.5±1.3		
anti-CD2 mAb	10.2±0.7	66.5	P < 0.005
anti-CD48 mAb	19.0±1.6	37.7	P < 0.005

[a]T cells were stimulated with anti-CD3 mAb in the presence of the indicated mAbs. After 48h of culture AK-T cells were assayed for their ability to conjugate to neutral red-labeled P815 target cells. Unstimulated T cells displayed 3.5±0.6% conjugation. Data are shown as the mean % conjugation of triplicate cultures +/- SD. Data are representative of three independent experiments.
[b]Statistical significance was determined by Student's t-test.

Figure 8. **CD2-CD48 interactions play a minor role in the adhesion of AK-T cells to P815 mastocytoma cells**

T cells were stimulated for 48h with anti-CD3 mAb to induce AK-T cells. CTL cytotoxicity against P815 tumor target cells labeled with $Na_2\ ^{51}CrO_4$ was assessed in the presence of saturating concentrations of the indicated mAbs (20 μg/ml). Data are shown as mean % specific lysis +/- SD for triplicate wells. Statistical significance was determined by Student's t-test for 50:1 and the 25:1 effector: target cell ratios. (*) denotes a statistically significant difference in comparison to controls. Data are representative of three independent experiments.

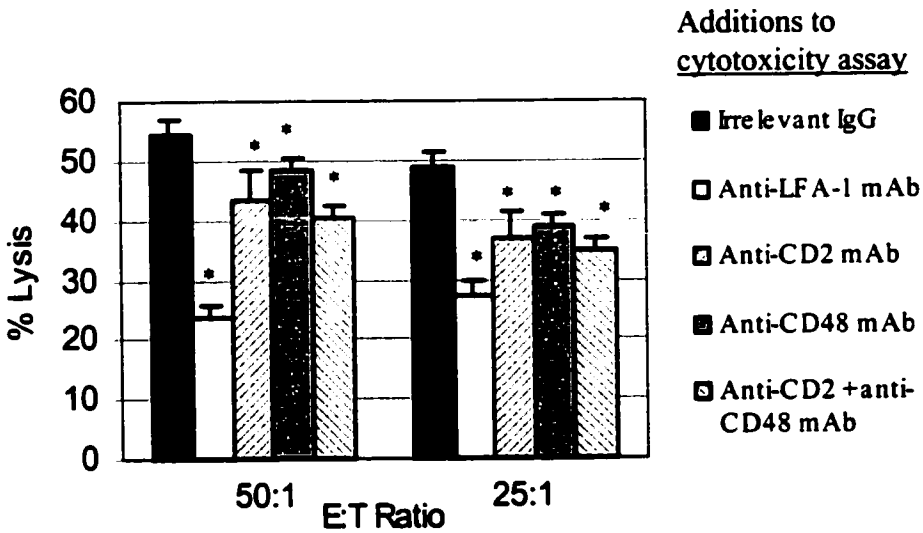
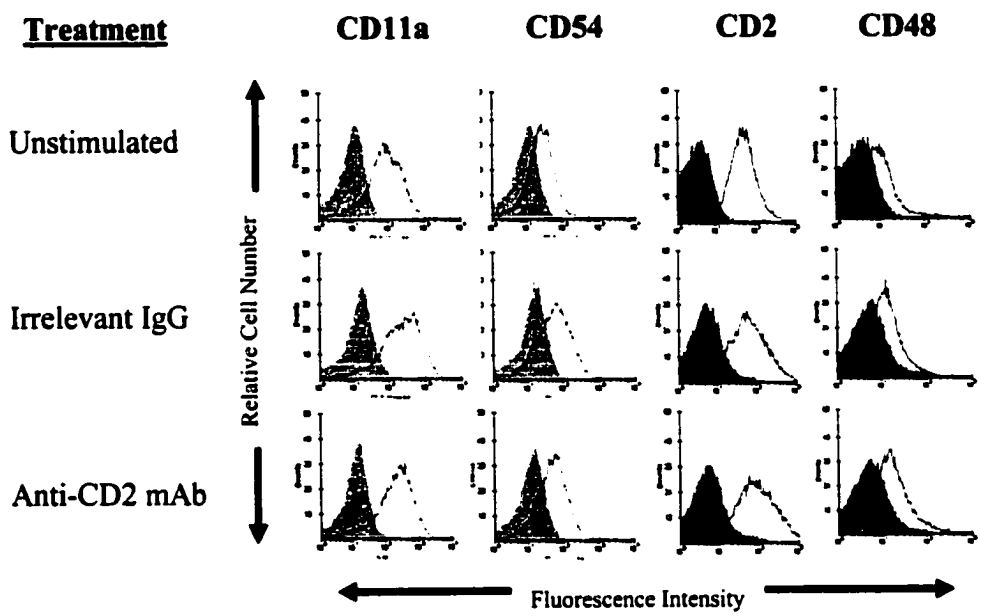

Additions to
cytotoxicity assay
Irrelevant IgG
Anti-LFA-1 mAb
Anti-CD2 mAb
Anti-CD48 mAb
Anti-CD2 +anti-CD48 mAb
% Lysis
60
50
40
30
20
10
0
50:1
25:1
E:T Ratio

Figure 9. **Effect of CD2 blockade on adhesion molecule expression**
Unstimulated T cells or T cell cultures stimulated anti-CD3 mAb in the presence or absence of the anti-CD2 mAb were examined by flow cytometry for CD11a, CD54, CD2, and CD48 expression at 48h of culture. Anti-CD11a mAb, anti-CD54 mAb, and anti-CD2 mAb or anti-CD48 mAb were added followed by mouse anti-rat IgG-FITC or mouse anti-hamster IgG-FITC antibody as appropriate. Histograms (X-axis, relative fluorescence intensity; Y-axis, relative cell number) show anti-CD11a, anti-CD54, CD2, or CD48 mAb staining (black line) relative to isotype control antibody staining (shaded gray region). Data are representative of three independent experiments.


Treatment
CD11a
CD54
CD2
CD48
Unstimulated
Irrelevant IgG
Anti-CD2 mAb
Relative Cell Number
Fluorescence Intensity

expression of CD2 remained similar in anti-CD2 mAb-treated and control cell cultures, indicating that *in vitro* treatment with anti-CD2 mAb does not modulate CD2 from the cell surface. Similarly, CD48 expression was not affected by the presence of anti-CD2 mAb during T cell activation.

3.8 IL-2 and IFN-γ production in AK-T cell cultures is downregulated in the presence of anti-CD2 or anti-CD48 mAb

Because of the importance of IL-2 and IFN-γ in the development of antigen-specific and non-specific cytotoxic T cells (Fitzpatrick *et al.* 1996; Maraskovsky *et al.* 1989), we determined the effect of blocking CD2-CD48 interactions on the production of IL-2 and IFN-γ in AK-T cell cultures. As shown in **Table 2**, IL-2 production was inhibited by 85% in AK-T cell cultures treated with anti-CD2 mAb and by approximately 50% in AK-T cell cultures treated with anti-CD48 mAb. In contrast, both anti-CD2 mAb and anti-CD48 mAb treatment inhibited IFN-γ production by approximately 50%. A similar inhibitory effect was observed on IL-2 and IFN-γ mRNA levels when AK-T cells were induced in the presence of anti-CD2 mAb (**Figure 10**).

3.9 CD25 expression is downregulated but CD119 expression by AK-T cells is enhanced in the absence of CD2 signaling

IL-2 and IFN-γ utilization in T cell proliferation and differentiation occurs through specific cell-surface cytokine receptors (Waldmann 1998; Farrar and Schreiber 1993). To examine the effect of CD2 blockade on AK-T cell expression of IL-2 and IFN-γ receptors, T

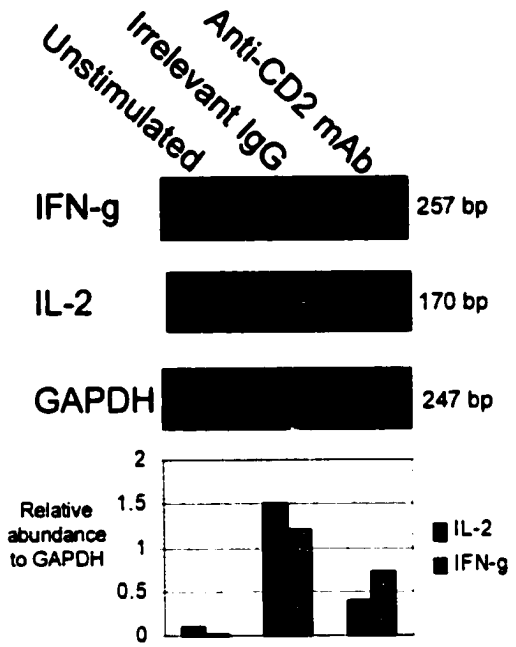
Table 2. **IL-2 and IFN-γ production in AK-T cell cultures is downregulated in the presence of anti-CD2 or anti-CD48 mAb**

Treatment[a]	IL-2 (pg/ml)	% Inhibition	Significance (P^b)	IFN-γ (pg/ml)	% Inhibition	Significance (P^b)
Irrelevant IgG	157±3			779±19		
anti-CD2 mAb	23±17	85	P < 0.005	366+44	53	P = 0.027
anti-CD48 mAb	78±10	50	P < 0.005	397+73	49	P = 0.032

[a]T cells were stimulated with anti-CD3 mAb in the presence of the indicated mAbs. After 24h and 48h of culture, IL-2 and IFN-γ production, respectively, was measured by standard sandwich ELISA as described in *Methods*. Data shown as mean concentration of IL-2 or IFN-γ in pg/ml of triplicate samples in three independent experiments +/- standard error.

[b]Statistical significance was determined Student's t-test.

Figure 10. **IL-2 and IFN-γ mRNA expression in AK-T cell cultures is downregulated in the presence of anti-CD2 mAb**
T cells were stimulated with anti-CD3 mAb in the presence or absence of anti-CD2 mAb. Following 12h of culture, total RNA was isolated and IL-2, IFN-γ, and GAPDH mRNA levels were determined by semi-quantitative RT-PCR as described in *Methods*. Data are representative of three independent experiments.


Unstimulated
Irrelevant IgG
Anti-CD2 mAb
IFN-g
257 bp
IL-2
170 bp
GAPDH
247 bp
Relative abundance to GAPDH
2
1.5
1
0.5
0
IL-2
IFN-g

lymphocytes were cultured in the presence of activating anti-CD3 mAb in combination with anti-CD2 mAb or isotype control antibody. Surface expression of CD25 and CD119 (IFN-γR α subunit) was determined by flow cytometry at 48h of culture. Following activation, T cells showed increased expression of CD25, which was subsequently downregulated following exposure to anti-CD2 mAb (**Figure 11**). Interestingly, CD119 expression was actually enhanced in AK-T cell cultures subjected to CD2 blockade.

3.10 Granzyme B and perforin expression is downregulated in AK-T cell cultures treated with anti-CD2 mAb, but is unaffected by anti-CD48 mAb treatment

We next determined whether CD2 blockade during the induction phase affected AK-T cell expression of cytolytic effector molecules. Granzyme B and perforin mRNA expression by AK-T cell cultures induced in the presence of anti-CD2 mAb, anti-CD48 mAb, or an isotype control antibody were assessed by RT-PCR. Perforin mRNA expression was nearly abrogated when AK-T cells were induced in the presence of anti-CD2 mAb (**Figure 12A**). Granzyme B mRNA expression was also reduced, although not as dramatically. Similar results were obtained when granzyme B and perforin protein expression was examined by western blotting (**Figure 13**). In contrast, AK-T cell induction in the presence of anti-CD48 mAb had no effect on expression of granzyme B and perforin mRNA (**Figure 12B**). FasL and TRAIL mRNA expression was unaffected when AK-T cells were induced in the presence of anti-CD2 mAb or anti-CD48 mAb (**Figure 12**).

Figure 11. **CD25 expression is downregulated but CD119 expression by AK-T cells is enhanced in the absence of CD2 signaling**
Unstimulated T cells or T cell cultures stimulated anti-CD3 mAb in the presence or absence of anti-CD2 mAb were examined by flow cytometry for CD25 and CD119 expression at 48h of culture. Anti-CD25 mAb-FITC or rat anti-CD119 mAb followed by mouse anti-rat IgG-FITC antibody were used for staining. Histograms (X-axis, relative fluorescence intensity; Y-axis, relative cell number) show anti-CD25 or anti-CD119 mAb staining (black line) relative to isotype control antibody staining (shaded gray region). Data are representative of three independent experiments.

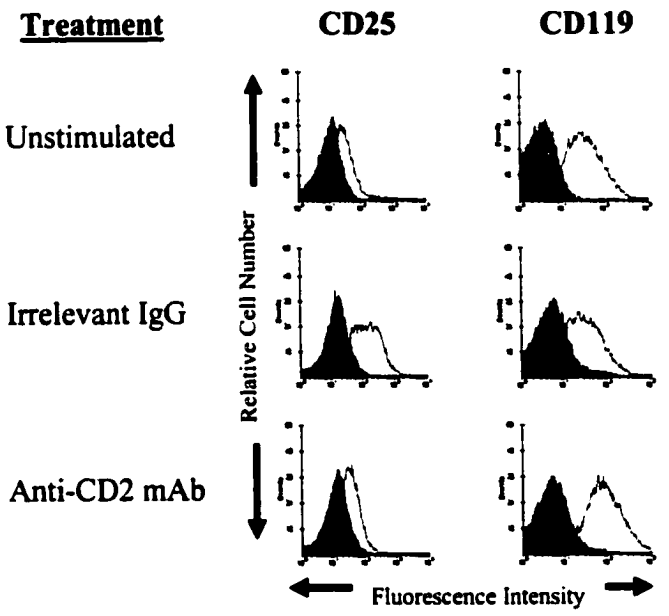
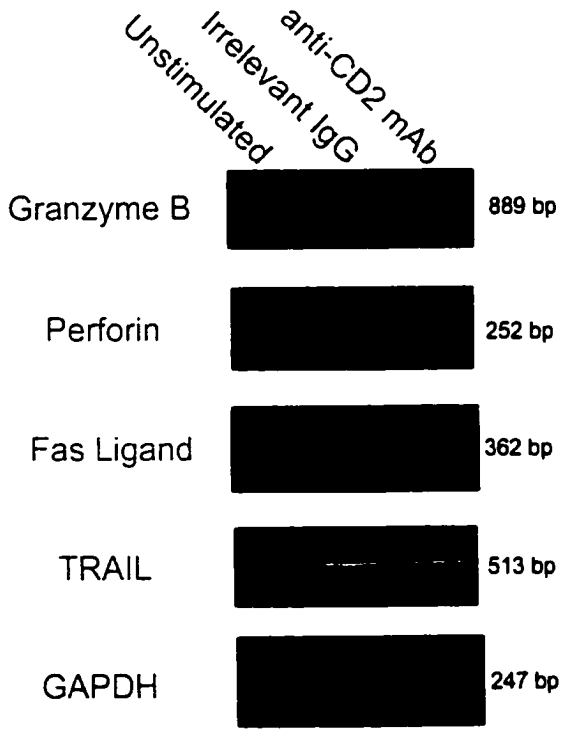

Treatment
CD25
CD119
Unstimulated
Irrelevant IgG
Anti-CD2 mAb
Relative Cell Number
Fluorescence Intensity

Figure 12. Granzyme B and perforin expression is downregulated in AK-T cell cultures treated with anti-CD2 mAb, but is unaffected by anti-CD48 mAb treatment

T cells were stimulated with anti-CD3 mAb in the absence or presence of (**A**) anti-CD2 mAb or (**B**) anti-CD48 mAb. Following 48h of culture, total RNA was isolated and granzyme B, perforin, and GAPDH mRNA levels were determined by semi-quantitative RT-PCR as described in *Methods*. Data are representative of three independent experiments.


Unstimulated
Irrelevant IgG
anti-CD2 mAb
Granzyme B
889 bp
Perforin
252 bp
Fas Ligand
362 bp
TRAIL
513 bp
GAPDH
247 bp

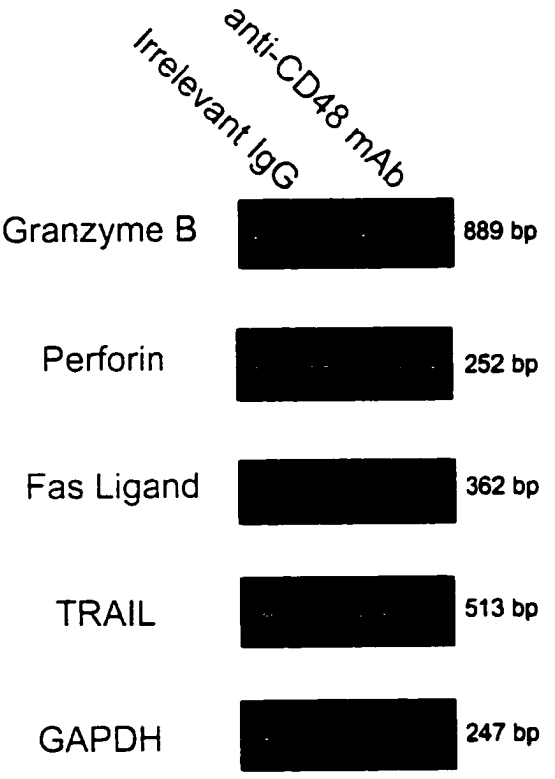
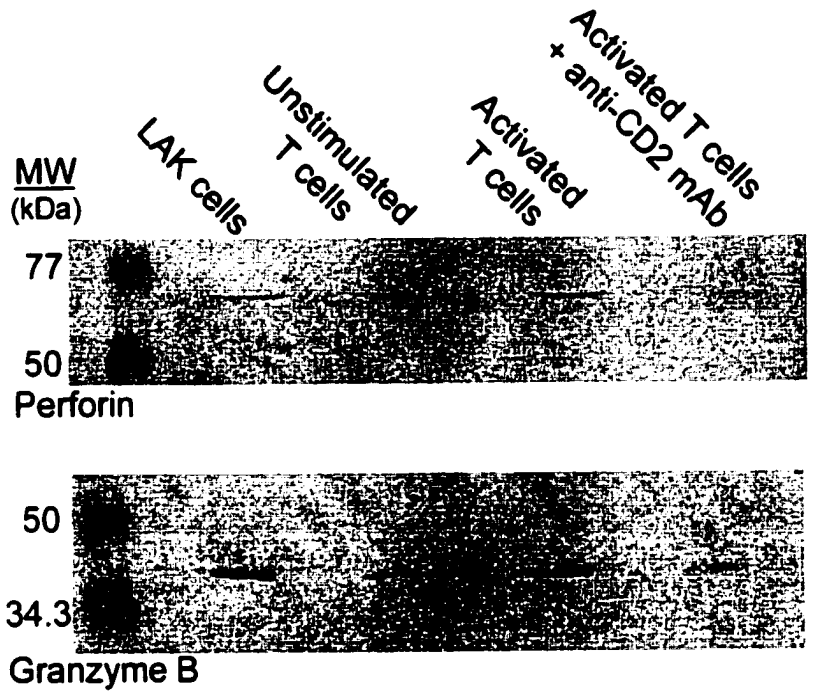

Irrelevant IgG
anti-CD48 mAb
Granzyme B
889 bp
Perforin
252 bp
Fas Ligand
362 bp
TRAIL
513 bp
GAPDH
247 bp

Figure 13. Granzyme B and perforin protein expression is downregulated in AK-T cell cultures treated with anti-CD2 mAb

Cell lysates were prepared from unstimulated T cells and from T cells stimulated with anti-CD3 mAb in the absence or presence of anti-CD2 mAb for 48h. The lysates were subjected to immunoblotting with a monoclonal anti-mouse granzyme B or anti-mouse perforin mAb as described in *Methods*. Data are representative of three independent experiments.


MW
(kDa)
LAK cells
Unstimulated T cells
Activated T cells
Activated T cells + anti-CD2 mAb
77
50
Perforin
50
34.3
Granzyme B

3.11 Perforin mRNA expression is downregulated in unstimulated T cells treated with anti-CD2 mAb

We next determined whether anti-CD2 mAb downregulates baseline perforin expression in resting T cells by examining perforin mRNA expression in unstimulated T cell cultures containing anti-CD2 mAb after 6h. RT-PCR analysis indicated that perforin expression was downregulated substantially in resting T cell cultures containing anti-CD2 mAb in comparison to control cultures containing an isotype control antibody (**Figure 14**). These data suggest that CD2 crosslinking may negatively regulate cytotoxic molecule expression.

3.12 Anti-CD2 mAb do not inhibit CTLL-2 cell proliferation

We next examined the effect of anti-CD2 mAb on the proliferative response of the IL-2 dependent CTLL-2 cell line to determine whether CD2 blockade interferes with IL-2R signaling. CTLL-2 cell proliferation was assessed by [^{3}H]TdR incorporation at 48h of culture. Anti-CD2 mAb had no effect on CTLL-2 cell proliferation (**Figure 15**), indicating that IL-2R signal transduction pathways are intact in the presence of anti-CD2 mAb.

3.13 Exogenous IL-2 and IFN-γ restore normal function to AK-T cells induced in the absence of costimulation through CD2

Since IL-2 and IFN-γ production are strongly inhibited in AK-T cell cultures induced in the presence of anti-CD2 or anti-CD48 mAb (**Table 2**), and these cytokines are important for AK-T cell development (Fitzpatrick *et al.* 1996), we next determined whether the addition of exogenous IL-2 and IFN-γ at levels similar to those normally present in AK-

Figure 14. **Perforin mRNA expression is downregulated in unstimulated T cells exposed to anti-CD2 (RM2.5) mAb for 6h**
Unstimulated T cells were incubated for 6h in the absence or presence of anti-CD2 mAb. Following 6h of culture, total RNA was isolated, and perforin and GAPDH mRNA levels were determined by semi-quantitative RT-PCR as described in *Methods*. Data are representative of three independent experiments.

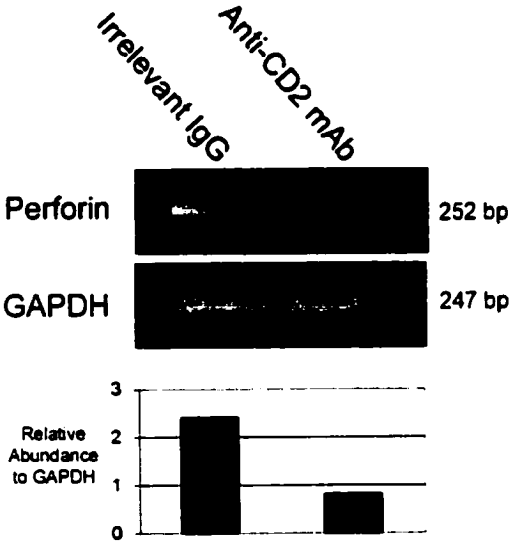
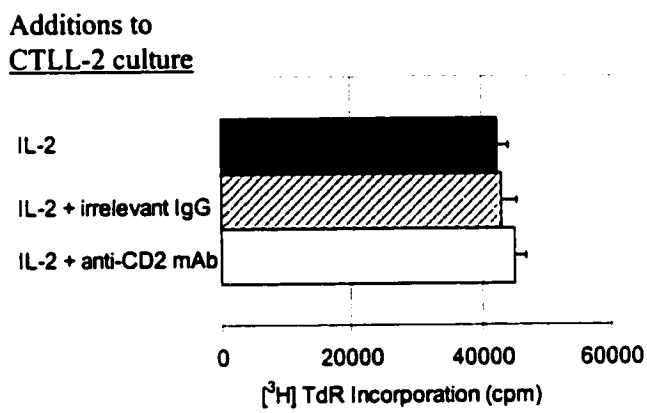

Irrelevant IgG
Anti-CD2 mAb
Perforin
252 bp
GAPDH
247 bp
Relative Abundance to GAPDH
3
2
1
0

Figure 15. Anti-CD2 mAb do not inhibit CTLL-2 cell proliferation
CTLL-2 cells were incubated in the absence or presence of anti-CD2 mAb for 48h without or with IL-2 (10 U/ml). Proliferation was assessed by [^{3}H]-TdR-incorporation as described in *Methods*. CTLL-2 cells alone, with irrelevant IgG, or with anti-CD2 mAb in the absence of exogenous IL-2 gave $97^{\pm}45$, $122^{\pm}12$, and $149^{\pm}72$ cpm, respectively. Data is shown as mean cpm of quadruplicate cultures +/- SD. Statistical significance was determined by Student's t-test. (*) denotes a statistically significant difference in comparison to controls. Data are representative of three independent experiments.


Additions to
CTLL-2 culture
IL-2
IL-2 + irrelevant IgG
IL-2 + anti-CD2 mAb
0
20000
40000
60000
[3H] TdR Incorporation (cpm)

T cell cultures could compensate for an absence of costimulation through CD2. Both proliferative responses (**Figure 16A**) and cytolytic activity (**Figure 16B**) were restored to normal levels when AK-T cells were induced in the presence of anti-CD2 mAb plus exogenous IL-2 and IFN-γ. On the other hand, proliferative responses and cytotoxic activity by AK-T cells were only partially restored by the addition of IL-2 or IFN-γ alone. However, in both cases IL-2 was more effective than IFN-γ.

3.14 Exogenous IL-2 and IFN-γ restore normal function to AK-T cells induced in the presence of anti-CD48 mAb

We next determined whether the addition of exogenous IL-2 and IFN-γ at levels similar to those normally present in AK-T cell cultures could compensate for an anti-CD48 mAb-mediated blockade of costimulation through CD2. Both proliferative responses (**Figure 17A**) and cytolytic activity (**Figure 17B**) were restored to normal levels when AK-T cells were induced in the presence of anti-CD48 mAb plus exogenous IL-2 and IFN-γ. In contrast, proliferative responses and cytotoxic activity were only partially restored by the addition of either IL-2 or IFN-γ alone to anti-CD48-treated AK-T cell cultures.

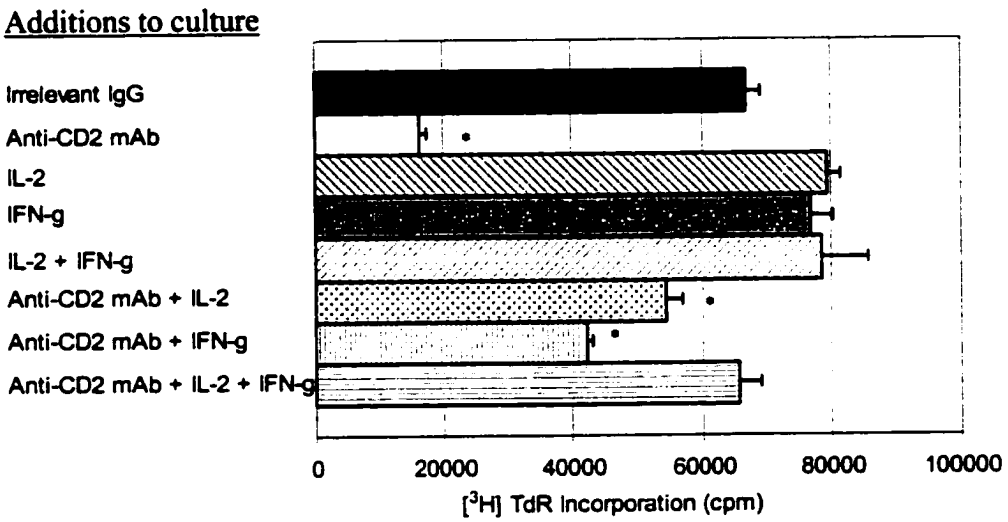
3.15 IL-2 is required for AK-T cells to gain the ability to conjugate to P815 target cells

Cell-mediated cytotoxicity requires that effector cells physically interact with target cells via adhesion molecules and their respective ligands (de Vries *et al.* 1989). Previous work has shown that AK-T cell interactions with P815 mastocytoma cells are largely dependent on LFA-1 binding to ICAM-1 (Hoskin *et al.* 1998), and that this process can be negatively impacted upon by blockade of CD2-CD48 interactions during AK-T cell

Figure 16. **Exogenous IL-2 and IFN-γ restore normal function to AK-T cells induced in the absence of costimulation through CD2**

T cells were stimulated for 48h with anti-CD3 mAb in the absence or presence of anti-CD2 mAb without or with exogenous IL-2 (150 pg/ml) and/or IFN-γ (800 pg/ml). (**A**) AK-T cell proliferation was assessed by [^{3}H]TdR incorporation and shown as the mean cpm of quadruplicate cultures +/- SD. Statistical significance was determined by Student's t-test. (**B**) P815 tumor target cells were labeled with $Na_2\ ^{51}CrO_4$ as described in *Methods*. Data are shown as mean % specific lysis +/- SD for triplicate wells. Statistical significance was determined by Student's t-test for 50:1 and the 25:1 effector: target cell ratios. (*) denotes a statistically significant difference in comparison to the IgG control. Data are representative of three independent experiments.

A


Additions to culture
Irrelevant IgG
Anti-CD2 mAb
IL-2
IFN-g
IL-2 + IFN-g
Anti-CD2 mAb + IL-2
Anti-CD2 mAb + IFN-g
Anti-CD2 mAb + IL-2 + IFN-g
0
20000
40000
60000
80000
100000
[3H] TdR Incorporation (cpm)

B

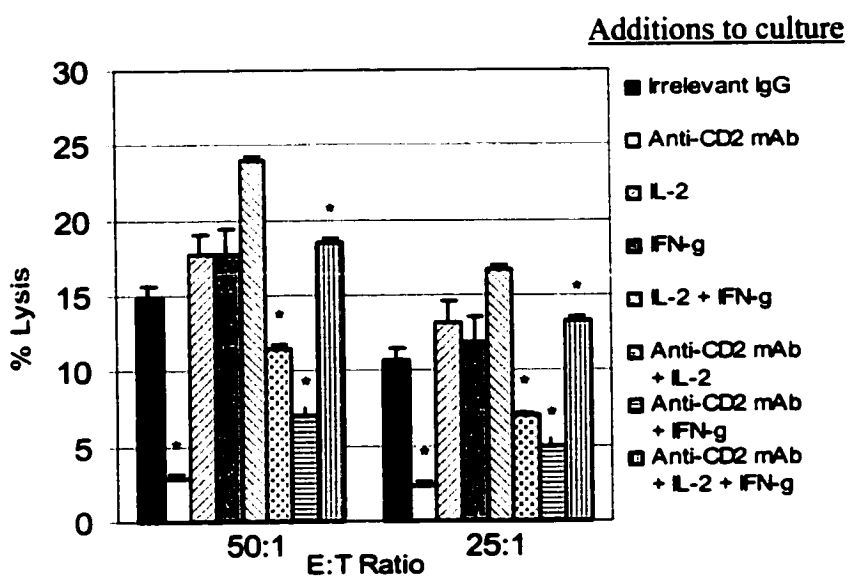
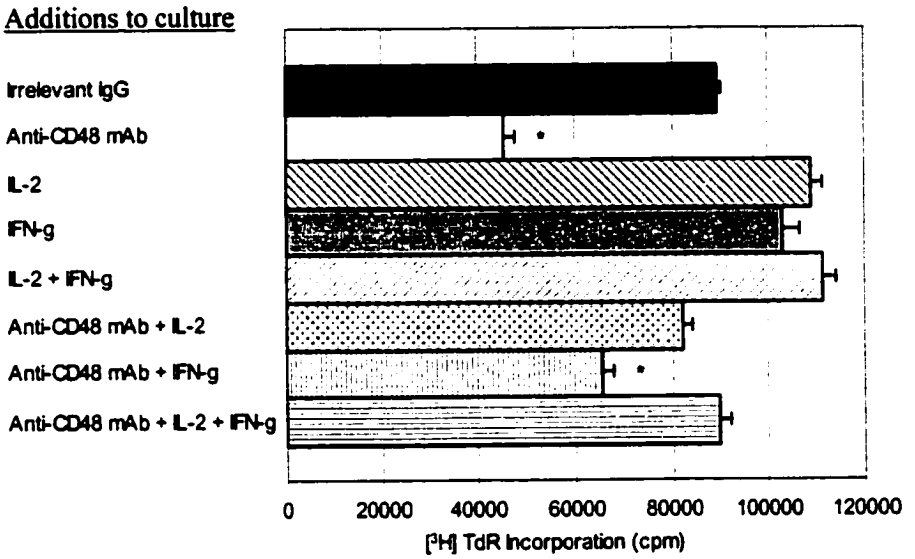

Additions to culture
30
25
20
15
10
5
0
% Lysis
50:1
25:1
E:T Ratio
Irrelevant IgG
Anti-CD2 mAb
IL-2
IFN-g
IL-2 + IFN-g
Anti-CD2 mAb + IL-2
Anti-CD2 mAb + IFN-g
Anti-CD2 mAb + IL-2 + IFN-g

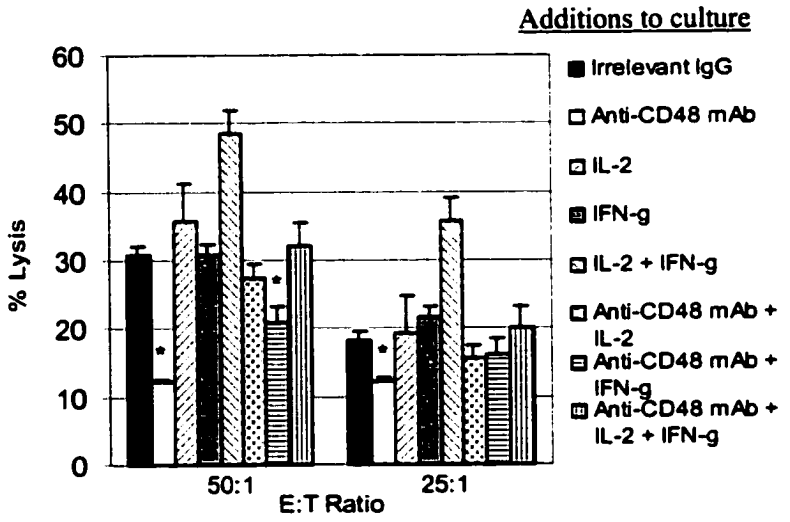
Figure 17. Exogenous IL-2 and IFN-γ restore normal function to AK-T cells induced in the presence of anti-CD48 mAb

T cell cultures were stimulated for 48h with anti-CD3 mAb in the absence or presence of the anti-CD48 mAb without or with exogenous IL-2 (150 pg/ml) and/or IFN-γ (800 pg/ml). (**A**) AK-T cell proliferation was assessed by [^{3}H]TdR incorporation and shown as the mean cpm of quadruplicate cultures +/- SD. Statistical significance was determined by Student's t-test. (**B**) P815 tumor target cells were labeled with $Na_2\ ^{51}CrO_4$ as described in *Methods*. Data are shown as mean % specific lysis +/- SD for triplicate wells. Statistical significance was determined by Student's t-test for 50:1 and the 25:1 effector: target cell ratios. (*) denotes a statistically significant difference in comparison to the IgG control. Data are representative of three independent experiments.

A


Additions to culture
Irrelevant IgG
Anti-CD48 mAb
IL-2
IFN-g
IL-2 + IFN-g
Anti-CD48 mAb + IL-2
Anti-CD48 mAb + IFN-g
Anti-CD48 mAb + IL-2 + IFN-g
0
20000
40000
60000
80000
100000
120000
[3H] TdR Incorporation (cpm)

B


Additions to culture
60
50
40
30
20
10
0
% Lysis
50:1
25:1
E:T Ratio
Irrelevant IgG
Anti-CD48 mAb
IL-2
IFN-g
IL-2 + IFN-g
Anti-CD48 mAb + IL-2
Anti-CD48 mAb + IFN-g
Anti-CD48 mAb + IL-2 + IFN-g

development (**Table 1**). To determine whether exogenous IL-2 and/or IFN-γ might restore the ability of AK-T cells to conjugate to P815 target cells, conjugation assays were performed following AK-T cell induction in the absence or presence of anti-CD2 mAb with or without exogenous IL-2 and/or IFN-γ. **Figure 18** shows that the addition of exogenous IL-2, but not IFN-γ, restored the ability of AK-T cells induced in the presence of anti-CD2 mAb to bind P815 target cells. The ability of anti-CD48 mAb-treated AK-T cells to conjugate with P815 target cells was restored by the addition of exogenous IL-2 but not IFN-γ (**Figure 19**).

3.16 Granzyme B and perforin expression is restored in anti-CD2 mAb-treated AK-T cells by the addition of exogenous IL-2 and IFN-γ

Since the addition of exogenous IL-2 and IFN-γ restored cytolytic activity in anti-CD2 mAb-treated AK-T cell cultures, we next determined whether the expression of granzyme B and perforin was also restored in the presence of exogenous IL-2 and IFN-γ. As shown in **Figure 20**, RT-PCR analysis of granzyme B and perforin mRNA expression by AK-T cell induced in the absence or presence of anti-CD2 mAb with or without exogenous IL-2 and/or IFN-γ revealed that anti-CD2 mAb treated AK-T cell cultures containing exogenous IL-2 and/or IFN-γ expressed granzyme B and perforin at a level which was comparable to controls.

3.17 2B4 mRNA is undetectable in AK-T cell cultures as assessed by RT-PCR

A recent report (Brown *et al.* 1998) that 2B4 is structurally very similar to CD2 (both are members of the Ig superfamily who share CD48 as a ligand), prompted us to

Figure 18. Exogenous IL-2 restores conjugation to AK-T cells induced in the absence of costimulation through CD2

T cell cultures stimulated by anti-CD3 mAb in the absence or presence of anti-CD2 mAb without or with exogenous IL-2 (150 pg/ml) and/or IFN-γ (800 pg/ml) were assayed for their ability to conjugate to neutral red-labeled P815 target cells following 48h of culture. Unstimulated T cells displayed $4.6^{\pm}0.1\%$ conjugation to target cells. Data are shown as the mean % conjugation of triplicate cultures +/- SD. Statistical significance was determined by Student's t-test. (*) denotes a statistically significant difference in comparison to the IgG control. Data are representative of three independent experiments.

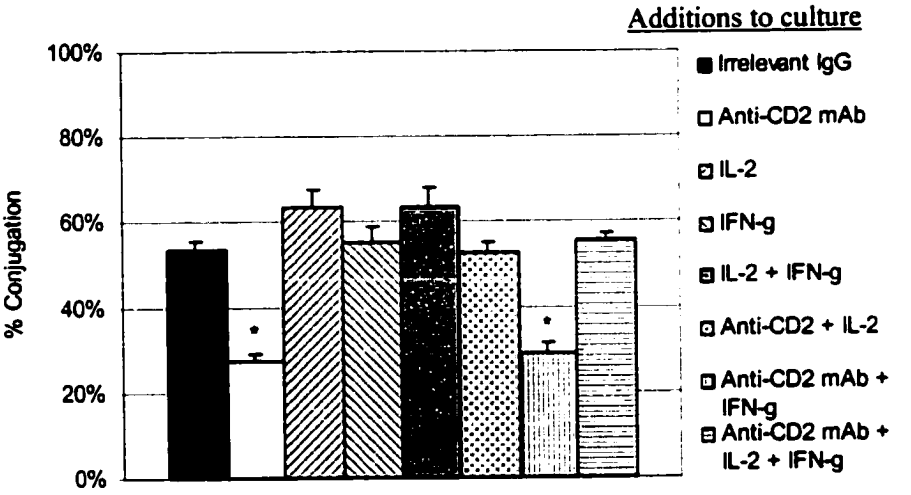

Additions to culture
100%
80%
60%
40%
20%
0%
% Conjugation
Irrelevant IgG
Anti-CD2 mAb
IL-2
IFN-g
IL-2 + IFN-g
Anti-CD2 + IL-2
Anti-CD2 mAb + IFN-g
Anti-CD2 mAb + IL-2 + IFN-g

Figure 19. Exogenous IL-2 restores conjugation to AK-T cells induced in the presence of anti-CD48 mAb

T cell cultures stimulated by anti-CD3 mAb in the absence or presence of anti-CD48 mAb without or with exogenous IL-2 (150 pg/ml) and/or IFN-γ (800 pg/ml) were assayed for their ability to conjugate to neutral red-labeled P815 target cells following 48h of culture. Unstimulated T cells displayed 4.4±0.1% conjugation to target cells. Data is shown as the mean of triplicate cultures +/- SD. Statistical significance was determined by Student's t-test. (*) denotes a statistically significant difference in comparison to the IgG control. Data are representative of three independent experiments.

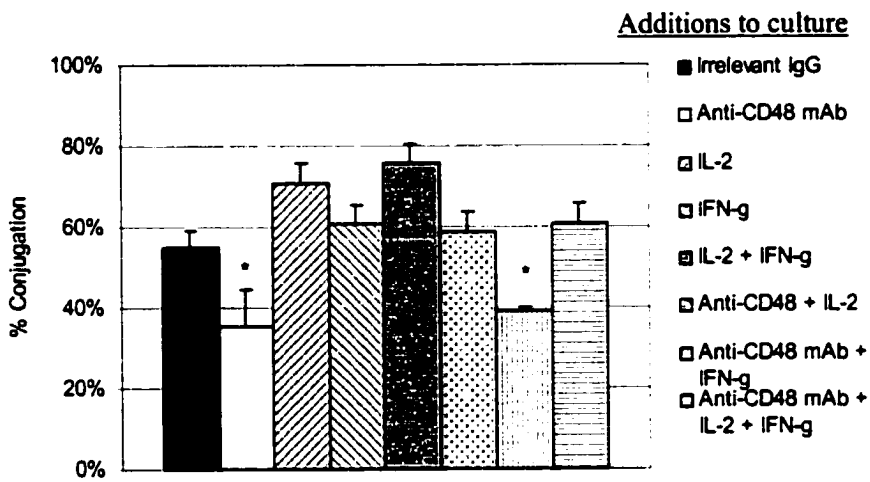
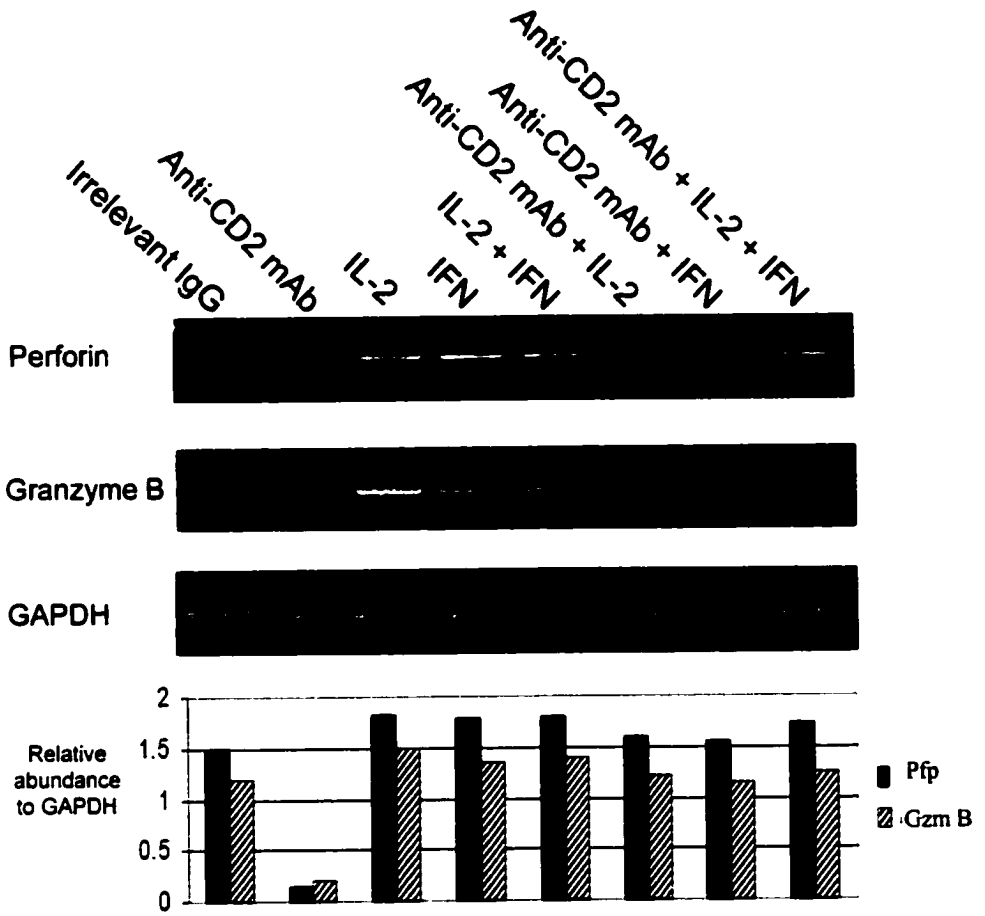

Additions to culture
100%
80%
60%
40%
20%
0%
% Conjugation
Irrelevant IgG
Anti-CD48 mAb
IL-2
IFN-g
IL-2 + IFN-g
Anti-CD48 + IL-2
Anti-CD48 mAb + IFN-g
Anti-CD48 mAb + IL-2 + IFN-g

Figure 20. **AK-T cell expression of granzyme B and perforin is restored by the addition of exogenous IL-2 and IFN-γ**

T cells were stimulated with anti-CD3 mAb in the absence or presence of anti-CD2 mAb without or with IL-2 (150 pg/ml) and/or IFN-γ (800 pg/ml). Following 48h of culture, total RNA was isolated and granzyme B, perforin, and GAPDH mRNA levels were determined by semi-quantitative RT-PCR as described in *Methods*. Data are representative of three independent experiments.


Irrelevant IgG
Anti-CD2 mAb
IL-2
IFN
Anti-CD2 mAb + IL-2
Anti-CD2 mAb + IFN
Anti-CD2 mAb + IL-2 + IFN
Perforin
Granzyme B
GAPDH
Relative abundance to GAPDH
2
1.5
1
0.5
0
Pfp
Gzm B

explore whether 2B4 is expressed in AK-T cell cultures. Furthermore, 2B4 has been shown to function as an activating receptor molecule on NK cells (Nakajima and Colonna 2000). Although LAK cell cultures demonstrated abundant expression of 2B4 mRNA, no 2B4 mRNA was detectable by RT-PCR in RNA isolated from AK-T cell cultures (**Figure 21**).

Section II: Signaling through CD2 enhances IL-2 and IFN-γ mRNA stability in T lymphocytes: Evidence that CD2 meets criteria for a costimulatory molecule

3.18 Blockade of CD2-CD48 interactions prevents proliferation in anti-CD3-activated T cell cultures

In our initial experiments, we investigated the role of CD2 costimulatory signaling in murine T cell activation by blocking signaling with anti-CD2 mAb during T cell activation with soluble anti-CD3 mAb. For purposes of comparison, CD28 costimulatory signaling was also blocked with a cocktail of anti-CD80 and anti-CD86 mAb. T cell proliferation was assessed by tritiated thymidine incorporation at 48h of culture. **Figure 22** shows that T cell proliferation was markedly decreased in cultures containing anti-CD2 mAb. A marginally greater inhibitory effect was observed in cultures containing anti-CD80 and anti-CD86 mAb. Anti-CD2 in combination with anti-CD80 and anti-CD86 mAb reduced T cell proliferation to baseline levels, suggesting that optimal T cell activation requires costimulation though both CD2 and CD28.

Figure 21. 2B4 mRNA is undetectable in AK-T cell cultures
T cells and erythrocyte-depleted splenocytes were stimulated with anti-CD3 mAb or anti-CD3 mAb + IL-2 (250 U/ml), respectively. Following 48h of culture, total RNA was isolated and 2B4 and GAPDH mRNA levels were determined by semi-quantitative RT-PCR as described in *Methods*. Data are representative of three independent experiments.

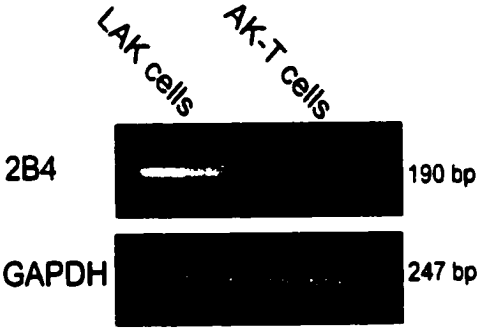
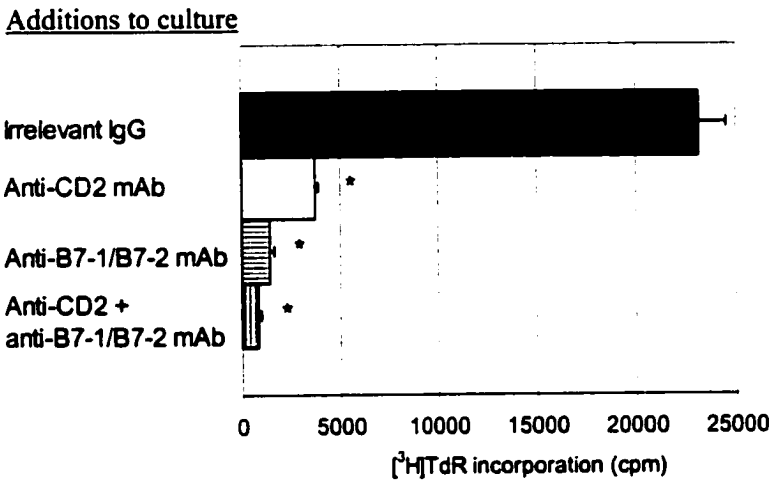

LAK cells
AK-T cells
2B4
190 bp
GAPDH
247 bp

Figure 22. CD2 and/or CD28 blockade inhibits anti-CD3 mAb-induced T cell proliferation

T cells were stimulated with anti-CD3 mAb in the absence or presence of the indicated mAbs. Following 48h of culture, proliferation was assessed by [^{3}H]-TdR-incorporation as described in *Methods*. Data is shown as mean cpm of quadruplicate cultures +/- SD and are representative of three independent experiments. Statistical significance was determined by Student's t-test. (*) denotes a statistically significant difference in comparison to the IgG control.


Additions to culture
Irrelevant IgG
Anti-CD2 mAb
Anti-B7-1/B7-2 mAb
Anti-CD2 +
anti-B7-1/B7-2 mAb
0
5000
10000
15000
20000
25000
[3H]TdR incorporation (cpm)

3.19 IL-2, IFN-γ, and CD25 mRNA levels are decreased but CD119 mRNA levels are unaffected in T cells activated in the absence of CD2 signaling

To determine the effect of CD2 blockade on activated T cell expression of mRNA coding for IL-2, IFN-γ, CD25 (the α chain of the high affinity IL-2 receptor), and CD119 (the α subunit of the IFN-γ receptor), T lymphocytes were activated with anti-CD3 mAb in the presence of anti-CD2 mAb or an isotype control antibody. IL-2, IFN-γ, CD25 and CD119 mRNA levels were measured by RT-PCR after 12h of culture. T cells activated in the presence of anti-CD2 mAb demonstrated reduced levels of IL-2, IFN-γ, and CD25 mRNA but no change in CD119 mRNA levels (**Figure 23**). IL-2, IFN-γ, and CD25, but not CD119, mRNA levels were also reduced in T cell cultures activated in the presence of anti-CD80 and anti-CD86 mAb, alone and in combination with anti-CD2 mAb.

3.20 IL-2 and IFN-γ mRNA half-life is reduced in the absence of CD2 signaling

Since IL-2, IFN-γ, and CD25 mRNA expression by activated T cells was downregulated in the presence of anti-CD2 mAb (**Figure 23**), we next examined the role of CD2 signaling in the stabilization of mRNA coding for these genes. T cells were stimulated with anti-CD3 mAb in the presence of anti-CD2 mAb or an isotype control antibody for 6h, at which point actinomycin D was added to block further mRNA transcription. RNA from replicate cultures was subsequently isolated at 0h to 4h after addition of actinomycin D and IL-2, IFN-γ, CD25, and CD119 mRNA levels were determined by RT-PCR. **Figure 24** shows that IL-2 and IFN-γ mRNA stability is decreased in the absence of CD2 signaling. T cells activated in the presence of anti-CD80 and anti-CD86 mAb displayed a similar reduction in IL-2 and IFN-γ mRNA stability. Combined blockade of CD2 and CD28

Figure 23. **Activated T cells express decreased levels of mRNA coding for cytokines and cytokine receptors in the absence of CD2 and/or CD28 signaling**

T cells were stimulated with anti-CD3 mAb in the absence or presence of the indicated mAbs. Following 12h of culture, total RNA was isolated and IL-2, IFN-γ, CD25, CD119, and GAPDH mRNA levels were determined by semi-quantitative RT-PCR as described in *Methods*. Data are representative of three independent experiments.

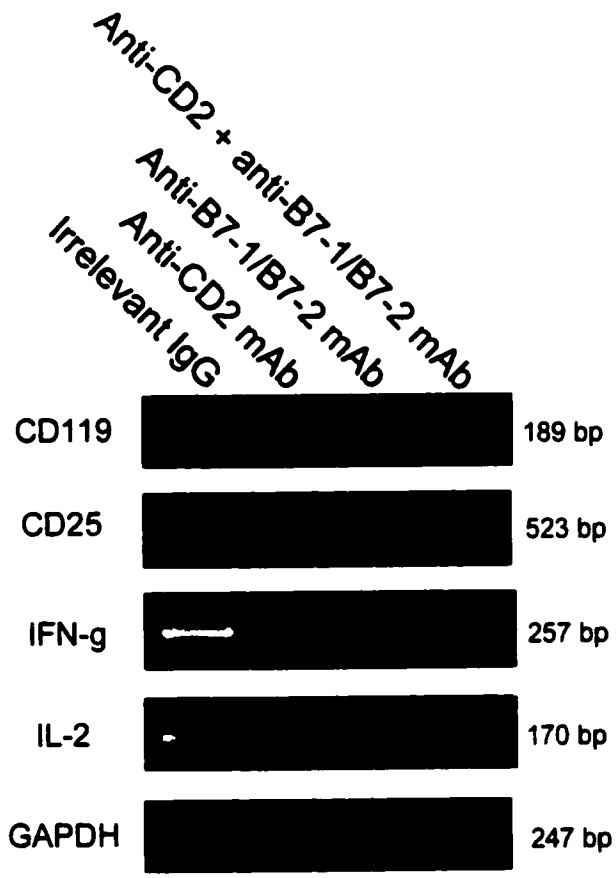
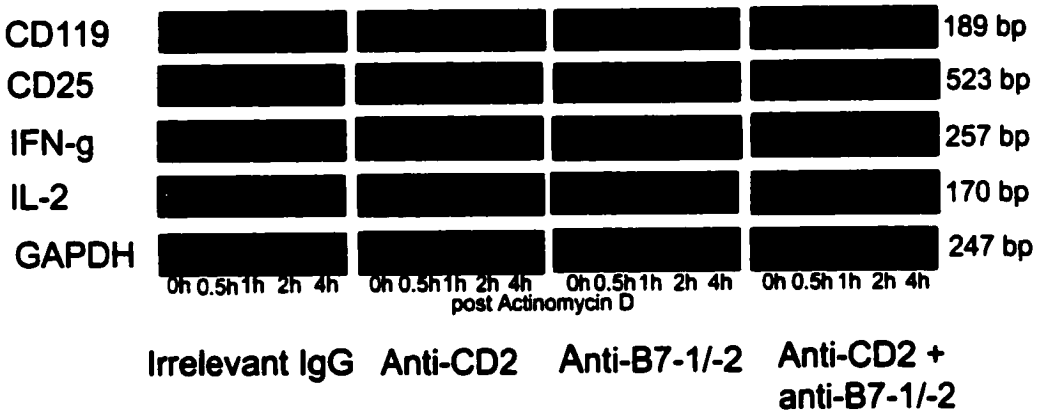

Anti-CD2 + anti-B7-1/B7-2 mAb
Anti-B7-1/B7-2 mAb
Anti-CD2 mAb
Irrelevant IgG
CD119
189 bp
CD25
523 bp
IFN-g
257 bp
IL-2
170 bp
GAPDH
247 bp

Figure 24. **CD2 blockade decreases IL-2 and IFN-gamma mRNA stability**
T cells were stimulated with anti-CD3 mAb in the absence or presence of the indicated mAbs. Following 12h of culture, actinomycin D (5 μg/ml) was added and total RNA was isolated at time points from 0h to 4h. IL-2, IFN-γ, CD25, CD119, and GAPDH mRNA levels were determined by semi-quantitative RT-PCR as described in *Methods*. Data are representative of three independent experiments.


CD119
CD25
IFN-g
IL-2
GAPDH
189 bp
523 bp
257 bp
170 bp
247 bp
0h 0.5h 1h 2h 4h
0h 0.5h 1h 2h 4h
0h 0.5h 1h 2h 4h
0h 0.5h 1h 2h 4h
post Actinomycin D
Irrelevant IgG
Anti-CD2
Anti-B7-1/-2
Anti-CD2 + anti-B7-1/-2

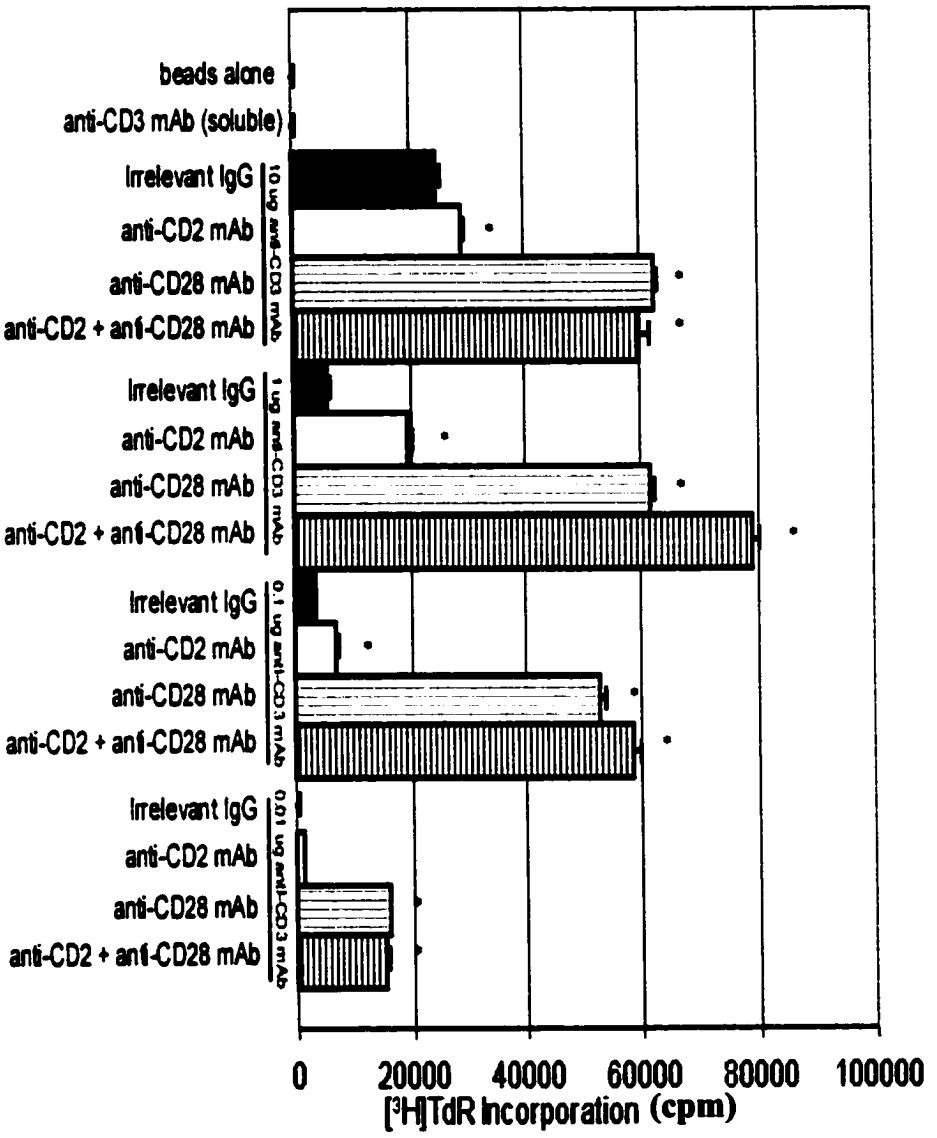
signaling yielded results which were similar to those obtained with blockade of either CD2 or CD28 alone. The stability of CD25 and CD119 mRNA was unaffected by blockade of CD2 or CD28 signaling.

3.21 CD2 signaling enhances T cell proliferation in response to sub-optimal stimulation with anti-CD3 mAb-coated microspheres

Following our examination of the effect of CD2 blockade on the induction of T cells, we next investigated the effect of CD2 costimulation via anti-CD3 and anti-CD2 mAb-coated microspheres on T cell activation, as reflected by proliferation assessed by tritiated thymidine incorporation at 48h of culture. T cell proliferation was increased in cultures containing anti-CD2 and anti-CD3 mAb-coated microspheres at all concentrations of anti-CD3 mAb employed (**Figure 25**). An increase in proliferation was also observed in cultures containing anti-CD3 and anti-CD28 mAb-coated microspheres. However, when 1 μg anti-CD3 mAb was used for coating, the increase in T cell proliferation by CD2 and CD28 signaling was most dramatic, enhancing T cell proliferation three-fold and ten-fold respectively over responses to microspheres coated only with anti-CD3 mAb. A combination of anti-CD2 mAb- and anti-CD28 mAb-coated microspheres resulted in an approximately additive enhancement of T cell proliferation. Based on this data (**Figure 25**), a concentration of 1 μg anti-CD3 mAb was used to coat microspheres for all further experiments. Both anti-CD2 and anti-CD28 mAb were used at 5 μg/ml to coat microspheres.

Figure 25. CD2 signaling enhances T cell proliferation
T cells were stimulated with bead-immobilized anti-CD3 mAb without or with bead-immobilized anti-CD2 (5 μg/ml) and/or anti-CD28 (5 μg/ml) mAb. Following 48h of culture, proliferation was assessed by [^{3}H]-TdR-incorporation as described in *Methods*. Data are shown as mean cpm of quadruplicate cultures +/- SD and are representative of three independent experiments. Statistical significance was determined by ANOVA and Bonferroni multiple comparison tests.

Culture Conditions



3.22 IL-2 and IFN-γ expression are augmented by CD2 signaling

We next examined the effect of CD2 signaling on T cell expression of mRNA coding for IL-2, IFN-γ, CD25 and CD119. T cells stimulated with anti-CD3 mAb-coated microspheres demonstrated low-level expression of IL-2 and IFN-γ mRNA, which was subsequently increased in T cells co-cultured with anti-CD3 and anti-CD2 mAb-coated microspheres (**Figure 26**). A marginally greater increase was observed in T cells treated with anti-CD3 and anti-CD28 mAb-coated microspheres, as well as with both anti-CD2 and anti-CD28 mAb in combination with anti-CD3 mAb. Interestingly, anti-CD3 and anti-CD2 mAb-coated microspheres did not increase CD25 mRNA expression but anti-CD3 and anti-CD28 mAb-coated microspheres did enhance CD25 mRNA levels in T cells. These data suggest that there is a difference in the means by which CD2 and CD28 signaling affect CD25 gene expression. CD119 mRNA levels were unaffected by the presence of anti-CD2 or anti-CD28 mAb on anti-CD3 mAb-coated microspheres.

3.23 IL-2 and IFN-γ mRNA half-life is enhanced by CD2 signaling

To further investigate the role of CD2 signaling in the stabilization of mRNA, T cells were stimulated with antibody-coated microspheres for 6h and then treated with actinomycin D. Replicate cultures were harvested from 0h to 4h after addition of actinomycin D and RNA was extracted for RT-PCR analysis. IL-2 and IFN-γ mRNA half-life is markedly prolonged by CD2 signaling in combination with TCR/CD3 triggering (**Figure 27**). A somewhat greater enhancement in mRNA stability is observed with TCR/CD3 and CD28 signaling in T cell cultures. However, combined CD2 and CD28 signaling plus TCR/CD3 signaling did not further enhance IL-2 or IFN-γ mRNA stability.

Figure 26. CD2 and CD28 signaling enhances expression of IL-2, IFN-gamma and CD25 mRNA by activated T cells

T cells were stimulated with bead-immobilized anti-CD3 mAb (1 μg/ml) without or with bead-immobilized anti-CD2 (5 μg/ml) and/or anti-CD28 (5 μg/ml) mAb. Following 12h of culture total RNA was isolated and IL-2, IFN-γ, CD25, CD119, and GAPDH mRNA levels were determined by semi-quantitative RT-PCR as described in *Methods*. Data are representative of three independent experiments.

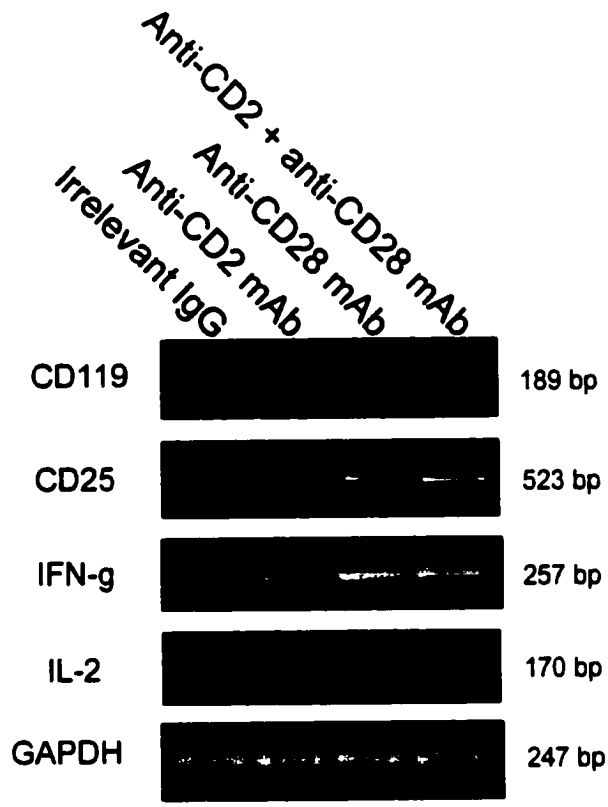
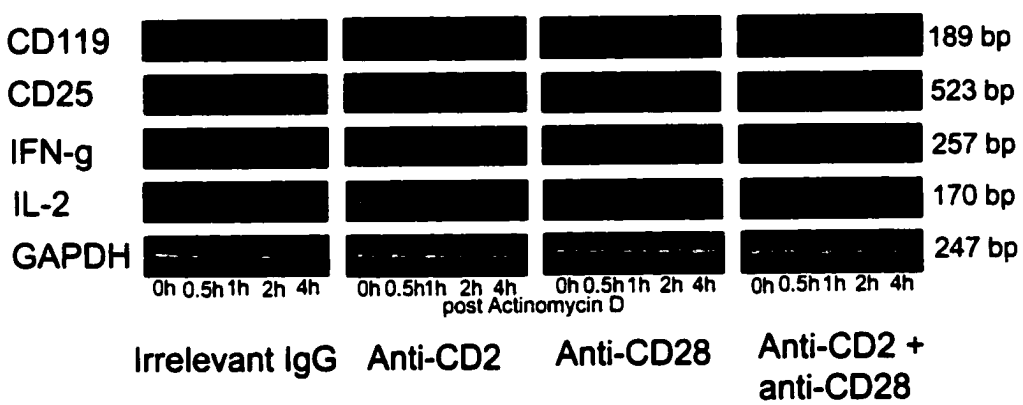

Anti-CD2 + anti-CD28 mAb
Anti-CD28 mAb
Anti-CD2 mAb
Irrelevant IgG
CD119
189 bp
CD25
523 bp
IFN-g
257 bp
IL-2
170 bp
GAPDH
247 bp

Figure 27. **IL-2 and IFN-gamma mRNA stability is enhanced by CD2 signaling** T cells were stimulated with bead-immobilized anti-CD3 mAb (1 μg/ml) without or with bead-immobilized anti-CD2 (5 μg/ml) and/or anti-CD28 (5 μg/ml) mAb. Following 12h of culture, actinomycin D (5 μg/ml) was added and total RNA was isolated at time points from 0h to 4h. IL-2, IFN-γ, CD25, CD119, and GAPDH mRNA levels were determined by semi-quantitative RT-PCR as described in *Methods*. Data are representative of three independent experiments.


CD119
CD25
IFN-g
IL-2
GAPDH
189 bp
523 bp
257 bp
170 bp
247 bp
0h 0.5h 1h 2h 4h
0h 0.5h 1h 2h 4h
0h 0.5h 1h 2h 4h
0h 0.5h 1h 2h 4h
post Actinomycin D
Irrelevant IgG
Anti-CD2
Anti-CD28
Anti-CD2 + anti-CD28

TCR/CD3 signaling plus CD2 or CD28 signaling, alone or in combination, did not enhance CD25 and CD119 mRNA stability.

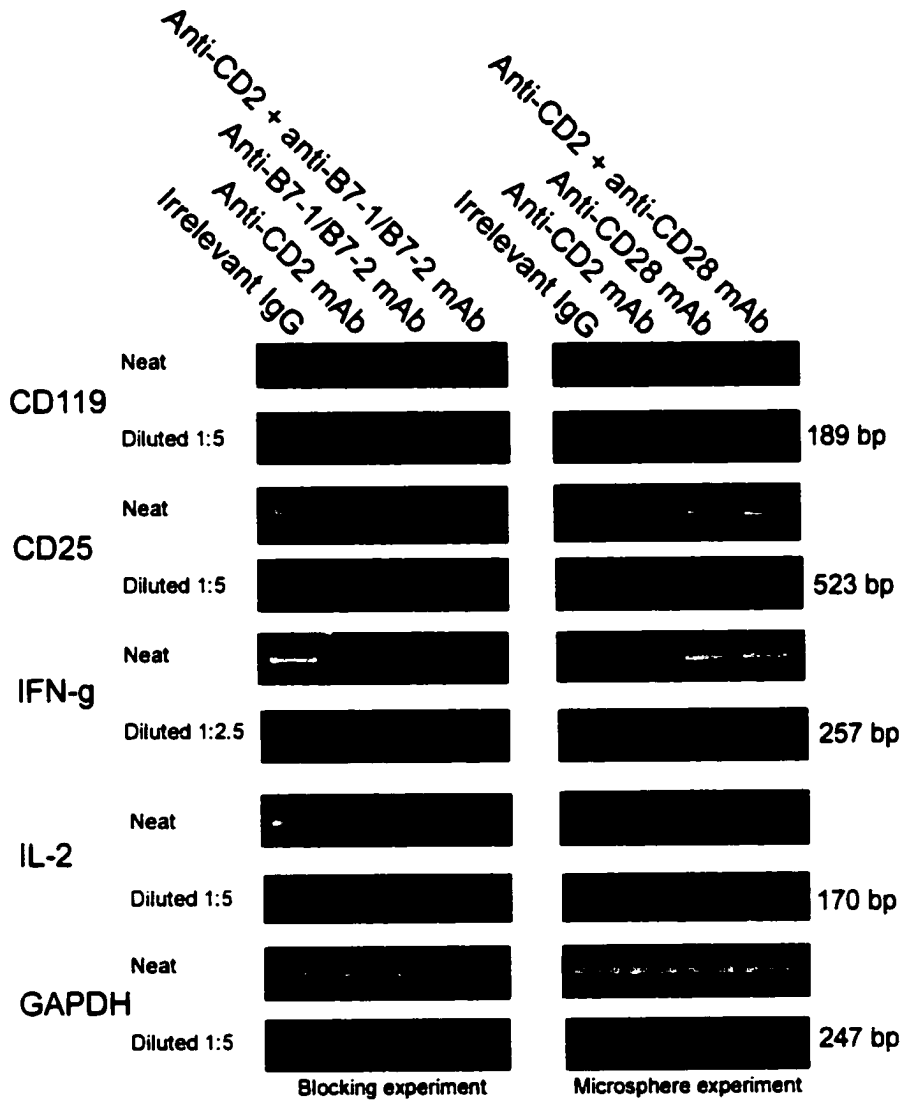
3.24 Dilution analysis of IL-2, IFN-γ, CD25, CD119, and GAPDH confirms RT-PCR results of neat cDNA samples was under non-saturating conditions

We next confirmed that the effects of anti-CD2 mAbs on T cell cytokine and cytokine receptor mRNA expression (**Figures 23** and **26**) were not an artifact of saturating concentrations of cDNA in the PCR. We diluted cDNA samples by 1:2.5 (IFN-γ) or 1:5 (IL-2, CD25, CD119, GAPDH) and repeated PCR. **Figure 28** shows that similar results were obtained when lower dilutions of mRNA were used for analysis of IL-2, IFN-γ, CD25, and CD119 mRNA expression, confirming that RT-PCR analysis was performed under non-saturating conditions. The observed effects of anti-CD2 mAbs are therefore not an artifact of RT-PCR analysis.

3.25 Determination of the appropriate number of PCR cycles for amplification of IL-2, IFN-γ, CD25, CD119, and GAPDH mRNA

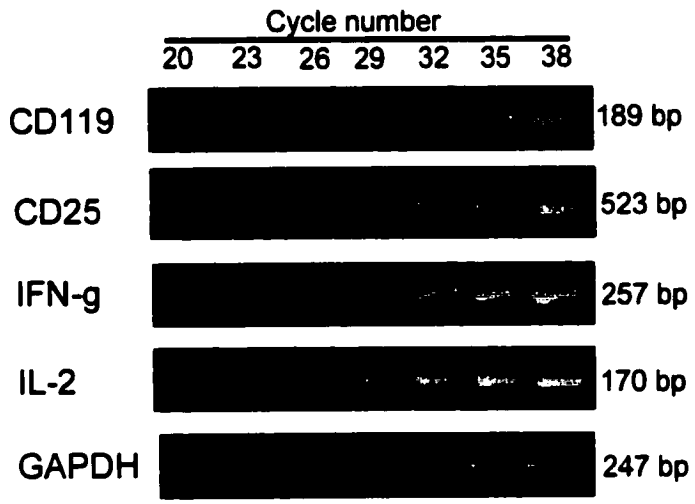
The number of PCR cycles selected for amplification of cDNA was determined based on the generation of a PCR product during the exponential phase of amplification. In order to determine the appropriate number of PCR cycles for determination of relative abundance of mRNA coding for IL-2, IFN-γ, CD25, CD119, and GAPDH, we investigated the amplification curve of each PCR product by examining a range of cycles from 20 to 38. The appropriate number of cycles was based on the number of cycles required to amplify a

Figure 28. **Dilution analysis of IL-2, IFN-γ, CD25, CD119, and GAPDH confirms RT-PCR of neat cDNA samples was under non-saturating conditions** T cells were stimulated with anti-CD3 mAb in the absence or presence of the indicated mAbs (Blocking experiment) or with bead-immobilized anti-CD3 mAb (1 μg/ml) without or with bead-immobilized anti-CD2 (5 μg/ml) and/or anti-CD28 (5 μg/ml) mAb (Microsphere experiment). Following 12h of culture, total RNA was isolated and neat cDNA samples and dilutions of 1:2.5 (IFN-γ) or 1:5 (all others) were subjected to PCR amplification of IL-2, IFN-γ, CD25, CD119, and GAPDH as described in *Methods*. Data are representative of three independent experiments.


Anti-CD2 + anti-B7-1/B7-2 mAb
Anti-B7-1/B7-2 mAb
Anti-CD2 mAb
Irrelevant IgG
Anti-CD2 + anti-CD28 mAb
Anti-CD28 mAb
Anti-CD2 mAb
Irrelevant IgG
CD119
Neat
Diluted 1:5
189 bp
CD25
Neat
Diluted 1:5
523 bp
IFN-g
Neat
Diluted 1:2.5
257 bp
IL-2
Neat
Diluted 1:5
170 bp
GAPDH
Neat
Diluted 1:5
247 bp
Blocking experiment
Microsphere experiment

product during the exponential phase without reaching a plateau of amplification. The appropriate number of cycles for amplification of IL-2, IFN-γ, CD25, CD119, and GAPDH was 28 (**Figure 29**).

Figure 29. Determination of the appropriate number of PCR cycles for amplification of IL-2, IFN-γ, CD25, CD119, and GAPDH mRNA
T cells were stimulated with anti-CD3 mAb for 48h of culture. Total RNA was isolated and cDNA samples were subjected to PCR amplification of IL-2, IFN-γ, CD25, CD119, and GAPDH for 20 to 38 cycles of amplification as described in *Methods*. Data are representative of three independent experiments.


Cycle number
20 23 26 29 32 35 38
CD119
189 bp
CD25
523 bp
IFN-g
257 bp
IL-2
170 bp
GAPDH
247 bp

4.0 DISCUSSION

Section I: CD2 signaling is required for non-specific cytotoxic T lymphocyte induction by anti-CD3 antibody

4.1 T cell activation and costimulation

During CTL induction, interactions of T cell costimulatory molecules with their respective ligands are critical for optimal induction of cytolytic activity to occur. In this regard, the well-characterized interaction of CD28 with B7-1 and B7-2 has been shown to costimulate both antigen-specific and non-specific CTL activation (Makrigiannis 1999; Greenfield 1998; Harris 1999). Although there is a considerable body of evidence regarding the many proposed functions of CD2, the exact role of CD2 in T cell activation is unknown. Initially, CD2 was found to function as an adhesion molecule since purified CD2 inhibits rosetting of T lymphocytes with sheep erythrocytes (Dustin *et al.* 1987b). Following this report, a series of studies demonstrated that anti-CD2 antibodies are able to induce antigen-independent T cell activation by an unknown mechanism, and that these antibodies result in the activation of CD2-associated intracellular signaling molecules in T cells following binding to CD2 (Hunig *et al.* 1987). Next, it was noted that antibodies blocking the interaction of CD2 with its ligand(s) prolong allograft survival *in vivo* (Chavin *et al.* 1992). This prolongation of allograft survival is due to T cell hyporesponsiveness induced by anti-CD2 antibodies.

CD2 signaling is also involved in T cell activation (Latchman and Reiser 1998; Li *et al.* 1996). This is consistent with the recent finding that CD2 is present in the immunological

synapse (Grakoui *et al.* 1999). CD2 signaling has been linked to the activity of several PTKs including ZAP-70, $p56^{lck}$ and $p59^{fyn}$ (Meinl *et al.* 2000; Hubert *et al.* 1996; Fukai *et al.* 2000). In addition, a recent study showed that $p62^{dok}$ tyrosine phosphorylation was increased by CD2 stimulation (Harriague *et al.* 2000). More recently, it was shown by several groups that CD2 appears to be responsible for regulating T cell responsiveness to cytokines such as IL-12, regulating the threshold for antigen-dependent T cell activation, and rescuing T cells from an anergic state (Gollob *et al.* 1995; Bachmann *et al.* 1999; Sasada and Reinherz 2001). However, the most convincing evidence to date of the importance of CD2 in T cell induction arose from a study on knockout mice which are deficient in both CD28 and CD2 (Green *et al.* 2000). In this important study, Green and colleagues clearly demonstrated that although mice deficient in either CD28 or CD2 display a nearly normal phenotype (Green *et al.* 1994; Shahinian *et al.* 1993; Killeen *et al.* 1992), mice deficient in both CD2 and CD28 are severely impaired in T cell function, underscoring the putative role of CD2 as a costimulatory molecule. These studies all suggest a costimulatory capacity for CD2. However, according to a model of costimulatory molecules proposed by Watts and DeBenedette (1999), CD2 still does not fulfill the criteria for being a true costimulatory molecule because there are no published reports of cytokine mRNA stability enhanced by CD2 signaling. The findings presented in this thesis indicate that CD2 displays the characteristics of a true costimulatory molecule, and thus, the current study elucidates the role of CD2 in T cell induction.

4.2 CD2 signaling in T cell activation

Following initial findings in dose-response experiments, the concentrations of anti-CD2 and anti-CD48 mAb required to achieve maximal inhibition of T cell proliferative and cytotoxic responses were determined to be 0.5μg/ml and 1.0μg/ml, respectively (Figures 1 and 2). In the present study, AK-T cells activated in the presence of anti-CD2 mAb exhibited greatly reduced levels of proliferation and cytotoxic activity against P815 murine mastocytoma cells. Inhibition of proliferation and cytotoxic activity was also observed under conditions in which CD2 interactions with CD48, the only known murine ligand of CD2, were blocked by anti-CD48 mAb. The effects of CD2 signaling blockade on T cell proliferation and cytotoxicity via anti-CD48 mAb are marginally less than those observed with anti-CD2 mAb. One possible explanation for this observation is that additional ligands for CD2 are present, and these ligands are able to compensate somewhat for blockade of CD48. This seems unlikely, however, considering the results from studies that have searched for additional ligands for CD2 in rodents (Brown *et al.* 1995). Although the low-affinity of CD2-CD48 interactions makes the detection of additional ligands more difficult, assays that are specifically designed for targeting low-affinity interactions between surface molecules have detected only CD48 as a CD2 ligand in rats (Brown *et al.* 1995). It is more likely that the anti-CD48 mAb used in this study bind murine CD48 with lower affinity than the anti-CD2 mAb bind CD2, thereby allowing residual CD2-CD48 interactions to occur in the presence of anti-CD48 mAb.

Lin et al. (Lin *et al.* 1995) found that differences in the ability of different anti-CD2 mAb to induce modulation of surface CD2 on T cells correlated with the ability of these mAb to inhibit proliferation. However, in our studies AK-T cell proliferation was strongly

inhibited despite the failure of anti-CD2 mAb to modulate CD2 from the T cell surface (Figure 11), indicating that CD2 modulation is not the mechanism for anti-CD2 mAb-mediated inhibition of T cell activation. Previous work in our lab (Butler, M.Sc. thesis 2000) has shown that inhibition of T cell activation may result from decreased CD28 expression on the surface of T cells. However, CD28 expression by T cells was unaffected by anti-CD2 mAb treatment, discounting the possibility that anti-CD2 mAb-mediated inhibition of T cell activation is the result of decreased CD28 expression (data not shown). In addition to participating in cell-cell adhesion through interactions with its ligand CD48, CD2 also possesses a cytoplasmic tail that can trigger a TCR-independent activation pathway in T cells (Meuer *et al.* 1984; Moingeon *et al.* 1989). This activation is mediated through Ca^{2+} mobilization and activation of PTKs such as $p56^{lck}$ and members of the mitogen-activated protein kinase (MAPK) superfamily (Danelian *et al.* 1992; Ueno *et al.* 2000), suggesting that CD2 plays a role in the early stages of TCR-mediated T cell activation.

4.3 Kinetics of $CD8^+$ T cell activation

In order to examine the nature of anti-CD2 mAb-mediated inhibition of T cell activation, we next examined whether blockade of CD2-CD48 interactions alters the kinetics of AK-T cell activation. As seen in Figure 7, peak proliferation was observed at 48h of culture in anti-CD2 mAb-treated cultures as well as control cultures, indicating that CD2-CD48 blockade does not alter the kinetics of AK-T cell activation. Because several recent reports have demonstrated differential responses of $CD4^+$ T cells and $CD8^+$ T cells to certain stimuli (Phu *et al.* 2001; Elloso and Scott 2001), we also examined the effects of CD2 blockade on $CD8^+$, as well as unfractionated AK-T cell cultures. Both

CD4-depleted and unfractionated AK-T cell cultures responded similarly to anti-CD2 mAb (Figure 3). Taken together, these data indicate that the observed effects on T cell activation and subsequent induction of cytotoxicity are not simply due to inhibited T helper cell activity, and suggest direct $CD8^+$ T cell-specific effects such as a lack of costimulation. This is interesting considering that $CD8^+$ CTL have been reported to become activated in the absence of $CD4^+$ T helper cells, and that T helper cell-independent activation of $CD8^+$ T cells is absolutely dependent on costimulatory signaling (Zhan *et al.* 2000). Taken together, these findings further support the importance of costimulatory signaling through molecules such as CD2 in immune activation.

4.4 Early requirements for T cell activation

Further examination of the mechanism of anti-CD2 mAb-mediated CTL inhibition revealed several intriguing pieces of information. We noted that CD2 signaling is required during the first 12h of anti-CD3 mAb-mediated T cell activation (Figure 6). Additionally, decreased tyrosine phosphorylation of several low molecular weight proteins as a result of CD2 blockade suggests that inhibition of T cell activation through blockade of CD2-CD48 interactions is the result of decreased PTK activity (Figure 4). It is also possible that an approximately 60 kDa protein seen in anti-CD2 mAb-treated AK-T cells at 60 min represents a dimer of the 30 kDa molecule that is absent in the same lane, but this has not been examined yet. Regardless, the observed decrease in tyrosine phosphorylation is in line with evidence for altered tyrosine phosphorylation of low molecular weight proteins following CD2 stimulation of human T cells which has been

obtained in past studies (Meinl *et al.* 2000). It is important to note that although the time points chosen for harvesting T cell lysates were 30 min and 60 min, other studies examining earlier time points (<10 min) have seen additional effects (Fukai *et al.* 2000; Harriague *et al.* 2000). In Figure 4, there is a distinct lack of noticeable phosphorylation in control and experimental lanes of 56, 59, and 70 kDa substrates which may correspond to p56lck, p59fyn, ZAP-70, respectively. Tyrosine phosphorylation of p59fyn has been reported to occur by 5 min and to be undetectable by 10 min post-stimulation (Fukai *et al.* 2000). Similarly, ZAP-70 and p56lck are phosphorylated by the 5 min time point (Harriague *et al.* 2000). Thus, it seems likely that 30 min and 60 min time points chosen in the present study were too late to detect differences in the phosphorylation state of p56lck, p59fyn, or ZAP-70, although the observed low molecular weight proteins whose tyrosine phosphorylation was affected by CD2 blockade may prove to be important once identified. Other studies have shown an altered tyrosine phosphorylation pattern resulting from CD2 signaling (Meinl *et al.* 2000), although only the ζ chain, p56lck, and p59fyn have been identified as candidates (Samelson *et al.* 1990; Danielian *et al.* 1992; Carmo *et al.* 1993). Although most signaling molecules that have been implicated in T cell activation are generally of higher molecular weight than those seen in Figure 4, possible candidates of the approximate size include members of the cyclin-dependent kinases (cdk). The kinase activity of cdk molecules is responsible for the transition from G1 phase to S phase of the cell cycle (Appleman *et al.* 2001). Thus, decreased cdk kinase activity could affect the ability of a T cell to successfully proceed through the cell cycle. Anti-CD2 mAb-treated CTL may arrest in cell cycle, possibly appearing as blast cells as noted in our studies (data not shown), but displaying reduced effector T cell function.

T cells found in the lung demonstrate several characteristics of recent activation but express low levels of IL-2R and CD2 and appear to be locked in G1 phase of the cell cycle (Strickland *et al.* 1996). Anergy generally entails the inability of T cells to produce IL-2 and proliferate in response to stimulation, usually resulting from a lack of costimulatory signaling. CD2 signaling is able to reverse an established anergic state in T cells and thus, blockade of CD2 signaling may lead to anergy in T cells (Boussiotis *et al.* 1994). To date, however, CD2 signaling has not been linked to the induction of anergy, but this aspect of the regulation of T cell costimulation through CD2 remains to be explored. Interestingly, adding back IL-2 restores CTL activity in anti-CD2 mAb-treated AK-T cell cultures (Figure 16). IL-2 signaling is required for cdk2 activation, and cdk2 is required for the accumulation of CD25 in TCR-stimulated T cells (Mohapatra and Pledger 2001), further suggesting that the effects of CD2 blockade may be related to decreased IL-2 production by T cells, perhaps indirectly due to reduced cdk2 activation.

One aspect which the current study failed to address is the possible effects of anti-CD2 mAb cross-linking on p27Kip1 expression and/or activity. p27Kip1 is a cdk-inhibitor that associates with the c-jun co-activator JAB1, inhibiting AP-1 activation and subsequently IL-2 gene transcription (Boussiotis *et al.* 2000). T cells normally enter S phase approximately 24h after TCR-stimulation and express little p27Kip1. However, in anergic T cells, p27Kip1 is expressed at a much higher level at 24 h post-stimulation (Jackson *et al.* 2001). If anti-CD2 mAb increased p27Kip1 expression in T cells, this could prevent cdk-mediated entry into the S phase of the cell cycle and maintain a non-responsive state. Costimulation through CD28 inhibits p27kip1 accumulation and thus facilitates the cdk-mediated transition into S phase (Appleman *et al.* 2001). It is not

currently known whether CD2 costimulation exhibits similar regulation of p27kip1 expression. Interestingly, rapamycin, an immunosuppressive macrolide derived from *Streptomyces* sp. commonly used to prevent T cell-mediated allograft rejection, appears to function in precisely this manner, terminating cell cycle progression at G1 phase (Seghal *et al.* 1975; Terada *et al.* 1995). This is accomplished via inhibiting the decrease in p27Kip1 expression, resulting in inhibition of cdk2 kinase activity, a crucial regulator of the G1 phase to S phase transition (Dumont and Su 1996). If anti-CD2 mAb function in a similar manner as rapamycin in inhibiting T cell progression from the G1 phase, this would further emphasize the therapeutic potential of anti-CD2 mAb in transplantation.

Activation of PKC by PMA and release of Ca^{2+} from intracellular stores triggered by ionomycin bypass the requirement for TCR stimulation in the activation of T cells (Zhang *et al.* 1999; Dedkova *et al.* 2000). Further supporting the importance of PTK activity in CD2 signaling, anti-CD2 mAb fail to inhibit T cell activation by combinations of PMA and ionomycin or PMA and anti-CD3 mAb (Figure 5). CD2 blockade therefore affects T cell receptor signaling upstream of PKC activation and Ca^{2+} mobilization. Altogether, although the precise signaling pathway utilized by CD2 is not yet known, our data implicates early signaling events in CD2-mediated costimulation, more specifically, during the first 12h of T cell activation. We also provide further evidence for the importance of the activation of intracellular signaling molecules through CD2 since anti-CD2 mAb result in reduced phosphorylation of tyrosine residues of several proteins. This data underscores the relevance of CD2 costimulatory function in T cell activation through signaling rather than simply as an adhesion molecule responsible for binding APC and target cells.

4.5 Enhanced cytokine production by CD2 signaling

Also notable was the dramatic decrease in IL-2 and IFN-γ production (Table 2), along with the decrease in expression of CD25 (Figure 11), a component of the high-affinity IL-2 receptor, which was observed when T cells were activated in the presence of anti-CD2 mAb. This data contrasts with an earlier study which showed that LFA-3 (the ligand for CD2 in humans) costimulation induced strong IFN-γ production but little IL-2 synthesis in SEA-triggered $CD8^+$ T cells (Parra *et al.* 1997). Thus, depending on the stimuli, costimulation through CD2 may mediate differential effects on T cells. T cells would also be rendered less responsive to IL-2 through decreased CD25 expression, and combined with decreased IL-2 production, it is therefore not surprising that the proliferative response of anti-CD2 mAb-treated T cells is greatly reduced (Figure 1A). Surprisingly, anti-CD2 treated T cells showed a slight upregulation of CD119, the α subunit of the IFN-γ receptor (Figure 11). This may be a means by which the T cell attempts to compensate for reduced IFN-γ synthesis and/or IL-2 bioactivity. Interestingly, although CD119 expression is upregulated, T cell responsiveness to exogenous IFN-γ is less than that of exogenous IL-2 (Figure 18), suggesting that IFN-γR signaling may not be intact following anti-CD2 mAb treatment in spite of increased surface expression of the IFN-γR. A second explanation for the apparent increase in CD119 expression by anti-CD2 mAb-treated T cells may be due to the internalization of the IFN-γR following binding of IFN-γ. Decreased IFN-γ present in anti-CD2 mAb-treated T cell cultures would result in reduced IFN-γ bound to IFN-γR with a corresponding decrease in the modulation of IFN-γR from the surface of T cells, since the IFN-γR is internalized following binding of IFN-γ (Bach *et al.* 1997). The observation that T cell CD119 mRNA

levels were unaffected by anti-CD2 mAb treatment are consistent with this possibility (Figures 23 and 26).

The addition of either IL-2 or IFN-γ alone to culture partially restored normal proliferative and cytotoxic responses to AK-T cells induced in the presence of either anti-CD2 mAb or anti-CD48 mAb while a combination of IL-2 and IFN-γ completely restored CTL function (Figures 16 and 17). In contrast, conjugation of AK-T cells to P815 target cells was restored only by addition of exogenous IL-2 but not IFN-γ in both anti-CD2 mAb- and anti-CD48 mAb-treated AK-T cells (Figures 18 and 19). Despite the cytokine-dependent nature of anti-CD2 mAb-mediated inhibition of T cell activation, anti-CD2 mAb did not inhibit the proliferative response of the IL-2-dependent T cell line, CTLL-2 (Figure 15). Moreover, combined with the finding that anti-CD2 mAb-mediated inhibition of AK-T cells is abrogated by adding back IL-2 (Figure 16), these data indicate that IL-2R signaling is intact in activated T cells following anti-CD2 mAb treatment. Because anti-CD2 mAb had no effect on CTLL-2 cell proliferation and the addition of exogenous IL-2 restores normal AK-T cell function in the presence of anti-CD2 mAb, we propose that anti-CD2 mAb-mediated inhibition of T cell activation is due to decreased IL-2 production in T cell cultures.

4.6 CD2 signaling and the induction of T cell cytotoxicity

Effector CTL induce apoptosis in target cells via both granule-dependent (granzyme B and perforin) and granule-independent (FasL and TRAIL) cytotoxic pathways (Kagi *et al.* 1994; Nagata and Golstein 1995; Mariani and Krammer 1998a). In this study, RT-PCR analysis revealed that anti-CD2 mAb-treated T cells express normal

levels of TRAIL and FasL mRNA, but exhibit reduced levels of perforin and granzyme B mRNA and protein. Taken together, these data imply that granzyme B and perforin expression in AK-T cells is CD2-dependent whereas FasL and TRAIL induction require only TCR signaling. Interestingly, resting T cells isolated from mice expressed substantial levels of perforin mRNA and protein, but this expression was absent from T cells treated with anti-CD2 mAb. The inhibitory effects of CD2 blockade on AK-T cell proliferation and cytotoxic activity were almost completely abrogated by the addition of exogenous IL-2 in combination with IFN-γ, indicating that the observed inhibitory effects are most likely cytokine-dependent. Furthermore, the restoration of granzyme B and perforin expression by the addition of exogenous IL-2 and IFN-γ to anti-CD2 mAb treated AK-T cell cultures is consistent with the cytokine-dependent nature of the granule-associated cytolytic machinery (Liu *et al.* 1989; Fitzpatrick *et al.* 1996).

Our findings are in marked contrast to a recent study by Sasada and Reinherz which suggested that CD2 deficiency fails to alter CTL effector function (Sasada and Reinherz 2001). However, it is important to denote that these investigators induced CTL from CD2-deficient mice in the presence of conditioned medium. Since our findings indicate that addback of IL-2 and IFN-γ abrogates the inhibitory effects of CD2-CD48 blockade on CTL induction (Figure 16), it is not surprising that CTL effector function from CD2-deficient mice would appear normal when CTL are generated in the presence of cytokine-rich conditioned medium. Although CD25 expression was downregulated following treatment of AK-T cells with anti-CD2 mAb (Figure 11), CD25-associated signaling mechanisms remained functional since treatment of the IL-2-dependent CTLL-2 cell line with anti-CD2 mAb had no effect on IL-2-driven proliferation (Figure 15).

Nevertheless, combined with the reduction in IL-2 production, reduced CD25 expression would be expected to severely impair the ability of T cells to develop into cytotoxic effector cells. Decreased CD25 expression occurs primarily for two reasons. Since CD25 expression is IL-2-dependent (Demaison *et al.* 1996), decreased IL-2 production observed in anti-CD2 mAb-treated T cell cultures would be expected to result in decreased CD25 expression. Also, TCR triggering in combination with costimulatory signaling is known to enhance CD25 expression, along with IL-2 production (Conlon *et al.* 1992). In light of the data presented in this study which favors a role for CD2 as a costimulatory molecule, it would be predicted that CD2 blockade would result in decreased CD25 expression on T cells.

4.7 Negative signaling through CD2

CD2 is a well-documented adhesion molecule that can mediate adhesion to cells bearing CD48, a molecule widely expressed on lymphocytes, macrophages, and dendritic cells (Moingeon *et al.* 1989; Davis and van der Merwe 1996). If the sole means by which anti-CD2 mAb inhibit P815 killing by AK-T cells was through blocking CD2-CD48 interactions during the induction phase of cytotoxicity, we would expect that the inhibition seen in both anti-CD2 mAb and anti-CD48 mAb treated AK-T cell cultures would be similar. However, as seen in Figures 1 and 2, that is not the case. An additional inhibitory effect was observed in anti-CD2 mAb treated AK-T cell cultures compared to anti-CD48 mAb treated cultures. Thus, it appears that activation of an additional negative regulatory pathway may occur following CD2 crosslinking by mAb in addition to a lack of costimulation caused by blocking CD2 and CD48 contact. In addition to participating in

cell-cell adhesion through interactions with its ligand CD48, CD2 also possesses a cytoplasmic tail that can trigger a TCR-independent activation pathway in T cells (Meuer *et al.* 1984; Moingeon *et al.* 1989), indicating that signaling molecules are associated with CD2. This finding is consistent with the idea that CD2 is able to activate a negative signaling pathway in T cells.

Although signaling through costimulatory surface proteins is required for T cell activation, a balance of opposing effects must be achieved to regulate activation through the synthesis of inhibitory proteins leading to inhibition of T cell responses or induction of apoptosis (Bluestone *et al.* 1997). Although many costimulatory proteins are known, only a handful of inhibitory proteins have been documented. These include the killer inhibitory receptors for HLA class I and CD94-NKG2A normally present on NK cells and some T cells (Ciccone *et al.* 1996), and CTLA-4 (CD152) normally present on T cells (Bluestone *et al.* 1997). Negative signaling by killer cell inhibitory receptors is dependent on the protein tyrosine phosphatase SHP-1 that has been shown to target SLP-76 in T cells and NK cells (Binstadt *et al.* 1998). Tyrosine-phosphorylated SLP-76 is required for optimal activation of cytotoxic T cells. CTLA-4 acts to negatively regulate T cell activation by activating a phosphatase that dephosphorylates molecules of the TCR-CD3 activation signaling pathway (Walunas *et al.* 1994). It has also been suggested that engagement of CTLA-4 halts progression through the cell cycle (Brunner *et al.* 1999).

The data from our current study strongly suggests that in addition to providing costimulation, CD2 is also able to activate an as yet undefined negative signaling pathway following crosslinking by certain anti-CD2 mAb. Downregulation of baseline perforin expression in anti-CD2 mAb treated AK-T cell cultures indicates that mAb-

crosslinked CD2 may transduce a negative regulatory signal that is separate and distinct from its costimulatory functions (Figures 12A and 13). Additionally, expression of perforin was downregulated substantially in T cell cultures containing only anti-CD2 mAb in the absence of activating mAb such as anti-CD3 mAb (Figure 14), further suggesting that CD2 possesses the capacity for negative regulation of T cell effector function independently of the TCR. It is important to note that two earlier studies have also suggested the presence of a negative signaling pathway through CD2 in rodents (Ohno *et al.* 1991; Sido *et al.* 1996). Our current data is consistent with this idea. A report by Li and associates demonstrated the coupling of a phosphatase (PEST) to CD2, which may provide a mechanism by which negative signaling is activated (Li *et al.* 1998). Also, CD45 is associated with CD2 (Schraven *et al.* 1990) and phosphatase activity of CD45 may be partially responsible for the putative negative signal induced by anti-CD2 mAb. There is also evidence that an alternative function of CD2 is to fine tune an immune response by regulating the responsiveness of T cells to antigen and also regulating T cell anergy (Bachmann *et al.* 1999; Boussiotis *et al.* 1994). Thus, it seems likely that CD2 plays a somewhat unique multi-faceted role in T cell activation due to its capacity both as a costimulatory molecule and a regulatory molecule.

4.8 T cell adhesion to target cells

CD11a and CD54 are important adhesion molecules that have been implicated in effector T cell function (Wang and Lenardo 1997). The observed decrease in AK-T cell conjugation to target cells following anti-CD3 mAb-mediated induction in the presence of anti-CD2 or anti-CD48 mAb cannot be explained simply by blockade of CD2-CD48

adhesion by residual mAb since directly blocking CD2-CD48 adhesion in cytotoxicity assays has only a minor inhibitory effect on AK-T cell conjugation to target cells. Anti-CD48 mAb do not affect expression of cytolytic molecules in AK-T cells (Figure 12B). Together with the observation that conjugation is markedly inhibited by anti-CD2 mAb (Table 1), this finding implies that the effect of anti-CD48 mAb on AK-T cell function is primarily at the level of T cell-target cell binding while anti-CD2 mAb affect both effector molecule expression and target cell binding. Hence, anti-CD2 mAb is more effective at inhibiting effector T cell function than anti-CD48 mAb. Moreover, we show here that CD2 signaling has little or no effect on CD11a and CD54 expression on AK-T cells. Although CD11a expression is largely constitutive, CD54 expression has been shown to be dependent on TCR signaling (Roebuck and Finnegan 1999; Dustin and Springer 1991). LFA-1 affinity for ICAM-1 is upregulated by increasing the affinity of the individual LFA-1 molecules whereas LFA-1 binding avidity is increased through clustering of LFA-1 molecules which occurs independently of LFA-1 expression (Gahmberg *et al.* 1997; Krauss *et al.* 1999). Thus, the reduction in AK-T cell conjugation to target cells observed when the effector cells were induced in the presence of anti-CD2 or anti-CD48 mAb may result from a failure to induce a conformational shift of individual LFA-1 molecules to the high affinity conformation or from a failure of LFA-1 molecules to cluster.

Unstimulated T cells express only the low-affinity form of LFA-1 (Marlin and Springer 1987). The shift to high affinity LFA-1 occurs rapidly following TCR triggering (Dustin and Springer 1989) and increased LFA-1 clustering and binding avidity is observed following treatment of T cells with phorbol ester (Stewart *et al.* 1998). Membrane rafts are also required for increased LFA-1 avidity for ICAM-1. Accordingly, LFA-1-mediated

binding of T cells to ICAM-1 can be rapidly induced by antibody-mediated clustering of membrane rafts, supporting the importance of rafts in T cell adhesion. A requirement for CD2 signaling for raft formation might, therefore, explain decreased target cell binding by AK-T cells induced under conditions of CD2 or CD48 blockade. Interestingly, addition of exogenous IL-2, but not IFN-γ, to AK-T cell cultures containing anti-CD2 mAb restores subsequent conjugation of AK-T cells to P815 target cells. This implies that IL-2 may increase the affinity and/or avidity of LFA-1 for ICAM-1. Note that LFA-1 and ICAM-1 expression were largely unaffected by CD2 blockade. Consequently, signaling through the IL-2 receptor may be involved in activating LFA-1 to a high affinity state for ICAM-1 or inducing LFA-1 clustering with an increase in binding avidity for ICAM-1. At present, the intracellular signaling pathways involved in the reorganization of LFA-1 into high-avidity clusters on the surface of T cells are not clear. It appears that the distribution of LFA-1 on unstimulated T cells is uniform over the entire cell surface, with LFA-1 maintained in its low affinity form anchored to the actin cytoskeleton (Petruzzelli *et al.* 1998; van Kooyk *et al.* 1999). Cleavage of the actin-anchor releases LFA-1 and allows it to form clusters (van Kooyk *et al.* 1999; Stewart *et al.* 1998). A change in conformation of LFA-1 results in its higher affinity for ICAM-1, and this conformational change has been shown to be induced by Mg^{2+} (Dransfield *et al.* 1992). There is also evidence that soluble lipids are involved in the induction of the high-affinity form of LFA-1 (Lee *et al.* 1994). Considering the importance of IL-2 in T cell activation, it is reasonable to suggest that IL-2R signaling may be involved in altering the binding capacity of LFA-1 for ICAM-1. The involvement of CD2 in the shift to high-affinity/avidity LFA-1 may, therefore, result from enhanced IL-2

production by T cells or raft formation and increased LFA-1 clustering via CD2 costimulatory signaling.

4.9 2B4 on T cells

2B4, a member of the Ig superfamily, is a newly described ligand for CD48, which is the only known murine ligand of CD2 (Brown *et al.* 1998; Latchman and Reiser 1998). 2B4 is a cell surface glycoprotein that is similar in size to CD2 (65 vs. 50 kDa), and as such could be a potential target for anti-CD2 mAb. 2B4 has been shown to function as an activating receptor molecule that is known to be expressed on NK cells (Nakajima and Colonna 2000). A concern is that 2B4, rather than CD2, could be the origin of the putative negative signal, or that the observed effects of anti-CD2 mAb could be due to the mAb cross-reacting with 2B4. Although there has been no evidence of negative signaling properties of 2B4 to date, this is an issue that needed to be addressed. RT-PCR with 2B4-specific primers was, therefore, performed on mRNA from AK-T cell cultures, as well as LAK cell cultures (as a positive control). Although LAK cell cultures demonstrated abundant expression of 2B4 mRNA, no 2B4 mRNA was detectable in AK-T cell cultures (Figure 21), thus confirming the specificity of the effects of anti-CD2 mAbs and discounting a role for 2B4 in the observed results.

Section II: Signaling through CD2 enhances IL-2 and IFN-γ mRNA stability in T lymphocytes: Evidence that CD2 meets criteria for a costimulatory molecule

4.10 CD2 and CD28 in T cell costimulation

It is well established that CD28 plays a major role in the costimulation of T cell activation (June *et al.* 1994). However, CD28 deficient mice are still capable of generating an effective immune response (Green *et al.* 1994; Shahinian *et al.* 1993), suggesting that other costimulatory molecules may be able to substitute for CD28. One such molecule is CD2. This possibility is supported by data from experiments with CD2 and CD28 knockout mice which demonstrate only slight defects in T cell activation when either molecule is absent (Green *et al.* 1994; Shahinian *et al.* 1993; Killeen *et al.* 1992). In contrast, a combined deficiency in both CD2 and CD28 results in profoundly deficient T cell activation (Green *et al.* 2000), supporting the hypothesis that combined CD2 and CD28 signaling is required for optimal T cell activation. The importance of combined CD2 and CD28 signaling is also evident in a study which demonstrated that combined blockade of CD2 and CD28 with anti-CD2 mAb and CTLA4Ig induced hyporesponsiveness and tolerance to cardiac allografts in mice (Woodward *et al.* 1996).

In this study we have compared the importance of CD2 and CD28 costimulation for mouse T cell activation and cytokine synthesis by using specific mAb to block the interactions of these costimulatory molecules and their ligands, as well as comparing the capacity of microspheres coated with anti-CD3 and anti-CD2 or anti-CD28 mAb to activate mouse T cells. We have shown that blockade of CD2-CD48 with anti-CD2 mAb interactions results in an inhibition of T cell activation with a substantial reduction in IL-

2, IFN-γ, and CD25 mRNA expression (Figures 22 and 23). In contrast, CD119 mRNA expression was unaffected by CD2 blockade. Highly purified APC-depleted T cells were also stimulated with bead-immobilized anti-CD3 and anti-CD2 mAb (Figures 25-27). This approach was used to mimic TCR and CD2 engagement by self MHC/antigen and CD48, respectively, on APC. Under these conditions, costimulation through CD2 results in greatly enhanced T cell proliferation, along with increased expression of IL-2 and IFN-γ mRNA. Costimulation was not due to APC contamination since highly purified T cells did not proliferate in response to soluble anti-CD3 mAb, indicating an absence of FcR^+ APC, which are required for T cell activation by crosslinking soluble anti-CD3 mAb. Although the effects of CD28 signaling on T cell activation were marginally greater than CD2 signaling, our data strongly denotes an important role for CD2 signaling in the optimal activation of T cells. This is most evident in Figure 22, where blocking CD2 signaling alone resulted in less than 20% of T cell proliferation compared to control T cell cultures.

4.11 Stabilization of cytokine mRNA via costimulation

Although CD2 signaling has been linked to increased synthesis of cytokines (Rosenthal-Allieri *et al.* 1995), to our knowledge, no data has yet implicated CD2 in the stabilization of cytokine mRNA. Crosslinking of CD2 by certain combinations of monoclonal antibodies triggers T cell activation, as well as cytokine secretion *in vitro* (Rosenthal-Allieri *et al.* 1995; Gonsky *et al.* 2000). Our data indicate an additional role for CD2 signaling in the stabilization of IL-2 and IFN-γ mRNA (Figures 24 and 27). Stabilization of IL-2 and IFN-γ mRNA likely facilitates the increase which was observed

in CD2 costimulated T cell cultures (Figure 26). Although decreased CD25 mRNA expression was also observed in T cell cultures induced in the presence of blocking antibody to CD2 (Figure 23) and increased CD25 mRNA expression was detected in CD2 costimulated T cell cultures, no stabilization of CD25 mRNA by costimulation through CD2 was detected. This data suggests that in addition to the effects on cytokine mRNA stability, costimulation through CD2 augments the rate of transcription of CD25. The effects of CD2 costimulation are similar to, though marginally less than those of CD28 signaling which is well known to prolong the half-life of cytokine mRNA including IL-2 and IFN-γ (Lindstein *et al.* 1989).

Control of mRNA stability is critical for immune regulation and, accordingly, some of the genes that are most labile include transcription factors and cytokines (Caput *et al.* 1986). A recent study demonstrated that CD2 costimulation stabilizes CD40L mRNA, thereby increasing CD40L protein expression on newly activated $CD4^+$ T cells (Murakami *et al.* 1999). CD28 signaling has also been shown to stabilize CD40L mRNA (Klaus *et al.* 1994). These data provide further evidence of the importance of CD2 in the regulation of T cell activation, and also make evident the similarities between CD2 and CD28. Although the precise mechanism of mRNA stabilization by costimulatory molecules such as CD28 and CD2 is not yet known, one hypothesis that is gaining acceptance involves altered protein binding to AU-rich elements in mRNA 3'untranslanted regions due to costimulatory signaling (Caput *et al.* 1986; Shaw *et al.* 1986).

It has been suggested that through selective activation of transcription factors, CD2 signaling is likely responsible for low-level production of IL-2, allowing for

autocrine T cell induction, whereas CD28 signaling is responsible for high-level production of IL-2, allowing for paracrine activation of other immune cells and long lasting T cell proliferation (Parra *et al.*1997). Our data supports this hypothesis since IL-2 and IFN-γ mRNA expression by T cells which received costimulation through CD2 was somewhat weaker than that observed following costimulation through CD28 (Figure 23 and 26). One group of investigators have reported that although CD2 stimulation can induce proliferation of naïve T cells, little IL-2 is produced by T cells stimulated with immobilized anti-CD3 and anti-CD2 mAb (Yashiro *et al.* 1998). In contrast, we observed an increase in IL-2 and IFN-γ mRNA transcription following CD2 costimulation (Figure 26). Moreover, a substantial decrease in IL-2 protein synthesis resulted from CD2 blockade (Table 2), as well as a decrease in IL-2 gene transcription which was comparable to the effect of blocking CD28 costimulation. It is noteworthy that Yashiro *et al.* stimulated T cells with plate-bound anti-CD3 and anti-CD2 mAb whereas in our study, stimulation was achieved with bead-immobilized anti-CD3 and anti-CD2 mAb. The apparent discrepancy in the effectiveness of CD2 costimulation may be the result of bead-immobilized antibody being a more effective means of stimulation since the beads better approximate the size and shape of APC compared to a flat plate. Since CD2 costimulation is marginally less potent than that of CD28, it is not surprising that seemingly minor differences may lead to substantial differences in the ability of T cells to respond to costimulation through CD2, especially considering that a postulated role for CD2 in T cell activation is setting the threshold required for full T cell activation (Bachmann *et al.* 1999).

4.12 Semi-quantitative RT-PCR

Because of the significance of RT-PCR results in the conclusions of this study, we felt that it was important to address several issues surrounding the semi-quantitative nature of this procedure. Used simply as a means of detecting the presence or absence mRNA for a given gene, PCR is arguably the most simple and effective means of detection. The number of PCR cycles selected for amplification of cDNA is determined based on the generation of a PCR product during the exponential phase. RT-PCR performed under these conditions allows for semi-quantitative analysis of mRNA levels if the difference is twofold or greater (Singer-Sam *et al.* 1990). However, when performing RT-PCR for the purpose of determining the relative expression of a given gene in various samples, it is critical to ensure that the appropriate controls are performed. As such, we sought to determine the appropriate number of PCR cycles for examining the relative abundance of mRNA coding for IL-2, IFN-γ, CD25, CD119, and GAPDH. By plotting the amplification curve of each PCR product through a range of cycles from 20 to 38, we were able to determine the appropriate number of cycles required to achieve a linear expansion of the PCR product without reaching a plateau of amplification, allowing termination of the reactions before reagents become limiting (Figure 29). In addition, through dilution analysis of cDNA samples, we confirmed that the effects of anti-CD2 mAbs on AK-T cell cytokine and cytokine receptor mRNA expression (Figures 23 and 26) were not an artifact of saturating concentrations of cDNA in the PCR. Figure 28 shows that the relative mRNA expression was similar in neat and diluted cDNA, indicating that the observed effects of the mAbs were not artifact of RT-

PCR analysis. Together, these controls increase the confidence in the data that are presented in this study.

Additionally, because RT-PCR is a sensitive means of detection of mRNA species, to further control for experimental variability we always examined the quality of RNA by gel electrophoresis. Degraded or poor quality mRNA would likely reduce the efficiency of RT reactions, and thus limit the effectiveness of the PCR reactions and subsequently introduce extra variability into our results. Because minute differences in quantities of RT-PCR reagents could also reduce reproducibility of results, we limited the variability by using a "master-mix" containing all required reagents (with the exception of individual mRNA samples) which was then aliquotted to each PCR reaction tube. Furthermore, in each PCR reaction, we included a sample containing the master mix and only pyrogen-free water to confirm the absence of any contaminating DNA. In all cases, no product was amplified in these control tubes, providing strong evidence that the observed products in other samples were the result of amplified T cell mRNA rather than contaminants. Furthermore, PCR primers were designed to span an intron of genomic DNA to prevent amplification of genomic DNA. By ascertaining the molecular size of the amplicons and checking it against the expected size of amplified mRNA versus amplified genomic DNA, we are confident that the observed amplicons represent mRNA rather than DNA. Finally, once the amplicons were resolved by gel electrophoresis, relative abundance of PCR products was determined by densitometric analysis of gel scans, allowing for an objective examination of the results. Taken together, these controls validate the effectiveness of the RT-PCR reactions for determining the relative abundance of mRNA species in different AK-T cell cultures.

Ideally, one would confirm the results obtained through RT-PCR by examining mRNA levels via northern blotting or RNAse Protection assays (RPA), or by introducing a competitive species of cDNA during PCR. However, previous attempts at measuring perforin and FasL mRNA expression by northern blotting were unsuccessful in our lab due to the low abundance of these transcripts in AK-T cells (A. Makrigiannis, personal communication). Furthermore, RT-PCR is a simple and effective technique that does not require the use of isotopes or relatively expensive reagents. In the case of the data presented in this study, changes in IL-2 and IFN-γ expression determined by RT-PCR were also confirmed via ELISA (Table 2, Figures 10 and 23). Western blotting was used to confirm RT-PCR data for granzyme B and perforin expression in AK- T cells, with similar results obtained by both procedures (Figures 12A and 13). Because protein measurements confirmed the RT-PCR results, we are confident that our RT-PCR data is a valid representation of gene expression in T cells activated under the experimental conditions described in this study. In conclusion, although RT-PCR is not a quantitative means of examining mRNA, it is nevertheless useful for semi-quantitative examination if the appropriate controls are in place and the pitfalls of this technique are recognized.

4.13 General conclusions

Based on the data presented in this study, one can conclude that the role of CD2 in T cell activation, in addition to regulating anergy and antigen-responsiveness in T cells, involves early costimulatory signaling events that serve to enhance TCR-mediated stimulation. The effect of CD2 blockade seems to inhibit T cell activation primarily in a cytokine-dependent fashion, as seen by decreased IL-2 and IFN-γ mRNA expression and

protein synthesis following CD2 blockade. This is confirmed by the restoration of AK-T cell effector function by cytokine addback to AK-T cell cultures. Although the precise signaling pathways activated by CD2-ligation are not yet known, the data in the present study emphasize the requirement for CD2-mediated costimulation in antigen non-specific AK-T cell induction and further support the role of CD2 as a costimulatory molecule.

Based on the data presented in this study, the significance of CD2 in T cell activation is comparable to that of CD28. The individual roles of CD2 and CD28 may overlap to some extent, especially considering that CD2 and CD28 have the ability to activate many similar signaling molecules. However, the capacity of CD2 and CD28 to activate these molecules is also somewhat different (Hutchcroft *et al.* 1998). The recent development of $CD2^{-/-}CD28^{-/-}$ mice will likely provide a means to elucidate the combined role of CD2 and CD28 in T cell activation and, by inference, also provide further information concerning the role of CD2 in this process. Our data is the first evidence, to our knowledge, that CD2 is able to enhance the stability of cytokine mRNA transcripts. This finding emphasizes the importance of CD2 signaling in T cell activation since IL-2 and IFN-γ are important for optimal activation of T lymphocytes.

Although the ability of CD2 signaling to prolong the half-life of cytokine mRNA may not have any immediately apparent direct clinical or immunotherapeutic applications, the role of CD2 as a costimulatory molecule in T cell activation is of great importance in basic T cell biology. The importance of CD28 in T cell costimulation is well documented (June and Bluestone 1994). Since the need for CD2 costimulation is comparable to the need for CD28 signaling in T cell activation, yet antigen-independent T cell activation has been demonstrated through CD2 but not CD28 (Rosenthal-Allieri *et*

al. 1995), it is feasible that the costimulatory role of CD2 is much greater than was once thought. Additionally, by delineating the costimulatory function of CD2 and associated signaling molecules, we will likely learn a great deal about the ability of certain T cell surface molecules to exert multiple effects on the T cell since CD2 is able to enhance T cell activation, yet also regulate T cell anergy and responsiveness to antigenic stimulation (Bachmann *et al.* 1999; Boussiotis *et al.* 1994). Finally, CD2-CD48 interactions are also relevant in the study of the nature of T cell interactions with APC and target cells during activation and effector cell function. CD2 is present in the immunological synapse, seemingly to position T cell receptors for optimal interactions with self MHC-peptide molecules (van der Merwe *et al.* 1995). The potential role for intracellular signaling through CD2 in immunological synapse formation is considerable, since CD28 has been shown to stimulate synapse formation (Viola *et al.* 1999). As is the case with LFA-1-ICAM-1 interactions, both CD2 and LFA-1 were once thought of exclusively as adhesion molecules, but have since been shown to possess the capacity for intracellular signaling leading to enhanced T cell activation (Ni *et al.* 2001). These issues all underscore the importance of studying CD2 to better understand the mechanism(s) of T cell activation and development of effector function.

Additionally, we have presented evidence that CD2-crosslinking may induce an unknown negative signaling pathway in T cells. Although the mechanism by which this putative negative signal exerts its effects is not known, the induction of a negative signal in T cells has considerable relevance in therapeutic applications. Since T cells are largely responsible for allograft rejection, and inhibition of T cell activation can lead to prolonged allograft survival and even tolerance, the ability of an antibody to specifically

inhibit T cell activation leads to possible therapeutic uses of anti-CD2 mAb to prolong allograft survival. In fact, anti-CD2 mAb are known to be quite effective at prolonging allograft survival, and when combined with CD28 blockade, immunological tolerance is achieved in treated animals (Chavin *et al.* 1992; Woodward *et al.* 1996). An alternative possibility for the *in vivo* relevance of activation of a negative signal through CD2 is its place in the tumor counter-attack model of immune activation. There are many documented cases of tumor-induced immune suppression ranging from secretion of inhibitory molecules such as adenosine and TGF-β to expression of death-inducing FasL on tumor cells (Igney *et al.* 2000; Chouaib *et al.* 1997; Blay *et al.* 1997). If tumor cells could, through interactions with CD2 expressed on T cells and CD48 or CD48-like structures expressed on tumor cells, induce a negative signal (and thus abrogate a T cell-mediated anti-tumor immune response), this activity would decrease the effectiveness of the cell-mediated anti-tumor immune response and favor tumor growth. Since preliminary experiments indicate that the ability of different anti-CD2 mAbs varies in their capacity to induce negative signaling in T cells, and this phenomenon is thought to be due to CD2 epitope-specific effects, altered CD48 structure (perhaps due to altered glycosylation pattern) on tumor cells may result in the engagement of a CD2 epitope which mediates specific inhibitory effects on tumor-specific T cell activation. Interestingly, a carbohydrate structure closely associated with CD15 on human myeloid cells (K562) was found to bind CD2 (Warren *et al.* 1996), suggesting that this scenario is entirely possible. Although the expression pattern of CD48, the lone ligand of rodent CD2, is more restricted than that of CD58, the primary ligand of human CD2 (Thorley-Lawson *et al.* 1982; Arvieux *et al.* 1986; Reiser 1990), CD48 is also widely expressed on

human cells, supporting the possibility of CD48-mediated triggering of inhibitory CD2 epitopes on human T cells. Interestingly, preclinical studies with murine anti-CD48 mAb have shown it to be a potentially useful therapeutic reagent in the treatment of leukaemia and lymphoma, suggesting that CD48 is expressed on tumor cells (Sun *et al.* 2000; Sun *et al.* 1998). Also, soluble CD48 has been detected in plasma of patients with lymphoid leukemias, suggesting that transformation may actually upregulate CD48 expression (Smith *et al.* 1997). Alternatively, tumors might induce T cell anergy through CD2-CD48 interactions, possibly through upregulation of p27Kip1 in tumor-specific T cells, again leading to downregulation of the cell-mediated anti-tumor immune response. It is also possible that altered CD58 structure on human cancer cells could also suppress tumor-specific T cells. Although an antibody to CD2 has not yet been shown to elicit a negative signal through human CD2, the homology of human and rodent CD2 suggest that human CD2 should be capable of negative signaling. If tumors could manipulate the negative signaling capacity of CD2 to their advantage, the effectiveness of T cell-mediated anti-tumor immune responses would likely be reduced, leading to increased tumor growth.

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