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EVIDENCE OF FUNDAMENTAL DIFFERENCES BETWEEN THY-1- AND T CELL RECEPTOR-ASSOCIATED PATHWAYS OF T CELL ACTIVATION

by

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Signature of Author

For my mother, the dearest

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ABSTRACT

Although the glycosylphosphatidylinositol-anchored protein Thy-1 has been implicated in the process of T cell activation, the exact function of Thy-1 in this context has not been established. A number of roles have been proposed for Thy-1, ranging from negative regulation to costimulation of T cell responses. Moreover, the relationship between Thy-1 and the T cell receptor (TCR) has remained highly controversial. This work was undertaken to address several unresolved issues regarding the role of Thy-1 signaling in the induction of T cell proliferative and cytotoxic responses in the presence or absence of TCR signaling and/or appropriate costimulation. I found that antibody-mediated blockade of Thy-1 prevented the induction of non-specific cytotoxic T lymphocytes (CTLs) in response to anti-CD3 monoclonal antibody. This indicates that Thy-1 plays an important role not only for the effector phase of T cell-mediated cytotoxicity as has previously been described for CTL clones, but also during the induction phase of a CTL response. Taking advantage of selective pharmacological inhibitors of intracellular signaling molecules, I demonstrated that the TCR- and Thy-1-associated signal transduction pathways are not identical, with the p38 mitogen-activated protein kinase being a noticeable point of difference. Finally, I discovered that antibody-mediated triggering of Thy-1 in the context of strong, CD28-mediated costimulation provided by dendritic cells, leads to robust T cell proliferation, interleukin-2 synthesis and the acquisition of cytotoxic effector molecules, but not cytotoxic effector function. This finding has led me to propose that Thy-1 triggering may provide a non-classical form of signal 1 for select features of T cell activation. Collectively, my results point to the existence of fundamental differences between the TCR- and Thy-1-driven signaling pathways in T lymphocytes, and also suggest a novel role for Thy-1 as a potential source of signal 1 for T cell activation.

ABBREVIATIONS

Ab	**Ab**ody
Ag	**Ag**en
AICD	**A**ctivation-**i**nduced **C**ell **D**eath
AIDS	**A**cquired **I**mmune **D**eficiency **S**yndrome
AP-1	**A**ctivator **P**rotein-**1**
AK-T	**A**nti-CD3-activated **K**iller **T**
APAF	**A**poptosis **P**rotease-**a**ctivating **F**actor
APC	**A**ntigen **P**resenting **C**ell
BCG	**B**acillus **C**almette-**G**uerin
β2M	β_2- **m**icroglobulin
B7h	**B7 h**omolog
B7-RP	**B7**-related **P**rotein
BSA	**B**ovine **S**erum **A**lbumin
CAM	**C**ell **A**dhesion **M**olecule
CD	**C**luster of **D**ifferentiation
CDC	**C**enters for **D**isease **C**ontrol
cDNA	**c**omplementary **DNA**
CHO	**C**hinese **H**amster **O**vary
CNS	**C**entral **N**ervous **S**ystem

ConA	**Con**canavalin **A**
CsA	**C**yclo**s**porine **A**
CTL	**C**ytotoxic **T** **L**ymphocyte
CTLA-4	**C**ytotoxic **T** **L**ymphocyte-associated **A**ntigen **4**
CTLp	**C**ytotoxic **T** **L**ymphocyte **P**recursor
DAG	**Dia**cyl**g**lycerol
DC	**D**endritic **C**ell
DD	**D**eath **D**omain
DED	**D**eath-**e**ffector **D**omain
DISC	**D**eath-**i**nducing **S**ignaling **C**omplex
DMSO	**Dim**ethyl **S**ulf**o**xide
DN	**D**ouble-**N**egative
DNA	**D**eoxyribo**n**uclecic **A**cid
dNTP	**d**eoxy**n**ucleotide **tri**phosphate
DP	**D**ouble-**P**ositive
EAE	**E**xperimental **A**llergic **E**ncephalomyelitis
ELISA	**E**nzyme-**L**inked **I**mmuno**s**orbent **A**ssay
ERK	**E**xtracellular Signal-**r**egulated **K**inase
Fab	**F**ragment of **a**ntigen **b**inding
FADD	**F**as-**a**ssociated **D**eath **D**omain
FasL	**Fas** **L**igand

FcR	**Fc R**eceptor
FBS	**F**etal **B**ovine **S**erum
FHL	**F**amilial **H**emophagocytic **L**ymphohistiocytosis
FITC	**F**luorescein **I**sothio**c**yanate
FLICE	**F**ADD-**l**ike **ICE**
FLIP	**FL**ICE **I**nhibitory **P**rotein
GAPDH	**G**lycer**a**ldehyde 3-**p**hosphate **deh**ydrogenase
gld	**g**eneralized **l**ymphoproliferative **d**isease
GM-CSF	**G**ranulocyte/**M**acrophage **C**olony **S**timulating **F**actor
GPI	**G**lycosyl**p**hosphatidylinositol
GVHD	**G**raft **V**ersus **H**ost **D**isease
GVL	**G**raft **V**ersus **L**eukemia
GZM	**G**ran**z**y**m**e
GzmA	**G**ran**z**y**m**e **A**
GzmB	**G**ran**z**y**m**e **B**
HIV	**H**uman **I**mmunodeficiency **V**irus
HDMEC	**H**uman **D**ermal **M**icrovascular **E**ndothelial **C**ell
ICAM	**I**nter**c**ellular **A**dhesion **M**olecule
ICOS	**I**nducible **Cos**timulator
IDDM	**I**nsulin-**d**ependent **D**iabetes **M**ellitus
IFN	**I**nter**f**ero**n**

Ig	**Immunoglobulin**
IgSF	**Immunoglobulin Superfamily**
IL	**Interleukin**
ILR	**Interleukin Receptor**
IP_3	**Inositol Triphosphate**
ITAM	**Immunoreceptor Tyrosine-based Activation Motif**
ITIM	**Immunoreceptor Tyrosine-based Inhibitory Motif**
JNK	**c-Jun N-terminal Kinase**
KDa	**Kilodalton**
KO	**Knockout**
KS	**Kaposi's Sarcoma**
LAT	**Linker for Activation of T Cells**
LFA	**Lymphocyte Function-associated Antigen**
lpr	**lymphoproliferation**
LPS	**Lipopolysaccharide**
mAb	**monoclonal Antibody**
MAPK	**Mitogen-activated Protein Kinase**
2-ME	**2-Mercaptoethanol**
MHC	**Major Histocompatibility Complex**
MLR	**Mixed Lymphocyte Reaction**
MS	**Multiple Sclerosis**

mRNA	**m**essenger **R**ibo**n**ucleic **A**cid
NFAT	**N**uclear **F**actor of **A**ctivated **T** Cells
NFκB	**N**uclear **F**actor **κB**
NGF	**N**erve **G**rowth **F**actor
NK	**N**atural **K**iller
NOD	**N**on**o**bese **D**iabetic
pAPC	**P**rofessional **A**ntigen **P**resenting **C**ell
PBS	**P**hosphate-**b**uffered **S**aline
PCR	**P**olymerase **C**hain **R**eaction
PD-1	**P**rogrammed **D**eath **1**
PE	**P**hyco**e**rythrin
PFN	**P**erfori**n**
PHA	**P**hyto**h**emagglutinin
PI-3K	**P**hosphatidyl**i**nositol **3**-**K**inase
PIP_2	**P**hosphatidyl**i**nositol bi**p**hosphate
PKC	**P**rotein **K**inase **C**
PLC	**P**hospho**l**ipase **C**
PMN	**P**oly**m**orpho**n**uclear
PNH	**P**aroxysmal **N**octurnal **H**emoglobinuria
PTK	**P**rotein **T**yrosine **K**inase
PWM	**P**oke**w**eed **M**itogen

RA	**R**heumatoid **A**rthritis
RT	**R**everse **T**ranscriptase
sAg	**S**uperantig**e**n
SLP-76	**S**H2-domain containing **L**eukocyte **P**rotein of **76** KDa
TAP	**T**ransporter Associated With **A**ntigen **P**rocessing
Tc	**T c**ytotoxic
TCR	**T C**ell **R**eceptor
Th	**T h**elper
TNF	**T**umor **N**ecrosis **F**actor
Tr	**T r**egulatory
TRAIL	**T**NF-**r**elated **A**poptosis-**i**nducing **L**igand
Ts	**T s**uppressor
TSST-1	**T**oxic **S**hock **S**yndrome **T**oxin **1**
VCAM-1	**V**ascular **C**ell **A**dhesion **M**olecule-**1**
WT	**W**ild-**t**ype

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1. General Introduction

1.1. T cells: a brief overview

T cells are an integral part of acquired immune responses and also participate in shaping innate defense mechanisms. T cell precursors originate from bone marrow and migrate to the thymus where they mature and learn how to specifically recognize and respond to foreign invaders without risking self components (Anderson *et al*, 1996). T cell precursors enter the thymus as double negative (DN, $CD4^-8^-$) cells, but evolve to double positive (DP, $CD4^+8^+$) thymocytes, followed by losing the expression of either CD4 or CD8, thereby giving rise to $CD8^+$ or $CD4^+$ single positive cells, respectively. T cell ontogeny in the thymus involves T cell receptor (TCR) gene rearrangement giving each T cell an antigen (Ag) receptor of unique specificity. T cells bearing αβ TCRs function as sentinels of adaptive immunity, whereas the exact function(s) of the highly conserved γδ T cells remains largely unknown despite reports of increased numbers of γδ T cells in a variety of infectious and autoimmune diseases (Carding and Egan, 2002). Mature, yet naïve single positive T cells that have successfully rearranged their TCR will eventually leave the thymus and migrate into secondary lymphoid organs or tissues such as lymph nodes, spleen and mucosal lymphoid tissues where they will await an encounter with their corresponding antigens.

During thymic education, immature double positive T cells expressing low levels of αβ TCR undergo stringent positive and negative selection processes. The former process is governed by the thymic epithelial cells residing in the

thymic cortex and ensures that only T cells that can recognize endogenous or exogenous peptides in the groove of a self major histocompatibility complex (MHC) molecule are allowed to survive and mature into single positive cells (Fowlkes and Schwighoffer, 1995). Positive selection thus implements the rule of "MHC restriction" according to which $CD4^+$ and $CD8^+$ T cells will eventually recognize antigenic peptides only if they are displayed in the context of self MHC class II and I molecules, respectively. T cells are then subjected to negative selection, which is mainly contributed by dendritic cells (DCs) present at the cortico-medullary junction, and also by macrophages scattered in both the cortex and the medulla. During negative selection, T cells exhibiting high affinities for self components are eliminated, while T cells with lower affinities for self components are spared (Kappler *et al*, 1987; Sprent and Webb, 1995). Negative selection thus avoids the induction of autoimmune reactions.

Major T cell subsets have been defined based on the functions they elicit *in vivo* and/or *in vitro*. T helper (Th) cells detect antigenic peptides in the context of MHC class II and, as their name implies, provide crucial help to other immunocytes. Th1 cells, which secrete interferon (IFN)-γ and tumor necrosis factor (TNF)-β, activate macrophages and promote cell-mediated immune responses. The Th2 cytokine profile includes, but is not restricted to, interleukin (IL)-4, IL-5, and IL-13. Th2 cells inhibit macrophage activation while favoring strong antibody (Ab) responses including those of the IgE isotype involved in precipitating allergic reactions (Mosmann and Sad, 1996). T cytotoxic (Tc) cells that are typically but not always $CD8^+$ are lethal towards neoplastic and virus-

infected cells. The type 1/type 2 paradigm has been extended to Tc cells to describe Tc1 and Tc2 cells with cytokine profiles analogous to Th1 and Th2 cells, respectively (Croft *et al*, 1994; Sad *et al*, 1995). However, no dramatic functional differences apparently exist between Tc1 and Tc2 cells (Li *et al*, 1997). More recent years have witnessed the reemerging study of T suppressor (Ts) cells with a $CD4^+CD25^+$ phenotype, which are now often referred to as regulatory T cells (Tr). Tr cells play an important role in maintaining immune homeostasis and dampening responses of an autoimmune nature (Shevach, 2000).

1.1.1 T cells in health and disease

T cells play a pivotal role in defense mechanisms against infectious agents and in immune surveillance against cancer. This is highlighted by the fact that animals or individuals with congenital or acquired T cell deficiencies display higher susceptibility to infections with viruses, fungi, intracellular bacteria and protozoa as well as greater risk for development of certain types of tumors. Congenital T cell immunodeficiencies are exemplified by nude (athymic) mice and patients with DiGeorge syndrome, both of which are prone to many types of infection (Rosen *et al*, 1995). Pneumonia caused by the opportunistic microorganism *Pneumocystis carinii*, and tumors like Kaposi's sarcoma (KS) are amongst the most common life-threatening complications of the acquired immunodeficiency syndrome (AIDS) in which $CD4^+$ T cells are targeted by the human immunodeficiency virus (HIV)(Miller, 1996). In fact, KS is an "AIDS-defining illness" according to the Centers for Disease Control (CDC) guidelines

(Dezube, 2002). All the above underline the very important role played by T cells in defense against infections and neoplasia.

Inappropriate or unwanted T cells responses may lead to adverse reactions with catastrophic consequences. The role of T cells in the pathogenesis of several autoimmune disorders such as insulin-dependent diabetes mellitus (IDDM), rheumatoid arthritis (RA) and multiple sclerosis (MS) is either established or highly likely (Van Noort and Amor, 1998). T cells are also involved in allograft rejection and in graft versus host disease (GVHD) encountered in bone marrow transplant recipients. However, alloreactive T cell responses are critical for graft versus leukemia (GVL) effect used to treat tumor hosts (Champlin *et al*, 1996).

Understanding the pathways of T cell activation and effector functions therefore has tremendous clinical significance, as many immunotherapeutic approaches attempt to either boost or suppress T cell responses in health and disease.

1.1.2 T cell receptor signal transduction

The engagement of the TCR by specific Ag presented in the context of a self MHC molecule typically initiates intracellular signaling cascades culminating in T cell activation. The TCR is a multisubunit complex consisting of the highly variable $\alpha\beta$ heterodimers non-covalently associated with the invariant CD3 γ, δ and ε chains, and the largely intracytoplasmic $\zeta\zeta$ homodimers. The $\alpha\beta$ heterodimers specifically bind the antigenic peptide:MHC complexes, while the CD3 proteins are required for surface expression of the $\alpha\beta$ heterodimers as well

as for signaling via the TCR complex (Malissen and Malissen, 1996). The co-receptor molecules CD4 and CD8 also cooperate with the TCR/CD3 complex by binding to the invariant parts of MHC II and I molecules, respectively (Zamoyska, 1998). The TCR complex, the co-receptor (CD4 or CD8) and the CD45 tyrosine phosphatase are brought together as a result of the binding of a peptide:MHC ligand to the TCR and co-receptor. This allows CD45 to remove inhibitory phosphate groups from *Src*-family protein tyrosine kinases (PTKs) *Fyn* and *Lck*, which are constitutively associated with the TCR complex and the co-receptor, respectively. These PTKs then phosphorylate the ζ chains, each of which contains three immunoreceptor tyrosine-based activation motifs (ITAMs). The consensus sequence for an ITAM is $YxxL(x)_{6-8}YxxL$, with Y denoting tyrosine, L denoting leucine, and x representing any amino acid (van Leeuwen and Samelson, 1999). For many years, tyrosine phosphorylation of CD3 molecules and ζ chains following TCR cross-linking has been postulated to be the principal trigger for T cell activation. However, a very recent report by Gil *et al* (2002) adds a novel and unexpected element to the story, namely an additional and slightly earlier triggering event involving a conformational change and subsequent exposure of a proline-rich region of the cytoplasmic tail of CD3ε. This allows for the binding of the adaptor protein *Nck* to CD3ε, which appears to be essential for proper T cell activation. Surprisingly, this event seems totally independent of early tyrosine phosphorylation and can be triggered via stimulation by monovalent Abs. The signal transduced by *Nck* recruitment to CD3ε and that generated by aggregation-dependent tyrosine phosphorylation of the TCR/CD3

complex on ITAMs by *Fyn* and *Lck* are likely to converge at some point downstream (Davis, 2002).

Once phosphorylated, ζ chain ITAMs provide docking sites for the cytosolic tyrosine kinase ZAP-70 whose subsequent activation via phosphorylation results in initiation of several downstream signaling pathways (van Oers *et al*, 1994). ZAP-70 phosphorylates the adapter proteins linker for activation of T cells (LAT) and SH2-domain containing leukocyte protein of 76 KDa (SLP-76) (Wardenburg *et al*, 1996; Zhang *et al*, 1998). LAT, which is a transmembrane protein, then becomes confined to membrane lipid rafts where it recruits several important signaling entities including phospholipase C γ1 (PLC-γ1) and phosphatidylinositol-3 kinase (PI-3K) via SH2 domain interactions (Xavier *et al*, 1998; Zhang *et al*, 1998). PLC-γ1 is phosphorylated and thus activated most likely by ZAP-70. Activated PLC-γ1 in turn cleaves phosphatidylinositol biphosphate (PIP_2) to yield diacylglycerol (DAG) and inositol triphosphate (IP_3). The former activates protein kinase C (PKC) leading to activation of the transcription factor nuclear factor κB (NFκB), while the latter induces a sudden increase in intracellular free Ca^{++}, which in turn activates the cytoplasmic phosphatase calcineurin. Calcineurin enables another transcription factor called nuclear factor of activated T cells (NFAT) to translocate to the T cell nucleus. The full transcriptional activity of NFAT also requires the action of activator protein (AP)-1 transcription factors.

PI-3Ks constitute a family of evolutionarily conserved lipid kinases that phosphorylate the D3 position of the inositol ring of phosphoinositides and

produce PI(3)P, PI(3,4)P_2, and PI(3,4,5)P_3 (Sasaki *et al*, 2002). Also mobilized by LAT, PI-3K is believed to be a key component of signaling pathways associated with the TCR/CD3 complex (Ward *et al*, 1992; Exley and Varticovski, 1997), CD28 (Pages et al, 1994) and the IL-2 receptor (IL-2R) of Th cells (Reif et al, 1997). This is consistent with the finding that signaling pathways associated with cytotoxic T lymphocyte (CTL) development in response to mitogenic anti-CD3 Ab involve PI-3K (Phu *et al*, 2001). Details on exactly how PI-3K fits into TCR-mediated signaling pathway are not, however, fully understood.

A third important signaling pathway initiated by ZAP-70 is the activation of Ras leading to the subsequent activation of a mitogen-activated protein kinase (MAPK) cascade, which in turn culminates in the activation of AP-1 (Dong *et al*, 2002).

NFκB, NFAT and AP-1 act together to initiate new gene transcription that eventually leads to T cell proliferation and differentiation into effector cells.

1.2 Outcomes of T cell activation

Productive T cell triggering typically involves TCR-mediated recognition of an antigenic peptide:MHC ligand in addition to a costimulatory signal provided upon certain molecular interactions between T cells and antigen presenting cells (APCs).

The decision of a T cell "to sense and to respond" to non-self, and also "to sense, live on, but not to respond" to self is a critical one. Self/non-self discrimination, which is arguably the hallmark of immunity, is largely achieved via translating differences in TCR affinity for foreign/altered versus self ligands into

intracellular signals that differ in quality, intensity, and/or duration (Germain and Stefanova, 1999). Quantitative and qualitative properties of TCR ligands, costimulatory interactions and cytokines comprise major factors in determining T cell fate and commitment. While the absence of costimulatory interactions during T cell priming leads to anergy rather than activation (Schwartz, 1990), the cytokine milieu in which T cells respond to Ag influences T cell polarization towards a Th1 or Th2 phenotype. In real life, signals involved in priming of naïve T cells are frequently provided by DCs, which can be viewed with some simplification as discrete packets of information that are decoded by T cells in secondary lymphoid organs. DCs are the most professional amongst professional APCs (pAPCs) owing to their expression of high levels of MHC and costimulatory molecules (Banchereau *et al*, 2002), and to their unique ability to bind to and form immunological synapses with naive T cells both *in vitro* and *in vivo* (Al-Alwan *et al*, 2001; Norbury *et al*, 2002). The immunological synapse is the specialized junction formed between a T cell and an APC, which accommodates a central cluster of TCRs surrounded by a ring of adhesion molecules (Grakoui *et al*, 1999). Once properly activated, T cells proliferate and differentiate into effector or memory T cells. Effector T cells produce certain cytokines and/or are armed with cytotoxic effector molecules, enabling them to lyse target cells following recognition of the target cell in an Ag-specific manner. Memory T cells remain viable most probably under the influence of IL-7 and IL-15 (Becker *et al*, 2002; Kieper *et al*, 2002; Schluns *et al*, 2002), and will be prepared for future antigenic encounters.

Differentiation, defined as changes that affect the future potential of the cells, probably occurs both initially and throughout full activation. However, whether all steps associated with T cell differentiation require cell division is unclear. There is likely to be a correlation between differentiation events with multiple rounds of division (Swain, 1999). Bird and coworkers (1998) found that the initial expression of IL-2 is cell cycle-independent, whereas effector cytokine expression is cell cycle-dependent. Moreover, IFN-γ expression increases with successive cell cycles, while IL-4 expression requires three cell divisions.

The expansion of T cell populations in response to Ag is often accompanied by cell death. It is now clear that the presence of IL-2, while contributing to cell division, can promote differentiation to a state where T cells are susceptible to activation-induced cell death (AICD). AICD is typically executed via Fas-Fas ligand (FasL) interactions (Lenardo, 1991; Refaeli *et al*, 1998), which will be discussed later.

1.3 Cytotoxic T Lymphocytes

One of the most important outcomes of T cell activation is differentiation of naïve CTL precursors (CTLp) into fully functional CTLs armed with cytotoxic effector molecules. CTLs provide a major line of host defense by clearing viral infections and by eradicating spontaneously arising neoplastic cells.

Most CTLs are $CD8^+$ and recognize antigenic peptides presented in the groove of a self MHC I molecule. In the case of viral infections, short peptide fragments are generated when proteins from replicating viruses are degraded in the cytosol by a large, multicatalytic protease complex called the proteasome

(Rotzschke et al, 1990; Yewdell *et al*, 1999). These peptides are then transported by the transporters associated with antigen processing (TAPs) into the lumen of the endoplasmic reticulum where they become stably bound with MHC class I molecules displaying degenerate specificity for a range of peptides of usually 8-10 amino acids in length (Shepherd *et al*, 1993). The peptide:MHC I complexes are then transported to the Golgi complex *en route* to the cell surface where they meet the CTLs or CTLps, each of which displays a TCR of unique specificity for the peptide:MHC complexes. The importance of the MHC I pathway of Ag presentation in CTL induction is evidenced by the failure of β_2-microglobulin (β2M)-deficient mice to mount significant virus-specific $CD8^+$ CTL responses (Zijlstra *et al*, 1990). β2M is a highly conserved polypeptide that non-covalently associates with MHC I α chain, and is required for the proper folding as well as cell surface expression of MHC I (Heemels and Ploegh, 1995). β2M-deficient mice do not express correctly folded MHC I molecules on the cell surface and, as a result, are defective in positive selection of $CD8^+$ T cells during thymic education. These mice thus have negligible numbers of $CD8^+$ T cells in the periphery (Koller *et al*, 1990; Zijlstra *et al*, 1990). In contrast, virus-specific $CD4^+$ CTLs are easily detectable in these mice. Although the contribution of MHC II-restricted, $CD4^+$ CTLs in anti-viral immunity is not fully appreciated, these T cells clearly play a role in immune regulation by targeting other immunocytes such as T and B cells and macrophages which express Fas (Hahn and Erb, 1999).

Tumor-specific CTLps are primed either directly by the tumor cells or by pAPCs such as DCs that have captured and processed tumor Ags in a process known as cross-priming (den Haan and Bevan, 2001). Cross-priming appears to be a more efficient mode of tumor Ag presentation since DCs often express much higher levels of costimulatory molecules than do tumor cells.

Several cytokines, including IL-2 and IFN-γ, promote CTL induction. It was traditionally believed that in order for $CD8^+$ CTLps to fully differentiate into effector CTLs, $CD4^+$ Th cells must provide IL-2 following interaction with APCs. However, $CD8^+$ CTLps are capable of developing into functional CTLs in the absence of $CD4^+$ Th cells (Buller *et al*, 1987; Rahemtulla *et al*, 1991). This is due to the ability of activated $CD8^+$ T cells to produce sufficient IL-2 to drive their own proliferation and differentiation in an autocrine manner.

Fully differentiated CTLs are armed with perforin (PFN), various granzyme (GZM) molecules, and FasL, which are regarded as the major effector molecules of the CTL cytotoxic arsenal (Berke, 1995). While target cell recognition by classical CTLs occurs in an Ag-specific fashion, cytotoxic effector molecules lack any specificity whatsoever for Ag. It is therefore surprising that no "innocent bystander" killing is observed when a CTL lyses its target (Kuppers and Henney, 1977). This may be due to the highly polarized nature of the release of cytotoxic effector molecules (Griffiths and Argon, 1995), and to the fact that these molecules are delivered within a closed cleft formed between the CTL and the target cell (Berke, 1995). Following the first encounter between a naïve CTLp and an Ag-bearing target cell, cytotoxic proteins are synthesized and/or loaded

into the CTL lytic granules. Ligation of the TCR expressed by an armed $CD8^+$ CTL also induces *de novo* synthesis of PFN and GZMs, so that the supply of lytic granules is replenished. This ensures that a single CTL can recycle and deliver the "kiss of death" to many targets in succession (Isaaz *et al*, 1995). It is noteworthy that most $CD8^+$ CTLs also release the cytokines IFN-γ, TNF-α and TNF-β, which can contribute to host defense in several other ways. IFN-γ, the main cytokine produced by CTLs, directly inhibits viral replication, and also activates and recruits macrophages to the sites of infection where they can serve as both effector cells and APCs. Furthermore, the expression of MHC I molecules on infected cells as well as some intracellular components of MHC class I pathway of Ag presentation are upregulated by IFN-γ, which increases the likelihood that virus-infected cells will be recognized as target cells for CTL attack. TNF-α and TNF-β synergize with IFN-γ in activating macrophages, and are directly lethal towards certain targets (Fruh and Yang, 1999; Ramshaw *et al*, 1992).

1.3.1 Pathways of T cell-mediated cytotoxicity

Following TCR-mediated recognition and cell adhesion molecule (CAM)-mediated conjugation to target cells, CTLs employ two major mechanisms to induce apoptotic cell death in target cells. The granule-dependent exocytotic pathway relies on the expression and function of PFN and GZMs, whereas the death receptor pathway is typically initiated by the cognate ligation of target cell membrane proteins belonging to the TNF receptor superfamily such as Fas. Although the two pathways often co-exist in CTLs, the general consensus is that

$CD8^+$ CTLs primarily use PFN and GZMs to eliminate virus-infected cells, while $CD4^+$ CTLs that participate in immune regulation and peripheral tolerance exhibit preference for the usage of FasL (Hahn *et al*, 1995).

1.3.2 Granule exocytosis

Granule-mediated cytotoxicity depends primarily on the synergistic actions of the pore-forming protein PFN, which is also known as cytolysin, and a family of granule-associated serine proteases termed granzymes (GZMs).

According to the accepted paradigm, a CTL is stimulated by a target cell to undergo vectorial exocytosis of granule contents into a tightly apposed, synaptosome-like space formed between the two cells (Berke, 1995). The released monomeric PFN is then inserted into the target cell membrane and polymerized to form "pores". PFN pores can cause osmotic lysis of the target cell, and also allow GZMs and presumably other granule components, such as granulysin (Stenger *et al*, 1999) to gain access to the target cell interior. GzmB can also enter the target cell independently of PFN, most probably through endocytosis. The cation-independent mannose 6-phosphate receptor has been demonstrated to function as a death receptor for GzmB cell surface binding, uptake, and the induction of apoptosis (Motyka *et al*, 2000). It is noteworthy that the internalized GzmB is ineffective in inducing apoptosis unless the target cell is also treated with PFN. PFN somehow causes the almost instant redistribution of GzmB from endocytic vesicles to the cytosol and nucleus (Jans *et al*, 1996). A cascade of events involving cysteine aspartases, or caspases for short, will eventually lead to DNA fragmentation and target cell death by apoptosis.

Exocytosis of the granules and insertion and polymerization of PFN all require the presence of extracellular Ca^{++}.

PFN is homologous to the C9 component of the complement system (Shinkai *et al*, 1988), and is expressed mainly by CTLs and natural killer (NK) cells, although PFN expression has also been reported in macrophages (Li *et al*, 1994), astrocytes (Gasque *et al*, 1998) and bone marrow progenitors (Berthou *et al*, 1995). Analysis of PFN knockout (KO) mice has confirmed the importance of PFN in both CTL- and NK-cell mediated cytotoxicity *in vitro*, as well as in immune responses to cancer (Smyth *et al*, 1999) and to certain intracellular pathogens *in vivo* (Stenger and Modlin, 1998). Accumulating evidence suggests a role for PFN in immunoregulatory phenomena, for instance in downregulation of T cell responses during chronic viral infection and autoimmunity (Matloubian *et al*, 1999). Interestingly, defective PFN was recently identified as the genetic basis for familial hemophagocytic lymphohistiocytosis (FHL), a lethal autosomal recessive disorder of immune dysregulation (Stepp *et al*, 1999). Histopathologically, FHL is characterized by the infiltration of the bone marrow, spleen, liver, lymph nodes, and central nervous system (CNS) by activated T cells and macrophages, and also by hemophagocytosis by hyperactivated macrophages. Patients with FHL fail to control certain infections and also mount an unregulated, self-destructive inflammatory response in the aftermath of such infections. The possible mechanisms underlying a role for PFN in immune regulation are beyond the scope of this introduction, and the interested reader is referred to a review by Stepp *et al* (2000).

Gene deletion studies in mice have provided compelling evidence that granzyme B (GzmB) is crucial for rapid induction of apoptosis, while other GZMs elicit a delayed apoptogenic response (Froelich *et al*, 1998). GrzmB, like the caspases themselves, cleaves substrates at aspartic acid residues, thereby mimicking and amplifying the effects of caspases (Trapani *et al*, 1999). CTLs obtained from mice lacking GzmB induce slowed DNA fragmentation in target cells *in vitro*. Nevertheless, these mice seem capable of dealing with many cytopathic and non-cytopathic viruses (Heusel *et al*, 1994). There is emerging evidence that other granule constituents, presumably other GZMs, can by and large overcome the absence of functional GzmB, pointing to the existence of impressive redundancy within the CTL killing machinery (Trapani *et al*, 1999).

1.3.3 Death receptor pathway

The death receptor pathway is initiated when distinct members of the TNF/nerve growth factor (NGF) receptor superfamily expressed by target cells are cross-linked as a result of interacting with their cognate ligands that are found on effector cells such as CTLs or NK cells. Certain agonistic Abs can also trigger death receptors thereby inducing cell death in target cells. The Fas/FasL pathway is considered to be the prototype of the death receptor pathways of cytotoxicity, and will be discussed in detail.

1.3.3.1 Fas/FasL pathway

Fas (CD95/Apo-1) is a type I transmembrane glycoprotein of 45-52 KDa that is widely expressed in lymphoid and non-lymphoid tissues including ovary, liver and heart (Walczak and Krammer, 2000). Soluble forms of Fas, which are

produced as a result of mRNA alternative splicing also exist. Expression of cellular Fas can be up-regulated by treatment with IFN-γ and TNF (Moller *et al*, 1994), certain anti-cancer drugs such as cisplatin, etoposide and antiestrogens (Williams *et al*, 1997; Haeryfar *et al*, 2000), or upon activation of lymphocytes (Klas *et al*, 1993). Interestingly, CTLs may induce the expression of Fas on Fas-negative target cells, perhaps by secreting IFN-γ, thereby rendering such target cells susceptible to CTL-mediated killing (Simon *et al*, 2000).

Cross-linking of Fas as a result of interaction with trimeric FasL or multivalent, agonistic anti-Fas Abs transmits an apoptotic signal. Paradoxically, Fas triggering can induce cell proliferation under certain circumstances (Alderson *et al*, 1993; Mapara *et al*, 1993). In fact, the term Fas, for **F**ibroblast-**as**sociated, was first coined following the observation that its triggering on fibroblasts causes a proliferative rather than apoptotic response (Aggrawal *et al*, 1995). Therefore, Fas may play different roles *in vivo* depending on the context of its cellular interactions. It is noteworthy that Fas expression by itself should not always be interpreted as denoting sensitivity to Fas-mediated killing. For instance, transfecting the Fas negative erythroleukemia cell line K562 with a Fas construct does not render these cells susceptible to Fas-mediated lysis (Montel *et al*, 1995). Therefore, Fas expression must simply be viewed as one element of a complex signaling pathway.

Autosomal recessive mutation of Fas is seen in naturally occurring *lpr* (lymphoproliferation) mice, and is accompanied by profound lymphadenopathy and lupus-like autoimmunity. The *lpr* mutation causes a splicing defect that

greatly decreases the expression of Fas (Watanabe-Fukunaga *et al*, 1992). In contrast, *lpr*^{cg} mice express Fas, but have a point mutation in the death domain (DD) of Fas, which results in abolished transmission of the apoptotic signal (Matsuzawa *et al*, 1990). Canale-Smith syndrome is the human equivalent of the murine *lpr* phenotype. Patients with this autosomal dominant syndrome have mutated Fas and display lymphadenopathy, hepatosplenomegaly, increased DN T cells, autoantibody-mediated hemolytic anemia and thrombocytopenia (Drappa *et al*, 1996).

FasL (CD95L/APO-1L) is a type II transmembrane protein with a much more restricted pattern of expression. Soluble FasL is not generated by mRNA alternative splicing, but is the product of the action of a zinc-dependent metalloprotease cleaving trimeric FasL from the cell surface (Mariani *et al*, 1995). Within the immune system, FasL is expressed by macrophages, NK cells, and activated T cells and B cells (Walczak and Krammer, 2000). Signaling through the TCR/CD3 complex up-regulates FasL expression on T cells (Anel *et al*, 1994; Vignaux *et al*, 1995). NK cells and CTLs employ FasL to destroy susceptible target cells. Upon T cell activation, FasL is delivered to the cell surface either directly (Vignaux *et al*, 1995) or via Ca^{++}-dependent, polarized degranulation of cytoplasmic vesicles, that serve as a storage compartment for the newly synthesized protein (Bossi and Griffiths, 1999). FasL-mediated killing also provides a mechanism by which T cells eliminate each other in order to control ongoing immune responses, for example during AICD. This process is also

known as "immune fratricide", meaning brother-killing (Walczak and Krammer, 2000).

Outside lymphoid tissues, FasL is expressed in the eyes, placenta, brain and testis where it acts as one of the means by which "immune privilege" prevails and autoimmune reactions are avoided (Bellgrau and Duke, 1999; Griffith *et al*, 1995; Guller and LaChapelle, 1999). FasL expression by the placenta contributes to the maintenance of immune tolerance towards the fetus, which would otherwise be rejected as a semi-allograft. Finally, some tumor cells also express and use FasL to kill immunocytes and to evade immune attack (O'Connel *et al*, 1999).

FasL deficiency is seen in *gld* (generalized lymphoproliferative disease) mice in which a point mutation in the FasL C-terminus impairs the ability of FasL to interact with its receptor (Takahasi *et al*, 1994).

Apoptotic signal transduction via Fas requires trimerization of Fas by FasL, which brings the Fas-associated death domains (FADDs) together. This leads to the formation of the so-called death-inducing signaling complex (DISC). First, the adaptor FADD protein (also known as Mort1) binds via its own DD to the DD of Fas. FADD contains an additional domain termed death-effector domain (DED), which recruits, via homologous interaction, the DED-containing procaspase-8, also known as FLICE (FADD-like ICE), into the DISC complex. This leads to the proteolytic activation of procaspase-8, followed by the release of active caspase-8 from the DISC into the cytoplasm. Active caspase-8 then cleaves various proteins including procaspase-3, thus initiating execution of the

apoptotic program (Krammer, 2000). It should also be noted that in several cell types (*e.g.*, Jurkat, and CEM T cell line), hardly any DISC is formed. Therefore, the caspase cascade cannot be propagated directly, and has to be amplified via the mitochondria. In these cells, caspase-8 cleaves the Bcl-2 family member *Bid.* Truncated *Bid* then triggers the release of pro-apoptotic molecules such as cytochrome *c* by the mitochondria. Cytochrome *c* associates with apoptosis protease-activating factor 1 (APAF1) and procaspase-9 to form a suborganellar structure known as the apoptosome. The caspase cascade is then activated and the end result will be cell death by apoptosis (Krammer, 2000; Scaffidi *et al*, 1998).

1.3.3.2 TRAIL pathway

TNF-related apoptosis-inducing ligand (TRAIL/APO-2L) is a more recently described death-inducing member of the TNF/NGF superfamily (Wiley *et al*, 1995). TRAIL is a 40 KDa type II transmembrane protein expressed by activated T and B lymphocytes, NK cells, DCs, and monocytes (Hoskin, 2000). Our group has shown that TRAIL expression by mouse T cells is up-regulated following Ab-mediated cross-linking of TCR/CD3 complex, but not by IL-2 signaling (Musgrave *et al*, 1999). Membrane-bound TRAIL can be shed as a result of proteolytic cleavage by cysteine proteases (Mariani and Krammer, 1998). TRAIL can bind two apoptosis-inducing receptors termed TRAIL-R1 (DR4) and TRAIL-R2 (DR5/TRICK2/KILLER), two additional membrane-bound receptors incapable of apoptogenesis, TRAIL-R3 (DcR1/LIT) and TRAIL-R4 (DcR2/TRUNDD), and also a soluble receptor named osteoprotegrin.

It is very interesting that TRAIL preferentially induces apoptotic cell death in a variety of transformed cells but not in normal cells (Walczak *et al*, 1999). TRAIL-R3 and TRAIL-R4 have been suggested to serve as "decoy receptors" that compete with TRAIL-R1 and TRAIL-R2 for binding to TRAIL, and thus protect normal cells from TRAIL-induced lysis (Pan *et al*, 1997). Recent experimental results, however, suggest that the decoy hypothesis alone is not adequate to explain regulation of TRAIL responsiveness. Differential expression of the intracellular apoptosis inhibitor FLIP (FLICE inhibitory protein) is currently believed to be important for the resistance of normal cells and some transformed cells to TRAIL-induced apoptosis (Kim *et al*, 2000). FIIP interferes with the generation of active caspase 8 at the receptor level, thereby preventing death receptor signaling (Walczak and Krammer, 2000).

The very fact that TRAIL-mediated killing spares normal cells makes TRAIL an attractive candidate for immunotherapy of cancer. Furthermore, the finding that tumors that have acquired resistance to chemotherapeutic agents via overexpression of the anti-apoptotic proteins *Bcl-2* or Bcl_{xL} remain sensitive to TRAIL adds more potential value to TRAIL therapy of cancer (Walczak *et al*, 2000).

1.3.3.3 TNF-α pathway

Carswell *et al* (1975) first reported that sera of *bacillus Calmette-Guerin* (BCG)-infected mice treated with endotoxin contained a substance, now known as TNF-α, which could cause haemorrhagic "necrosis" of the sarcoma Meth A and other transplanted tumors. It is now evident that in addition to triggering

"apoptosis" of certain tumor cells, TNF-α takes part in inflammatory responses and regulates immune function (Chen and Goeddel, 2002). Inappropriate production of TNF-α or sustained activation of TNF-α signaling has been implicated in pathogenesis of a broad spectrum of diseases including sepsis, diabetes, cancer, osteoporosis, allograft rejection, and autoimmune disorders such as multiple sclerosis, rheumatoid arthritis, and inflammatory bowel diseases.

TNF-α signals through two distinct membrane receptors, namely TNF-R1 and TNF-R2. Numerous experimental approaches have revealed that signaling through TNF-R1 is responsible for the majority of biological activities attributed to TNF-α.

TNF-mediated killing has been implicated as a minor pathway of cytolysis. Both CTLs and NK cells can utilize TNF-α to induce apoptosis in susceptible target cells in a relatively slow process (Tite, 1990; Vujanovic *et al*, 1996). However, the role of TNF-α in lymphocyte-mediated cytotoxicity *in vivo* is not entirely clear.

1.4 Anti-CD3-activated killer T cells

In vitro stimulation of mouse or human T cells with anti-CD3 monoclonal antibodies (mAbs) is known to mimic Ag-mediated activation of T cells through the TCR, leading to, among other effects, phosphorylation of serine and tyrosine residues on CD3 chains, rapid elevation of cytosolic Ca^{++} concentration, as well as PKC activation (Hoskin *et al*, 1998; Van Wauwe *et al*, 1980; Leo *et al*, 1987). T cell signal transduction pathways triggered by anti-CD3 Ab eventually result in

cytokine synthesis, polyclonal T cell proliferation and differentiation into non-specific cytotoxic cells termed anti-CD3-activated killer T (AK-T) cells. Although several differences exist between AK-T cells and classical Ag-specific CTLs, AK-T cells provide a valuable model to study CTL induction from naïve CTLps. The applicability of this model can be further emphasized by the fact that a small, but still significant percentage of CTLs exhibit MHC-unrestricted cytotoxicity (Stotter and Lotze, 1990).

Both murine and human AK-T cells display MHC-unrestricted cytolytic activity towards a broad range of tumor targets (Stankova *et al*, 1989; Watanabe *et al*, 1992). MHC-unrestricted CTLs including AK-T cells are non-specific killers in the sense that they do not utilize the TCR to "specifically" recognize their target cells (Brooks and Holscher, 1987). In fact, AK-T cells fail to express substantial levels of TCR/CD3 complex on their surface due to the rapid internalization of the complex following T cell stimulation with anti-CD3 Ab (Boyer *et al*, 1991; Stankova *et al*, 1989). It is not presently clear what surface molecules determine target cell selectivity of AK-T cells.

Similar to Ag-specific CTL induction, AK-T cell development requires cell surface interactions of a costimulatory nature. CD28, CD2 and CD8 are among T cell surface molecules delivering costimulatory signals once engaged by their corresponding ligands on accessory cells (Hoskin *et al*, 1998). Accessory cells are needed to be present in AK-T cell cultures not only to provide costimulatory signals, but also to cross-link anti-CD3 mAb via their Fc receptors (FcRs)(Okamoto *et al*, 1995). In the absence of accessory cells, T cells can be

activated by a combination of plastic-immobilized anti-CD3 and anti-CD28 mAbs (June *et al*, 1987).

Anti-CD3-mediated stimulation of T cells triggers an early burst of cytokine synthesis, including IL-2, IL-3, IL-4, IL-6, IFN-γ and TNF-α, some of which contribute to AK-T cell development (Hoskin *et al*, 1998). Cytokine synthesis in AK-T cell cultures precedes the up-regulated expression of cytotoxic effector molecules such as PFN and GzmB, as well as the induction of non-specific cytotoxic activity, suggesting that the killing machinery of AK-T cells is activated by one or more cytokine receptor signal transduction pathways. Murine AK-T cells express abundant levels of PFN, GzmB (but not GzmA), FasL and TNF-α mRNA transcripts. Both PFN/GZM and Fas/FasL pathways can be employed by AK-T cells to destroy susceptible target cells (Hoskin *et al*, 1998). Although anti-CD3-stimulated T cells have been shown to secrete biologically active TNF-α (Stankova *et al*, 1989; Steffen *et al*, 1988), the relative contribution of TNF-α to AK-T cell-mediated killing is yet to be determined.

1.5 Two-signal model of T cell activation

The introduction of the conceptual "two-signal" hypothesis for lymphocyte activation dates back over 3 decades when Bretscher and Cohn (1970) proposed that the recognition of a foreign Ag by an Ab-producing cell (signal 1) in the absence of a putative second signal results in paralysis of the Ab-producing cell. It is now known that signal 2 in the context of B cell activation can be provided by Th cell products. The two-signal theory has been extended to also address essential signals for T cell activation. Recent years have witnessed intense

investigations aiming at identification of novel costimulatory interactions and their applications in designing immunotherapeutic modalities, as well as elucidation of the mechanisms underlying costimulation.

1.5.1 Signal 1 for T cell activation

TCR-mediated recognition of an antigenic peptide presented in the context of a self MHC molecule constitutes signal 1 for T cell activation, which is central to the development of adaptive T cell-mediated immunity, and confers specificity on T cell responses. Alternatively, mitogenic Abs to the TCR/CD3 complex can mimic Ag-mediated signal 1.

Peptide:MHC complexes have, by and large, low affinity for TCRs but can nonetheless deliver sustained stimulation to T cells. This has prompted many investigators to search for mechanisms by which the overall avidity of these interactions is amplified. The formation of TCR and co-receptor arrays that interact with peptide:MHC complexes, and the more recent demonstration that TCRs are enriched within the central region of the immunological synapse (Grakoui *et al*, 1999), have been viewed as a means to generate stable molecular complexes that would sustain the signal over time. An alternative theory suggests that T cell signaling is an ongoing process that is sustained through serial engagement and triggering of TCRs by peptide-MHC ligands (Lanzavecchia and Sallusto, 2001). This view is supported by the observation that although the synapse reaches its stable conformation within approximately five minutes (Grakoui *et al*, 1999), TCR triggering and down-regulation remains continuous over a period of several hours and leads to the consumption of a

number of TCRs that far exceed the number of peptide:MHC ligands offered (Valitutti *et al*, 1995). Recent studies have identified that as few as two peptide-MHC complexes are sufficient to generate all the qualitative aspects of TCR signaling, while additional complexes enhance TCR signaling only quantitatively (Bachmann *et al*, 1998; Bachmann and Ohashi, 1999; Cochran *et al*, 2000). Furthermore, the immunological synapse *per se* is highly dynamic since T cells that have been activated following their encounter with an APC can form new synapses, within minutes, with another APC offering more peptide:MHC ligands (Valitutti *et al*, 1996a). The low affinity, high off-rate nature of peptide:MHC-TCR interactions (Davis *et al*, 1998; Weber *et al*, 1992) appears to ensure that peptide:MHC complexes can disengage from triggered TCRs and become available for reuse by fresh TCRs. Indeed, it has been demonstrated that in the case of optimal agonists, as few as 100 peptide:MHC ligands can trigger and down-regulate up to 20,000 TCRs during a 5-h period (Itoh *et al*, 1999; Valitutti *et al*, 1995; Viola and Lanzavecchia, 1996).

1.5.2 Signal 2 for T cell activation

Although necessary, the Ag-specific signal 1 is not usually sufficient for productive T cell activation. Naïve T cells by and large require high levels of Ag and are absolutely dependent on costimulation for biological responses. In contrast, effector and memory T cells respond to lower amounts of Ag with enhanced biological responses and are relatively independent of costimulation (Croft and Dubey, 1997). A number of "accessory" molecules found on APCs are known to participate in the process of T cell activation. Some of these molecules

are involved in the "physical embrace" between T cells and APCs and are regarded as "adhesion" molecules. Several accessory molecules that are identified as "costimulatory" molecules play a more distinct role in T cell activation in that T cell triggering in their absence results in anergy, a state of unresponsiveness to subsequent Ag stimulation (Schwartz, 1990).

Several criteria have been proposed in order to ascribe costimulatory function to an accessory molecule. Among these are the abilities to induce high levels of IL-2, to promote T cell survival, and to prevent apoptosis of T cells (Watts and DeBenedette, 1999). Although the above effects are eminent when T cells are costimulated by CD28 ligation, the mentioned criteria are not absolute and may exclude several molecular interactions that are regarded by many as efficient costimulatory systems. For instance, CD2-CD48/CD58 interactions not only play a role in cell-cell adhesion but also function in synergy with the TCR to stimulate IL-2 production (Bierer *et al*, 1988). Yet, a combination of anti-CD3 and anti-CD2 mAbs fails to rescue T cells from death, arguing against CD2 ligation supplying a complete signal 2 (Yashiro *et al*, 1998). The author therefore believes that until our understanding of costimulatory pathways of T cell activation is more complete, it is the safest to define costimulation in a broader sense based on the ability of cell surface interactions to prevent T cell unresponsiveness that would otherwise ensue when T cells are triggered through their TCR alone.

1.5.3 CD28-B7 costimulatory interactions

CD28-B7 interactions are by far the most intensely studied system of costimulation and believed to be the principal player in supplying naïve T cells with the requisite signal 2. T cells obtained from CD28-deficient mice show strong impairment of proliferation *in vitro* following stimulation with Abs to the TCR/CD3 complex, alloantigen and specific Ag (Lucas *et al*, 1995; Shahinian *et al*, 1993). CD28 deficiency reportedly results in reduced severity in murine models of arthritis (Tada *et al*, 1999), experimental allergic encephalomyelitis (EAE)(Girvin *et al*, 2000) and mucosal lung inflammation (Mathur *et al*, 1999).

CD28 is a disulfide-linked homodimeric, type 1 transmembrane protein and a member of the immunoglobulin (Ig) gene superfamily (IgSF). The costimulatory property of CD28 was first demonstrated in 1992 by the finding that Ab-mediated ligation of CD28 on T cells was able to induce T cell proliferation and IL-2 production in response to suboptimal doses of plate-bound anti-CD3 mAb. A similar response was induced by the presence of syngeneic lipopolysaccharide (LPS) blasts in culture, which could subsequently be inhibited by an Fab fragment of anti-CD28 Ab, suggesting that the LPS blasts also acted upon CD28 to induce T cell activation (Harding *et al*, 1992). In a separate study, CD28 ligation was shown to also costimulate proliferation and IL-2 synthesis by human tetanus toxoid-specific T cells in response to the corresponding Ag (Jenkins *et al*, 1991).

Like cytotoxic T lymphocyte-associated antigen 4 (CTLA-4/CD152), the second member of the CD28 family (Brunet *et al*, 1987), CD28 interacts with B7-1 (CD80/B7/BB1) and B7-2 (CD86), which are expressed on the APC in response to

activating signals that result from, for example, CD40 engagement (Bromley *et al*, 2001).

B7-1 was discovered by Freedman *et al* (1987) to be the first ligand for CD28. However, Abs to B7-1 proved inefficient in inhibiting primary T cell responses *in vitro*, whereas CTLA-4-Ig was extremely effective (Freeman *et al*, 1993a). In addition, CTLA-4-Ig could readily stain B cells from B7-1-deficient animals, suggesting the existence of an alternative receptor for CD28/CTLA-4 (Coyle and Gutierrez-Ramos, 2001). These observations led to the identification of B7-2 in 1993 (Freeman *et al*, 1993a,b). B7-1 and B7-2 belong to the IgSF, and are expressed on pAPCs, with B7-2 being expressed earlier during activation. Several new B7 homologues have recently been identified that are expressed on cells other than pAPCs, suggesting their involvement in regulation of T cell responses in peripheral tissues (Sharpe and Freeman, 2002). These new members of the B7 family will be discussed later.

CTLA-4 is a member of the IgSF, which is expressed on T cells only after a period of activation, and functions to down-regulate the ongoing T cell responses (Walunas *et al*, 1994). The importance of CTLA-4 function can be highlighted by the fact that the CTLA-4 affinity and avidity for B7-1/B7-2 are 10 fold and more than 500 fold higher than those of CD28, respectively (Mueller, 2000; van der Merwe *et al*, 1997). It is therefore not surprising that CTLA-4 KO mice develop profound autoimmunity and massive lymphoproliferation leading to splenomegaly, lymphadenopathy and fatal multiorgan tissue destruction (Tivol *et al*, 1995; Waterhouse *et al*, 1995). The CD28-antagonizing property of CTLA-4 results not

only from competition with CD28 for binding to B7 ligands, but also involves negative signaling on the part of CTLA-4. The intensity of the available ligands appears to be a determining factor in this regard. Carreno *et al* (2000) demonstrated that Ab-mediated CTLA-4 engagement in the absence of B7 inhibits IL-2 production. This effect requires the CTLA-4 cytoplasmic region. In contrast, the similar inhibitory effect that can be seen as a result of CTLA-4 engagement under B7-dependent costimulation is directly proportional to CTLA-4 cell surface levels and does not require the CTLA-4 cytoplasmic region. It is therefore possible that early in the course of T cell responses when B7 and CTLA-4 expression is minimal, CTLA-4-mediated negative signaling would serve to inhibit T cell activation. At later stages, however, the expression of B7 and CTLA-4 is up-regulated, and both negative signaling through CTLA-4 and B7 sequestration would function to suppress the ongoing immune response (Carreno *et al*, 2000).

The above results can also be viewed as new evidence that Ab-mediated ligation of a costimulatory or counter-stimulatory molecule does not necessarily represent the cognate engagement by the naturally occurring ligand under physiological circumstances. This notion is further supported by the finding that, unlike microbeads coated with anti-CD3 plus anti-CD28 mAbs, anti-CD3+B7-1-Ig- or anti-CD3+B7-2-Ig-coated beads caused $CD4^+$ T cells to produce high levels of Th2 cytokines IL-4, IL-10 and IL-13. In contrast, similar levels of T cell proliferation and IL-2 synthesis were induced by all three sets of microbeads (Broeren *et al*, 2000). It would be interesting to examine the cytokine profile of T cells stimulated by a combination of anti-CD3, B7-1-Ig and B7-2-Ig on the same microbead.

Despite the fact that several studies have addressed a possible role for B7-1/B7-2-mediated costimulation in induction of Th1 versus Th2 cytokine profile, whether costimulation through one or the other B7 molecule can preferentially skew T cell responses towards either Th1 or Th2 phenotype remains controversial. Initially, treatment of proteolipid protein-immunized mice with anti-B7-1 mAb was reported to reduce the incidence of EAE and induce the synthesis of Th2 cytokines, while treatment with anti-B7-2 increased disease severity and up-regulated Th1 cytokine production. This observation was used to argue for preferential costimulation of Th1 and Th2 cells by B7-1 and B7-2, respectively (Kuchroo *et al*, 1995). However, this conclusion is challenged by the finding that treating nonobese diabetic (NOD) mice with anti-B7-1 Ab accelerates the development of IDDM, another apparently Th1-dominated autoimmune disease, while anti-B7-2 treatment prevents the development of the disease (Lenschow *et al*, 1995).

Analysis of B7-1/B7-2 double-deficient mice has revealed that B7-1 and B7-2 may exert some overlapping compensatory functions, for example in the context of IgG responses, isotype switching and germinal center formation (Borriello *et al*, 1997). B7-1/B7-2 double-deficient mice provide a valuable tool in experimental systems where B7-1 is likely to compensate for B7-2 and *vice versa*. Such a likelihood cannot be addressed and/or ruled out by using $CD28^{-/-}$, $CTLA^{-/-}$ or even CD28/CTLA-4 double KO mice in light of the recent description of B7-dependent T cell costimulation in CD28/CTLA-4 double KO mice by Mandelbrot and coworkers

(2001), suggesting the existence of an additional receptor for B7 molecules, which is neither CD28 nor CTLA-4.

1.5.4 New members of the B7 family

New B7 family members and new signaling pathways have recently been identified that appear to be particularly important for regulating the responses of previously activated, rather than Ag-inexperienced, T cells (Sharpe and Freeman, 2002).

B7 homolog (B7h/GL-50/ICOS-L) was first described in 1999 as a TNF-α-inducible costimulatory molecule (Swallow *et al*, 1999). The murine homolog of B7h was subsequently cloned and termed B7-related protein 1 (B7RP-1)(Yoshinaga *et al*, 1999). B7RP-1 pairs with inducible costimulator (ICOS), the third member of the CD28 family (Hutloff *et al*, 1999). The "inducible" expression of ICOS following TCR triggering together with preferential induction of IL-10 by ICOS stimulation, suggests that ICOS may regulate T cell responses (McAdam *et al*, 2000). Recent evidence indicates that B7RP-1-ICOS interactions deliver a critical signal that facilitates Th2 cytokine production and inhibits Th1 responses. $ICOS^{-/-}$ T cells primed *in vivo* and restimulated *in vitro* with specific Ag produce minimal levels of IL-4, but remain fully competent to produce IFN-γ (Tafuri *et al*, 2001). Furthermore, $ICOS^{-/-}$ mice show enhanced susceptibility to EAE, indicating that ICOS has a protective role against Th1-dominated inflammatory autoimmune disease (Dong *et al*, 2001). Interestingly, CD28-B7-1/B7-2 interactions seem to be required for the optimal expression of ICOS, opening up the possibility that some of the functions attributed to the CD28-B7-1/B7-2 pathways, including those

involving Th cell differentiation into Th1 or Th2, may actually be mediated through ICOS (Coyle and Gutierrez-Ramos, 2001; McAdam *et al*, 2000).

B7-H1 (PD-L1)(Dong *et al*, 1999) and B7-DC (PD-L2)(Latchman *et al*, 2001) are two other new members of the B7 family, which bind to programmed death 1 (PD-1) and function to oppose T cell activation and to attenuate cytokine production. B7-H1 and B7-DC are expressed in both lymphoid and non-lymphoid tissues. B7-H1 is also expressed on many human tumors including carcinomas of the breast, lung, ovary and colon, but not on corresponding normal tissues (Latchman *et al*, 2001). Tumor-associated B7-H1 was recently shown to interact with CTLs through one or more receptors other than PD-1, thereby promoting the apoptotic cell death of effector CTLs via induction of FasL (Dong *et al*, 2002).

PD-1 is a member of the CD28 family, whose expression, unlike that of other CD28 family members, is not restricted to T cells. PD-1 can be found on activated T cells, but not on resting T cells, B cells and myeloid cells (Agata *et al*, 1996). PD-1 contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic tail and functions as a negative regulator of immune responses (Carter *et al*, 2002). Consistent with this notion, PD-1 KO mice develop late-onset progressive arthritis, and lupus-like glomerulonephritis and myocarditis (Nishimura *et al*, 1999a; Nishimura *et al*, 2001).

Finally, B7-H3, also known as B7-RP-2, is the latest member of the B7 family, and has been implicated in costimulation of T cell proliferative and cytotoxic responses, as well as selective IFN-γ production in the presence of simultaneous

TCR signaling (Chapoval *et al*, 2001). To the best of my knowledge, the cognate ligand for B7-H3 is not identified yet.

1.5.5 Other costimulatory interactions

Although CD28-B7 interactions are regarded as the most prominent source of costimulation, T cells from $CD28^{-/-}$ mice are capable of mounting a significant, albeit suboptimal, proliferative response following TCR stimulation (Kundig *et al*, 1996; Shahinian *et al*, 1993). This seems to indicate the existence of redundancy within the costimulatory machinery of T cells.

In addition to the CD28-B7 pathway, a number of different interactions between T cell signaling molecules and their corresponding ligands have been suggested to result in costimulatory activity. Among these interactions are Lymphocyte function-associated antigen (LFA)-1-intercellular adhesion molecule (ICAM)-1, CD2-CD48/CD58 (LFA-3), 4-1BB (CD137)-4-1BBL, OX40 (CD134)-OX40L, CD27-CD70 (CD27L), and CD40/CD40L interactions. A number of T cell surface molecules have also been implicated in the costimulation of T cell responses based solely on the ability of Abs recognizing these molecules to augment T cell proliferation and/or cytokine synthesis in response to TCR triggering (Watts and DeBenedette, 1999). CD5 and CD9 may best exemplify these molecules (Ledbetter *et al*, 1985; Tai *et al*, 1996). Whether or to what extent T cell responses to Ab-mediated ligation of such molecules correlates with the outcomes of T cell activation via engagement of these molecules with their natural ligands has yet to be elucidated.

1.5.6 Mechanisms of T cell costimulation

Several mechanisms have been documented or proposed to explain how costimulatory molecules in general, and CD28 in particular, work hand in hand with the TCR to induce T cell activation. Costimulation can have quantitative as well as qualitative effects in the context of T cell responses. CD28-mediated costimulation has been suggested to amplify Ag-specific signal 1 simply by tuning/lowering the TCR threshold for the Ag. When challenged with Ag in the absence of costimulation, naïve T cells are not usually activated despite down-regulating their TCRs. However, in the presence of a costimulatory signal, T cells respond to minimal doses of Ag that induce only modest TCR down-regulation (Cai *et al*, 1997; Iezzi *et al*, 1998; Viola and Lanzavecchia, 1996). In addition, the fact that several aspects of CD28 costimulation can be compensated for by increasing the dose of Ag or the duration of stimulation argues for the quantitative effects of costimulation (Iezzi *et al*, 1998; Kundig *et al*, 1996). CD28 recruits PI-3K (Pages *et al*, 1994), and can activate the *Src*-family PTK *Lck* via certain motifs present in its cytoplasmic domain (Holdorf *et al*, 1999). These biochemical properties are not unique to CD28 and are shared by the TCR, which leads to the concept that CD28-driven costimulation can intensify and prolong biochemical signals transduced by the TCR.

CD28 also participates qualitatively in the process of T cell activation. CD28 ligation results in up-regulation of the Bcl-xL anti-apoptotic protein, thereby leading to extended T cell survival upon activation (Boise *et al*, 1995). In addition, Bcl-xγ, another member of the Bcl-x family that is expressed only by activated T cells

requires CD28-dependent ligation for full expression (Ye *et al*, 2002). T cells from Bcl-x$\gamma^{-/-}$ mice display defective proliferative and cytokine responses to CD28-dependent costimulatory signals, and do not develop EAE following proteolipid protein peptide injection. Also importantly, forced expression of Bcl-xγ was recently shown to replace the requirement for B7-dependent ligation of CD28. Therefore, Bcl-xγ cytosolic protein is an essential component of the CD28-dependent signaling pathway that underlies T cell costimulation (Ye *et al*, 2002).

A key consequence of CD28-mediated costimulation is the enhanced production of IL-2, a major T cell growth and survival factor. CD28 signaling operates at both transcriptional (Fraser *et al*, 1991) and post-transcriptional levels (Lindstein *et al*, 1989) to up-regulate IL-2 mRNA synthesis and to prolong the stability of the synthesized IL-2 mRNA, respectively.

Recent studies suggest that costimulatory signals function to stabilize the immunological synapse. CD28 interaction with CD80 occupies the central cluster of the immunological synapse and is colocalized with the engaged TCR (Grakoui *et al*, 1999). Viola *et al* (1999) demonstrated that CD28 engagement promotes the recruitment of intracellular and membrane rafts to the TCR contact site when microbeads coated with anti-CD3 and anti-CD28 mAbs are used as surrogate APCs. The simultaneous engagement of the TCR/CD3 complex and CD28, but not that of TCR/CD3 complex alone, also augmented and sustained tyrosine phosphorylation of intracellular substrates and proteolytic degradation of *Lck*

Lipid rafts are specialized, cholesterol-rich microdomains that contain many crucial signaling elements and are regarded as membrane platforms for

signaling through immune recognition receptors including TCRs (Haeryfar and Hoskin, 2002). Costimulatory molecules other than CD28 such as murine CD2, CD5, CD9 and CD44 are present within lipid rafts where they may play a role in T cell activation (Yahiro-Ohtani *et al*, 2000). Unlike mouse CD2, human CD2 is not constitutively expressed within lipid rafts. However, human CD2 can be recruited into lipid rafts upon cross-linking by specific Abs or by CD58, its physiological ligand (Yang and Reinherz, 2001). Ab-mediated blockade of CD2-CD58 costimulatory interactions between cultured human umbilical vein endothelial cells and peripheral blood lymphocytes was recently shown to prevent lipid raft aggregation at the interface between the two cells, indicating that costimulation through CD2 is important for organization of the immunological synapse (Mestas and Hughes, 2001). It should be noted that in the above study, IFN-γ pretreatment was used to induce MHC II expression on endothelial cells, and a superantigen was used as the primary stimulus. Although this mode of T cell activation is clearly far from relevant to most physiological conditions where "professional" presentation of cognate Ag to Ag-specific T cells takes place, the finding that lipid rafts also play a role in costimulation mediated by non-professional APCs is notable.

1.6 Non-classical means of T cell activation

TCR-mediated detection of specific Ags leads to "clonal expansion" of T cells, typically with each clone displaying a TCR of unique specificity recognizing one single antigenic determinant or epitope. In contrast, allogeneic T cell responses usually involve more than just one T cell clone and can be assessed *in*

vitro by one-way or two-way mixed lymphocyte reaction (MLR). In one-way MLR, "responder" T cells recognize alloantigens displayed by "stimulator" cells that are not capable of proliferating as a result of being subjected to γ irradiation or treatment with mitomycin C, a DNA cross-linking agent. In two-way MLR, intact T cells from each party can act as both responder and stimulator cells to generate bulk cell proliferation in culture.

T cells can also be activated non-specifically by a variety of agents known as mitogens or polyclonal T cell activators. Polyclonal activators are often used to evaluate general lymphocyte responses in order to screen for immunodeficiency disorders. The most routinely used experimental means of polyclonal T cell activation include Abs to the TCR/CD3 complex or certain glycosylphosphatidylinositol (GPI)-anchored proteins such as Thy-1 (CD90), lectins, microbial products such as superantigens (sAgs) and ionomycin, and chemical reagents (*e.g.* phorbol esters). Induction of T cell proliferation by reagents such as anti-TCR$\alpha\beta$ or anti-CD3 Abs, the lectins phytohemagglutinin (PHA) and concanavalin A (ConA), and staphylococcal sAgs requires the presence of non-T cells as accessory cells in culture. Accessory cells provide FcR-mediated cross-linking of stimulatory Abs and/or costimulatory signals. In the case of staphylococcal enterotoxins, appropriate MHC II molecules need to be contributed by accessory cells in order for T cells to be activated (Kruisbeek and Shevach, 1994).

The presence of FcR-bearing accessory cells is an absolute requirement for T cell activation when mitogenic Abs to the TCR/CD3 complex are used in

suspension. The presence of accessory cells can be compensated for by immobilizing these Abs on plastic surfaces such as those of microtiter plates. However, the proliferative responses induced under these conditions may be only suboptimal (Jenkins *et al*, 1990). This could be due to the absence of important costimulatory interactions, although T cells can presumably costimulate each other, albeit less efficiently. The absence of significant costimulation in these experimental set-ups can be overcome by co-coating with anti-TCR/CD3 and anti-CD28 mAbs. Recently, the use of cell-sized microbeads as a matrix for coating mAbs has gained tremendous popularity among investigators. Ab- or ligand-coated beads may be superior to other forms of coated plastic surfaces due to the spherical shape of microbeads, which would increase the chances of interactions with responding T cells and better mimic the physiological interactions between T cells and APCs. One advantage of using microbeads over accessory cells is that coating with known doses of appropriate Abs helps zero in on the molecular interactions in question without possible interference from other cell surface molecules. However, one should be aware that plastic-bound Abs do not necessarily induce the same response as the corresponding natural ligands. Moreover, the use of Ab-coated beads as surrogate APCs eliminates the possible participation of T cell partners which may prove essential for certain features of T cell responses. For instance, DCs have been shown to "actively" participate in forming synapses with naïve T cells by rearranging their actin cytoskeleton (Al-Alwan *et al*, 2001).

Lectins are plant-derived proteins that can bind certain carbohydrate moieties. ConA and PHA, both extracted from jack beans, cross-link all TCRs irrespective of their Ag specificity and thus activate T cells non-specifically (Kruisbeek and Shevach, 1994; Novogrodsky and Katchalski, 1971). Pokeweed mitogen (PWM) is another lectin that can activate both human T cells and B cells indiscriminately (Mellstedt, 1975).

Superantigens are bacterial or viral proteins that can induce vigorous proliferation by both $CD4^+$ and $CD8^+$ $\alpha\beta$ T cells and sometimes $\gamma\delta$ T cells. Unlike conventional Ags, sAgs bind as unprocessed proteins to invariant regions of MHC class II molecules on the surface of APCs and to particular segments of the V_β region of TCR$\alpha\beta$ outside of the antigen-binding cleft, and stimulate, at nano- to picogram doses, up to 10-30 % of the host T-cell repertoire. The magnitude of sAg-mediated responses is overwhelming when compared with the activation of only one in every 10^5-10^6 T cells (0.01-0.0001 %) in response to any given conventional antigenic peptide binding to TCR. T cell activation by sAgs therefore leads to massive release of pro-inflammatory and other cytokines, which play a pivotal role in the pathogenesis of the diseases provoked by sAg-producing microorganisms and may bear disastrous consequences such as shock and death (Field *et al*, 1996; Muller-Alouf *et al*, 2001). These cytokines cause systemic toxicity on one hand and general suppression of adaptive immune responses on the other hand. Staphylococcal sAgs such as staphylococcal enterotoxins and the toxic shock syndrome toxin –1 (TSST-1), and mycoplasma arthritidis mitogen (MAM) are the best examples of bacterial sAgs.

Staphylococcal enterotoxins and TSST-1 are directly responsible for many symptoms of staphylococcal food poisoning and toxic shock syndrome, respectively. The role of viral sAgs is less clear. Endogenous viral sAgs are common in mice and have a profound impact in shaping the TCR repertoire since some of these sAgs function during thymic education to delete thymocytes expressing TCRs containing certain V_β regions. The endogenous sAgs of mice are typically encoded by mouse mammary tumor virus (MMTV) genes that have become integrated into the host genome. These sAgs generate strong primary T cell responses when T cells from a mouse strain lacking the sAg are stimulated by B cells from MHC-compatible mice that express it (MacDonald *et al*, 1988).

Non-specific activation of T cells can also be achieved by phorbol 12-myristate 13-acetate (PMA) in combination with ionomycin or other ionophores such as A23187. Phorbol esters, which were originally identified as tumor promoting agents, mimic the action of DAG resulting in the activation of PKC. Ionomycin is an antibiotic derived from *Streptomyces conglobatus* that carries calcium through the plasma membrane to increase the calcium concentration in the cytoplasm. The combination of PMA and ionomycin thereby bypasses the requirement for TCR cross-linking and is commonly used in T cell activation protocols (Miller, 1986).

Finally and most related to the subject of this research, mAbs targeting certain epitopes of Thy-1 can induce T cell activation, as manifested by proliferation and IL-2 production (Gunter *et al*, 1984; Kroczek *et al*, 1986a). Although the T cell proliferative responses induced by anti-Thy-1 mAbs are of

relatively low magnitude, these responses can be dramatically and synergistically amplified by concomitant stimulation with PMA (Kroczek *et al*, 1986a). Therefore, most T cell activation protocols involving Thy-1 employ a combination of anti-Thy-1 mAb and PMA. How Thy-1 cross-linking leads to T cell activation is not precisely clear and will be discussed in great detail later.

1.7 Thy-1: history, structure and distribution

The ever-enigmatic Thy-1 (CD90) was initially described by Reif and Allen more than three decades ago as a cell differentiation marker expressed mainly in the brain and the thymus of mice (Reif and Allen, 1964). Thy-1 is found on the surface of a variety of normal cell types. In addition, many tumor cells originating from Thy-1$^+$ or Thy-1$^-$ normal cells also express Thy-1. In all species studied thus far, Thy-1 is present on brain cells, predominantly neuronal cells, and also on fibroblasts (Pont, 1987; Firer *et al*, 1995). With the exception of the brain and perhaps fibroblasts, different species exhibit remarkable diversity in terms of Thy-1 expression and tissue distribution. Thy-1 is very abundant on mouse and rat thymocytes, but only a small percentage of human thymocytes residing in the thymic cortex are positive for Thy-1. Also, while Thy-1 is routinely used as a mouse T cell marker, it is almost completely absent from rat and human peripheral T cells (Crawford and Barton, 1986; Mansour *et al*, 1987; Firer *et al*, 1995). In the thymus, Thy-1 is probably the most abundant glycoprotein on the surface of thymocytes, being present at about one million copies per cell (compared with ~ 15,000 copies of CD4 per cell). By one calculation, Thy-1 occupies 10-20 % of the entire thymocyte surface area (Mason and Williams,

1980; Killeen, 1997). In contrast, the amount of cell surface Thy-1 on murine lymph node cells has been estimated at 200,000 copies per cell (Carlsson and Stigbrand, 1983).

Thy-1 expression is inducible on certain cell types. For instance, PC12 rat pheochromocytoma cells up-regulate Thy-1 upon treatment with NGF, phorbol ester, and the calcium ionophore A23187 (Doherty *et al*, 1988). Phorbol ester or TNF can also induce Thy-1 expression on human dermal microvascular endothelial cells (HDMECs)(Mason *et al*, 1996; Saalbach *et al*, 2000).

The mouse and human Thy-1 loci map to chromosomes 9 and 11, respectively (Pont, 1987; Seki *et al*, 1985). The Thy-1 locus in the mouse includes two alleles termed Thy-1^a and Thy-1^b, which code for Thy-1.1 and Thy-1.2 glycoproteins, respectively (Pont, 1987). Thy-1.1 and Thy-1.2 alloantigens differ solely in amino acid position 89, which is occupied by Arginine in Thy-1.1 and by Glycine in Thy-1.2. Thy-1.2 is expressed by most mouse strains, and Thy-1.1 is an alloantigen of the AKR/J and PL strains. In addition to alloantigenic determinants, species-specific, as well as species cross-reacting determinants also exist on Thy-1 (Firer *et al*, 1995).

Thy-1 is the smallest member of the IgSF, a GPI-anchored resident of lipid rafts, and an integral glycoprotein of 24-25 KDa in which a core protein of 111 amino acid is N-glycosylated at three sites. Unlike rodent Thy-1, human Thy-1 contains two instead of three glycosylation sites (Seki *et al*, 1985). Thy-1 is one of the most heavily glycosylated membrane glycoproteins with a carbohydrate content comprising up to 30 % of its molecular weight (Pont, 1987). The main

structural differences between brain and thymic Thy-1 reside in their carbohydrate compositions, which are also responsible for differences in apparent molecular weights. For instance, galactosamine is present only on brain Thy-1. In contrast, the amounts of sialic acids on thymic Thy-1 far exceed those of brain Thy-1 (Barclay *et al*, 1976). Furthermore, comparative studies between thymocytes and lymph node cells have revealed that the sialic acid content of Thy-1 increases in parallel with T cell maturation (Hoessli *et al*, 1980).

Research on Thy-1 over the years has been extremely fruitful. Thy-1 was the first lymphocyte surface Ag shown to be restricted to a functional subset of lymphocytes, *i.e.* T cells in the mouse. Anti-Thy-1 antisera provided immunologists with the first reagents that could discriminate mouse T cells from B cells (Raff, 1971). Secondly, structural studies on Thy-1 helped establish the foundation of the IgSF (Williams and Gagnon, 1982). Thirdly, the structural analysis of Thy-1 led to the first biochemical description of a vertebrate glycosylphosphatidylinositol (GPI) anchor (Low and Kincade, 1985; Tse *et al*, 1985). Paradoxically, despite this lengthy and rewarding history, the definite function(s) of Thy-1 itself has remained a mystery.

1.7.1 Thy-1: outstanding questions

A major open question with regard to Thy-1 biology is whether or not Thy-1 has a ligand/coreceptor. Although Thy-1 has been reported to bind or associate with several molecules, no ligand for Thy-1 has been defined in firm molecular terms. This is particularly important in the context of the signaling properties of Thy-1 as most of our knowledge on this issue stems from experiments using

cross-linking Abs to Thy-1 whose triggering action is postulated to mimic that of the presumed ligand for Thy-1.

In the nervous system, astrocytes seemingly express a putative Thy-1 ligand that prevents neurite outgrowth. In an adhesion assay system using the EL-4 thymoma cells that express high levels of Thy-1, Thy-1 was shown to interact with β_3 integrin on astrocytes (Leyton *et al*, 2001).

Human polymorphonuclear (PMN) leukocytes and monocytes, but not lymphocytes, also appear to express a putative Thy-1 ligand that may be involved in monocyte and PMN binding to Thy-1-expressing activated endothelial cells and fibroblasts (Saalbach *et al*, 2000). A Thy-1 ligand was also detected on melanoma cells, which interacts with HDMECs in cell adhesion assays (Saalbach *et al*, 2002). Interestingly, Thy-1 expression by endothelial cells is stimulated *in vitro* following treatment with soluble factors derived from melanoma cells. These observations clearly suggest a role for Thy-1 in the context of tumor metastasis.

In T cells, a physical association between Thy-1 and MHC I has been previously reported (Volarevic *et al*, 1990). Moreover, T cells obtained from β_2M KO mice showed reduced proliferation in response to anti-Thy-1 mAb, but not to anti-CD3 mAb (Amirayan *et al*, 1995). A similar association between Thy-1, TCR, and CD45, a transmembrane tyrosine phosphatase, was reported by Volarevic *et al* (1990). In this study, immunoprecipitated Thy-1 or TCR from lysates of cross-linked T cells also contained CD45 tyrosine phosphatase activity, leading to the speculation that CD45 may be the physical and functional link between TCR and Thy-1 (Volarevic *et al*, 1990). From a functional point of view, T cell clones

deficient in CD45 fail to respond to anti-Thy-1 mAb, but do respond to PHA, ConA, or a combination of PMA and ionomycin (Pingel *et al*, 1994).

Thy-1 also co-immunoprecipitates with p100, a transmembrane protein, which may be required for signaling through Thy-1 (Lehuen *et al*, 1995). This notion is suggested by the finding that only $p100^+$ CD4 T cells proliferate and synthesize cytokines in response to Thy-1 triggering, while cell proliferation in response to either anti-CD3 mAb or ConA is virtually identical in $p100^+$ and $p100^-$ CD4 T cell subsets.

The second major issue that remains unresolved is the exact function of Thy-1 in general, and in the context of immune responses in particular. As we will see in the following section, even modern and powerful tools such as Thy-1-deficient or –overexpressing cells or clones, and Thy-1 KO mice have failed to clearly define the exact biological role(s) of Thy-1 in the context of T cell activation. Nevertheless, future findings on how T cell responses are modulated are expected to fuel interest in the role of Thy-1 in the context of T cell biology.

1.7.2 Thy-1 and T cell activation: current controversies

A role for Thy-1 has long been implicated in the process of T cell activation. Cross-linking of Thy-1 by certain mAbs results in IL-2 production and proliferation by T cells (Gunter *et al*, 1984; Kroczek *et al*, 1986a) as well as apoptotic cell death in thymocytes (Fujita *et al*, 1997). However, the function of Thy-1 in this context is far from clear. A number of roles have been proposed for Thy-1, ranging from negative selection of thymocytes (Nakashima *et al*, 1991; Hueber *et al*, 1994) and negative regulation of T cell responses (Hueber *et al*,

1997; Killeen 1997) to fulfilling an accessory/costimulatory function during T cell activation (Johnson *et al*, 1993; Leyton *et al*, 1999). The observations that ectopic expression of Thy-1 in inappropriate cells or tissues such as B cells and kidney tubular epithelia leads to proliferative abnormalities (Chen *et al*, 1987; Kollias *et al*, 1987) support a role for Thy-1 in growth control. However, Thy-1 is not required for T cell development since the number and the phenotype of thymocytes and mature T cells in Thy-1-deficient mice are normal (Hueber *et al*, 1997).

The fact that Thy-1, which does not even possess a classical transmembrane portion or a cytoplasmic tail, could act as a signaling molecule for T cell activation has puzzled immunologists for many years. Signaling through neighboring molecules (Gunter *et al*, 1987), an indirect effect of Thy-1 on TCR signal transduction imposed by the highly abundant expression of Thy-1 (Killeen, 1997), and formation of membrane rafts that would consequently sequester signaling molecules such as PTKs (Ilangumaran *et al*, 2000) have all been proposed as likely mechanisms to account for Thy-1 signaling.

A possible interplay between the TCR/CD3 complex and Thy-1 has been suspected since the requirement for coexpression of an intact, functional TCR/CD3 complex in Thy-1-mediated activation was reported in several different studies using TCR/CD3-deficient or -altered T cells (Gunter *et al*, 1987; Sato *et al*, 1990; Schmitt-Verhulst *et al*, 1987). However, the finding that human B cells transfected with mouse Thy-1 are capable of responding to anti-Thy-1 mAb with a rise in cytoplasmic Ca^{++} (Kroczek *et al*, 1986b) casts doubt on the above-

mentioned findings, and seems to indicate that at least some aspects of Thy-1 signaling are independent of TCR and other T cell specific molecules.

Reciprocally, T cells deficient in GPI-anchored proteins including Thy-1 show impaired early events in TCR-mediated signal transduction, including tyrosine phosphorylation of TCR-ζ chain and ZAP-70, and activation of TCR-associated *Src* kinases *Fyn* and *Lck* (Leyton *et al*, 1999; Romagnoli and Bron, 1997; Yeh *et al*, 1988). However, these observations are clearly contrary to the finding that murine T lymphocytes pretreated with phosphatidylinositol-specific phospholipase C to remove GPI moieties fail to exhibit a reduced proliferative response to anti-CD3 mAb (Presky *et al*, 1990). Consistently, T cell development as well as T cell activation in response to anti-CD3ε mAb or ConA, as judged by increased CD25 and CD69 expression, IL-2 secretion and proliferation, is unaffected in mice lacking GPI-anchored proteins exclusively on T lymphocytes (Takahama *et al*, 1998). These animals were established by crossing mice carrying *lox-P* sites within their phosphatidylinositol glycan-class A (*PIG-A*) gene with mice carrying the *Cre* recombinase gene driven by the T cell specific $p56^{Lck}$ proximal promoter. The offspring exhibited specific disruption of *PIG-A* in thymocytes and almost complete loss of GPI-anchored proteins on peripheral T cells.

At least five genes including *PIG-A* are involved in biosynthesis of the GPI anchor. PIG-A encodes a protein involved in the first step of GPI anchor biosynthesis, namely the transfer of N-acetyl-glucosamine from UDP-N-acetyl-glucosamine to phosphatidylinositol (Marmor and Julius, 2000). The *PIG-A* gene

mutations are encountered in all patients with paroxysmal nocturnal hemoglobinuria (PNH), and are believed to be the sole reason for GPI deficiency in PNH (Nishimura *et al*, 1999b; Rosse and Ware, 1995). Because *PIG-A* is X-linked, a single mutation of this gene is sufficient to abolish cell surface expression of GPI-anchored proteins.

PNH is an acquired clonal hematopoietic stem cell disorder characterized by hemolysis with acute nightly exacerbation, pancytopenia of varying severity, and a tendency for thrombosis. The affected hematopoietic cells are deficient in GPI-anchored proteins, most importantly decay accelerating factor (DAF/CD55) and CD59 (Nishimura *et al*, 1998). Absence of these complement regulatory proteins in PNH results in complement-mediated hemolysis and hemoglobinuria. Interestingly, T cell responses in PNH vary considerably depending on the nature of the stimuli. GPI-deficient and GPI-expressing T cells obtained from the same patients respond similarly to TCR triggering by inducing Ca^{++} flux, proliferating, and producing IFN-γ. In contrast, GPI^- T cells responded less vigorously than GPI^+ T cells to PHA (Schubert *et al*, 1992). Moreover, T cell responses to alloantigen stimulation were diminished in GPI-deficient T cells (Schubert *et al*, 1995).

The results of studies with Thy-1 KO mice have also yielded contradictory conclusions. A negative regulatory function for Thy-1 was suggested based on the hyper-responsiveness of Thy-$1^{-/-}$ thymocytes to TCR triggering (Hueber *et al*, 1997), whereas peripheral T cells from Thy-$1^{-/-}$ mice exhibit a diminished Ca^{++} influx and reduced PTK activity following TCR stimulation (Beissert *et al*, 1998).

Finally, Thy-1 has been implicated in providing or regulating T cell costimulation. Several studies have assumed a costimulatory function for Thy-1. For instance, anti-CD3 mAbs only moderately activated EL-4 thymoma or 2B4 hybridoma cell lines, while costimulation with anti-Thy-1 mAb strongly enhanced TCR signaling (Leyton *et al*, 1999). On the other hand, a role for T cell-associated Thy-1 was suggested in negatively regulating B7-2 expression on spleen-derived APCs, as well as interfering with the costimulatory activity of spleen APCs and lipopolysaccharide (LPS)-activated B cells (Wu *et al*, 1995).

All the above findings emphasize the controversy that exists within the literature with regard to the function(s) of Thy-1 in initiation and modulation of various aspects of T cell biology. Despite the existing confusions and controversies, most, if not all, immunologists seem to have no doubt that Thy-1 and other GPI-anchored proteins are amongst the major players in the initiation and regulation of T cell-mediated immune responses.

1.8 Rationales, objectives and specific aims

Current information and existing controversies regarding Thy-1 justifies continued research on this enigmatic molecule. Although, with the exception of neuronal cells, fibroblasts and certain thymocytes, Thy-1 is not expressed by human T cells, the studies of Thy-1, as a prototype GPI-anchored protein, may reveal new biological function(s) for similar GPI-anchored proteins. In addition, the role of Thy-1 may be assumed by alternative membrane proteins such as CD7 in human, a protein that shares several characteristics with murine Thy-1 such as cell distribution and gene sequence, and is hence regarded as the

human homolog of Thy-1 (Firer *et al*, 1995; Schanberg *et al*, 1991). Thy-1 is a lipid raft marker, and studies of Thy-1 hold promise of further elucidating the importance of these membrane microdomains in the initiation, perpetuation and amplification of signal transduction pathways, especially those triggered in T cells.

As elaborated previously, numerous studies have been conducted, albeit to little avail, to identify a "definite" role for Thy-1 in the context of T cell responses. Interestingly, the effect(s) of Thy-1 triggering on certain aspects of T cell responses have not been addressed or are only vaguely understood. For instance, the contribution of Thy-1 to CTL induction, the downstream intracellular elements of Thy-1 signaling, and the effect of strong costimulation on Thy-1-stimulated T cell activation have not yet been investigated.

The objective of this work is to examine the role of Thy-1 signaling in induction of T cell proliferative and cytotoxic responses in the presence or absence of appropriate costimulation.

The specific aims are: I) *to determine the effect(s) of Ab-mediated blockade of Thy-1 during the induction phase of AK-T cell development* II) *to compare and contrast TCR- and Thy-1-driven signal transduction pathways in terms of downstream signaling requirements using selective pharmacological inhibitors* III) *to examine the effect of CD28-B7 costimulatory interactions in modulation of Thy-1-stimulated T cell responses.*

1. Materials and Methods

1.1 Mice

Adult (6-8-weeks old) mice were used in all experiments. C57BL/6 mice were purchased from Charles River Canada (Lasalle, PQ). Fc receptor γ-chain KO mice ($FcR\gamma^{-/-}$) on BALB/c background (model # 000584-M) and the wild-type (WT) controls ($FcR\gamma^{+/+}$) matched for age and sex were purchased from Taconic Farms (Germantown, NY). BALB/c mice carrying the I-A^d-restricted rearranged TCR transgene TgN (DO11.10) were obtained from the National Institutes of Health (Bethesda, MD). The transgenic TCR expressed by these mice recognize amino acids 323-339 of ovalbumin (Murphy *et al*, 1990). The expression of the transgenic TCR by the majority (typically greater than 82%) of TgN T cells was confirmed via staining by the mAb KJ1-26 (Caltag Laboratories, Burlingame, CA). Mice were housed in the Carleton Animal Care Facility of Dalhousie University and maintained on standard rodent chow and water supplied *ad libitum*.

1.2 Culture medium

RPMI 1640 medium (ICN Biomedicals Canada Ltd., Mississauga, ON) was supplemented with 5% heat-inactivated fetal bovine serum (FBS)(Life Technologies, Burlington, ON), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 mM HEPES buffer (pH 7.4), and will, hereafter, be referred to as complete medium.

1.3 Cell lines

The murine mastocytoma cell line P815 (H-2^d), the B lymphoma cell line A20 (H-2^d), the T lymphoma cell line EL4 (H-2^b), and the IL-2-dependent T cell

line CTLL-2 were obtained from the American Type Culture Collection (ATCC; Manassas, VA). The Fas-transfected P815 cell line (Lee *et al*, 1996) was kindly provided by Dr. E. Podack (University of Miami, Miami, FL). P815, P815-Fas, A20 and EL4 cell lines were all maintained in complete medium. CTLL-2 cells were grown in complete RPMI 1640 medium containing 10% FBS and 20 U/ml (~4 ng/ml) recombinant mouse IL-2 (PeproTech, Inc., Rocky Hill, NJ).

2.4 Antibodies and reagents

Antibodies used in this research have been listed in Table 2.1. Purified anti-CD3-Fos (also known as 145-2C11-Fos) was a generous gift from Dr. J. Tso (Protein Design Labs, Inc., Mountain View, CA). Anti-CD3-Fos is a genetically engineered $F(ab')_2$-like anti-murine CD3ε Ab. The variable (V) regions of this chimeric protein are from hamster anti-mouse CD3ε mAb (145-2C11) joined to the C_κ and CH_1 constant (C) regions and hinge region all adopted from mouse IgG_{2a}. The leucine zipper Fos is fused to the hinge, and functions to bring the two Fab' fragments together. FITC-conjugated mouse anti-rat IgG $F(ab')_2$ used in flow cytometry analyses as a secondary Ab reagent was from Jackson ImmunoResearch Laboratories (West Grove, PA).

Rat IgG_{2b}, hamster IgG, fluorescein isothiocyanate (FITC)-rat IgG_1 and phycoerythrin (PE)-hamster IgG isotype controls were purchased from Cedarlane Laboratories (Hornby, ON). Rat IgG_{2c} isotype control (clone A23-1 with unknown specificity) was obtained from BD Pharmingen (Mississauga, ON).

Ovalbumin peptides $OVA_{323-339}$ (NH_2-ISQAVHAAHAEINEAGR-COOH) and $OVA_{324-334}$ (NH_2-SQAVHAAHAEI-COOH) were from Bethyl Laboratories, Inc.

(Montgomery, TX). $OVA_{323-339}$ and $OVA_{324-334}$ were used as cognate and control peptides, respectively, in experiments addressing Ag-specific T cell responses. $OVA_{324-334}$ binds I-A^d but does not induce responses from transgenic T cells expressing the DO11.10 TCR (Murphy *et al*, 1990). The purity of the peptides was greater than 98% as assessed by high performance liquid chromatography according to the supplier.

Herbimycin A was purchased from Life Technologies (Burlington, ON). LY294002 and calphostin C were from Research Biochemicals Int. (Natick, MA). Cyclosporine A (CsA) was purchased from Sigma Chemical Co. (St. Louis, MO). PD98059 and SB203580 were a generous gift from Dr. Bill Pohajdak of Dalhousie University. SK&F 86002 was obtained from Calbiochem (San Diego, CA). CsA was initially dissolved in absolute ethanol, while stock solutions of other inhibitors were made in dimethyl sulfoxide (DMSO; Sigma Chemicals Co.) and stored at -20°C or at 4°C in the case of SK&F 86002. Working solutions, which always contained less than 0.1 % DMSO, were prepared in complete medium immediately before use.

2.5 T cell isolation

Mice were sacrificed by cervical dislocation, and spleen cell suspensions were prepared in ice-cold phosphate-buffered saline (PBS, pH 7.2) using a tissue homogenizer. Cell preparations were then depleted of erythrocytes via hypo-osmotic shock and passaged through nylon wool (Polysciences, Warrington, PA) columns to enrich for T lymphocytes (Julius *et al*, 1973). Nylon wool-non-adherent cells were subsequently treated with polyclonal rabbit anti mouse-asialo

GM1 antiserum (at a 1:40 final dilution) and Low-Tox rabbit complement (1:12, Cedarlane) to eliminate NK cells. This was to avoid the potential generation of confounding data due to the reported expression of Thy-1 by NK cell subsets (Hurme and Sihvola, 1984), constitutive expression of cytotoxic effector molecules by NK cells (Arase *et al*, 1995; Mori *et al*, 1997), and secondary activation of NK cells exposed to cytokines produced by stimulated T cells (Stankova *et al*, 1989). The resulting cell preparations typically contained 81% $CD3^+$ and 83% Thy-1.2^+ cells by flow cytometry. The majority of non-T cells in these preparations were B cells (typically 12% $B220^+$), which are needed to provide costimulation and Fc receptor (FcR)-mediated cross-linking of soluble anti-CD3 mAb (Okamoto *et al*, 1995). For experiments requiring highly purified T cell preparations, spleen cells were passaged through nylon wool twice and incubated in a plastic Petri dish at 37°C for 1 hr to remove plastic-adherent macrophages. This was followed by a two-step treatment with anti-B220 mAb (1:2 dilution of hybridoma supernatant) and anti-asialo-GM1 antiserum plus complement to deplete B cells and NK cells, respectively. Alternatively, T cell recovery column kits (Cedarlane) were used to isolate total or $CD4^+$ T cells. The cell preparations thus obtained typically contained 95-99 % T cells by flow cytometry.

In several experiments, nylon-wool non-adherent T cells were treated with anti-CD4 (GK1.5) or anti-CD8 (3.155) mAb, both at a 1:2 dilution of hybridoma supernatant, plus complement to obtain highly enriched $CD8^+$ or $CD4^+$ T cell preparations, respectively.

2.6 Dendritic cell preparation

Dendritic cells were generated from bone marrow precursors as described by Lutz *et al* (1999). Precursor cells were flushed out of femurs and tibias and washed using cold PBS. Erythrocytes were eliminated by hypo-osmotic shock and marrow cells were plated into sterile petri dishes at 2×10^6/10 ml complete medium containing 10% FBS, 200 U/ml recombinant mouse granulocyte macrophage-colony stimulating factor (GM-CSF)(Cedarlane), and 50 μM 2-mercaptoethanol (2ME). On day 3 of the culture, fresh medium containing 200 U/ml GM-CSF was added into the plates, followed by replacing 10 ml of the medium on day 6. On day 9 of the culture, plastic-nonadherent cells were collected and resuspended in 10 ml of complete medium containing 10% FBS, 2ME, 100 U/ml GM-CSF plus 1 μg/ml LPS (Sigma). Cells were finally harvested on day 10 and used in our activation protocol. The harvest typically consisted of more than 95% terminally differentiated dendritic cells as assessed by CD11c, MHC II and B7-2 expression.

2.7 B cell isolation

Erythrocyte-free spleen cells were depleted of T cells by a two-step treatment with anti-Thy-1.2 mAb and rabbit complement (1:12, Cedarlane). The resulting cell suspension was subsequently incubated on a sterile plastic petri dish for 1 hr at 37°C to remove macrophages. Plastic-nonadherent cells, which were highly enriched for B cells, were then harvested and used immediately.

2.8 T cell activation

Between 2-2.5 × 10^5 T cells in a total volume of 200 μl/well in U-bottom microplates (Sarstedt Inc., Newton, NC) were stimulated by an optimal concentration of soluble anti-CD3 mAb (1:20 dilution of hybridoma supernatant), G7 anti-Thy-1 mAb (6 μg/ml), G7 (6 μg/ml) plus PMA (10 ng/ml), or a combination of PMA (15 ng/ml) and ionomycin (500 ng/ml). When larger T cell yields were required, 8-10 × 10^6 T cells/2ml/well were stimulated using the above stimuli in 24-well flat-bottom plates (Sarstedt).

DCs, B cells or LPS-activated B lymphoblasts were used as accessory cells in a number of experiments in which highly purified T cells were stimulated with anti-CD3 or anti-Thy-1 mAb. In several experiments, highly purified T cells were combined in flat-bottom microplates (Sarstedt) at a 1:1 ratio with cell-sized (10 μm) microbeads (Polysciences) coated with indicated mAbs used as surrogate APCs.

Antigen-specific activation of $CD4^+$ TCR transgenic T cells was induced with cognate peptide $OVA_{323-339}$ (300 nM) plus syngeneic DCs. The $OVA_{324-334}$ peptide was always used in parallel as a negative control. All cultures were incubated at 37°C and 5% CO_2 in a 95% humidified atmosphere for indicated time periods.

2.9 B cell activation with LPS

B cells were seeded in 25-cm^2 tissue culture flasks at 5 × 10^6 cells/ml in complete medium and incubated for 72 h in the presence of LPS (5 μg/ml). LPS-

activated B lymphoblasts were then harvested, washed extensively and used as accessory cells in T cell activation protocols.

2.10 Trypan blue dye exclusion

Viable T cell recovery was always determined at the end of cultures by counting T cells in an isotonic preparation of trypan blue, a vital dye that live cells are able to exclude. T cell numbers were always corrected for viability prior to use in functional analyses such as cytotoxicity and conjugation assays.

2.11 Proliferation assay

T cells were pulsed with 0.5 μCi of tritiated thymidine ([^{3}H]TdR; specific activity 60 Ci/mmol; ICN Canada) during the final 6 or 18 h of the cultures. The cells were then harvested onto glass fiber filter mats (Skatron, Sterling, VA) using a Titer-Tek multiple sample harvester, and [^{3}H]TdR uptake was determined by liquid scintillation counting as a measure of DNA synthesis.

2.12 ^{51}Cr release assay

T cells were harvested, washed, resuspended in complete medium, and seeded into wells of a 96-well, V-bottom plate (Sarstedt). Target cells were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ (ICN Canada) for 1 h at 37°C, washed extensively, and added to the plate at 5 $\times 10^3$ cells/well. The plates were centrifuged at 400 × g for 5 min at the end of a 4-hr incubation at 37°C. A 100-μl aliquot of supernatant was then harvested from each well and the ^{51}Cr content of the samples was determined with a Beckman γ-counter. Specific lysis of the target cells was determined using following formula: % Specific lysis = [(ER-SR)/(TR-SR)] ×100, where ER (experimental release) is obtained from wells

containing both T cells and target cells, while TR (total release) and SR (spontaneous release) are determined from wells receiving only target cells and medium or 10% sodium dodecyl sulfate, respectively. A cytolytic anti-mouse Fas mAb (clone Jo2) was used as a positive control to induce cell death in Fas-expressing target cells. In some experiments, anti-CD3 or anti-Thy-1 mAb was present in the effector phase of killing for the assessment of redirected lysis of the target cells.

2.13 Conjugation assay

P815 tumor cells were incubated in filter-sterilized complete medium containing 1 mg/ml neutral red dye (Sigma) at 37°C for 30 min. Neutral red stains the cytoplasmic granules of P815, thereby making P815 cells easily distinguishable from T cells. T cells and stained P815 cells were thoroughly washed and resuspended in complete medium at 5×10^6 cells/ml. A 100-µl aliquot of T cells was mixed with an equal volume of P815 cells in 12 × 75 mm round-bottomed polystyrene tubes (Becton Dickinson, Franklin Lake, NJ). The cells were then pelleted by centrifugation at 100 × g for 5 min followed by incubation at 37°C for 45 min. The tubes were subsequently placed on ice and conjugates were gently resuspended immediately before counting. At least 100 T cells were counted under a light microscope and the percentage of T cells conjugated to P815 was determined in triplicate tubes for each treatment group.

2.14 Semiquantitative RT-PCR

Total RNA was extracted at 4, 18 or 48 h post-stimulation using TRIzol reagent. Approximately 1 µg RNA was then reverse-transcribed using 200 U of

Moloney murine leukemia virus (MMLV) reverse transcriptase (RT) in the presence of 0.5 mM deoxynucleotide triphosphates (dNTPs), 1 μg of random hexanucleotide primers and 10 mM dithiothreitol (DTT). Complementary DNA (cDNA) samples were diluted 1:10 in pyrogen-free water and subjected to polymerase chain reaction (PCR) in an automatic thermal cycler (MJ Research, Inc., Watertown, MA). Each PCR reaction mixture also included 2.5 U of native Taq DNA polymerase, 0.2 mM dNTPs, and primer pairs at 0.5 μM each in a 1:10 dilution of a PCR buffer containing 500 mM KCl, 100 mM Tris-HCl (pH 9.0) and 15 mM $MgCl_2$. The amplification of equal amounts of cDNA for different treatment groups was ensured through normalization based on the steady-state expression of the house-keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). All primers were designed to bind intron-bridging exons of the genes in question so possible contamination with genomic DNA would not escape our attention. Primer sequences were the following (the amplicon size is given after the reverse primer):

GAPDH (F) 5'-ACTCACGGCAAATTCAACGGC-3'

GAPDH (R) 5'-ATCACAAACATGGGGGCATCG-3' (246 bp)

PFN (F) 5'-TCAATAACGACTGGCGTGTGG-3'

PFN (R) 5'-GTGGAGCTGTTAAAGTTGCGG-3' (252 bp)

GzmB (F) 5'-GCCCACAACATCAAAGAACAG-3'

GzmB (R) 5'-GAGAACACATCAGCAACTTGGG-3' (889 bp)

FasL (F) 5'-ATGGTTCTGGTGGCTCTGGT-3'

FasL (R) 5'-GTTTAGGGGCTGGTTGTTGC-3' (362 bp)

IL-2 (F) 5'-TGATGGACCTACAGGAGCTCCTGAG-3'

IL-2 (R) 5'-GAGTCAAATCCAGAACATGCCGCAG-3' (170 bp)

The amplification protocols for GAPDH (28 cycles), PFN (32 cycles), and FasL (30 cycles) were as follows: denaturation at 92°C for 30 sec, annealing at 57°C for 30 sec, and primer extension at 72°C for 90 sec. For GzmB amplification (30 cycles), primer extension at 72°C was performed for 2 min instead. For IL-2 (28 cycles), the above steps were followed at 94°C for 30 sec, 57°C for 30 sec, and 72°C for 60 sec, respectively. The number of PCR cycles was initially determined to yield PCR products during the exponential phase of amplification. RT-PCR performed under these conditions provides reliable detection of differences equal to or greater than two fold in mRNA levels (Singer-Sam *et al*, 1990). PCR products were resolved on Tris-acetate-EDTA (TAE) agarose gel containing ethidium bromide and visualized by UV light illumination in parallel with a 100-bp DNA ladder. All the reagents used for RNA isolation and RT-PCR were from Invitrogen Canada, Inc. (Burlington, ON) except for Taq DNA polymerase and 10X PCR buffer which were purchased from Amersham Biosciences (Baie d'Urfé, PQ).

2.15 Cytokine quantification by ELISA

T cell culture supernatants were isolated at 24 h post-stimulation. The cytokine contents of the supernatants were immediately measured by sandwich enzyme-linked immunosorbent assay (ELISA) using capture and detection mAbs, recombinant cytokines and assay protocols all supplied by BD Pharmingen.

2.16 IL-2 bioassay

CTLL-2 cells were washed twice and resuspended in complete medium (containing 10% FBS) without IL-2 followed by incubation at 37°C for 1 h. Subsequent to IL-2 deprivation, cells were washed and seeded into wells of a 96-well flat-bottomed plate at 10^4 cells/well. Cell-free supernatants were collected from 24-h T cell cultures and immediately added at 50-μl aliquots into wells and the final volume was adjusted to 200 μl/well. Recombinant mouse IL-2 (1 U ≅ 200 pg) was used in parallel as positive control. The CTLL-2 cultures were then incubated at 37°C for 20-24 h with [^{3}H]TdR being present at 0.5 μCi for the final 8 h. [^{3}H]TdR uptake by CTLL-2 cells was then determined as an indirect measure of IL-2 bioactivity present in T cell culture supernatants.

2.17 Flow cytometric analysis

Approximately 1 × 10^6 resting or activated T cells were placed in 12 × 75 mm round-bottomed polystyrene tubes and washed once with cold PBS and twice with PBS containing 1% bovine serum albumin (BSA) and 0.2% sodium azide, which will, hereafter, be referred to as FACS buffer. Cells were then incubated with FITC- or PE- labeled mAbs to B220, CD3ε, CD11a (LFA-1), IL-2Rα (CD25) or TCRαβ at 1 μg/100 μl FACS buffer/tube. Rat IgG_{2a}-PE, hamster IgG-PE, rat IgG_{2a}-FITC, rat IgG_1-FITC and hamster IgG-PE (all from Cedarlane) were used in parallel as isotype controls, respectively. Tubes were placed on ice and incubated in dark for 30 min. T cells were then washed three times with FACS buffer and fixed with 1% paraformaldehyde.

The expression of FcγRs by freshly isolated DCs was assessed by two-step staining using rat anti-mouse CD16/CD32 (clone 2.4G2) as the primary Ab and FITC-conjugated mouse anti-rat IgG $F(ab')_2$ (Jackson ImmunoResearch Laboratories, West Grove, PA) as the secondary Ab reagent. Background staining was obtained from tubes receiving no primary Ab.

Cell surface expression of the above markers was then determined by analysis of 10^4 cells with a FACScan (Becton-Dickinson, Mississauga, ON).

2.18 Immunofluorescence staining for Thy-1

Cell-sized microbeads were incubated at 37°C with PBS, anti-CD3 mAb (1 μg), anti-CD3 mAb (1 μg) plus hamster IgG (10 μg), or a combination of anti-CD3 mAb (1 μg) and anti-CD28 mAb (10 μg). After 2 h, beads were washed with PBS three times and with complete medium once. Highly purified T cells and beads were then mixed at a 1:1 ratio (5×10^5 each) in a total volume of 0.5 ml complete medium in eppendorf tubes. The content of each tube was gently mixed by inversion for 20 min using a shaker placed inside a 37°C incubator. A 50-μl aliquot of the mixtures was subsequently placed on poly-L-lysine-coated slides and left to dry overnight before staining for Thy-1.

Slides were subsequently fixed for 2 min with 3.7% paraformaldehyde containing sodium periodate, washed with PBS three times and blocked with PBS containing 2% BSA for 2 h. Anti-Thy-1.2 mAb (clone 30-H12) was then used as primary reagent at a 1:100 dilution, while slides corresponding to negative controls received either rat IgG or no primary reagent at all. Following a one-hour incubation at room temperature, slides were washed with PBS, covered by FITC-

conjugated mouse anti-rat IgG $F(ab')_2$(Jackson ImmunoResearch) at 1:50 and left in dark for 1 h. Slides were then washed three times with PBS, mounted with PBS-glycerol and stored in dark at 4°C prior to examination under an ultraviolet microscope.

2.19 Statistical analysis

Results are representative of data obtained from at least 3 independent experiments yielding similar findings unless otherwise specified. All results are expressed as mean ± standard deviation (SD) in triplicate or quadruplicate wells of independent experiments. The Instat statistics program (GraphPad Software, Inc., San Diego, CA) was used to analyze data. Statistical comparisons were performed using Student's *t*-test, or one-way analysis of variance (ANOVA) for dose response experiments followed by the Bonferroni multiple comparisons test. *P* values less than 0.05 were considered to be statistically significant. NS (not significant) denotes a *p* value of > 0.05, while *, **, and *** indicate *p* values of <0.05, <0.01 and <0.001, respectively.

Table 2.1: a list of antibodies used in this research

Antibody	Clone, host species, isotype	Source	Reference (if applicable)
α-asialo-GM1	Polyclonal, rabbit antiserum	Wako, Richmond, VA	
α-B220	RA3-3A1/6.1, rat IgM	ATCC, Manassas, VA	
α-B220-PE	RA3-6B2, rat IgG_{2a}, κ	BD Pharmingen, Mississauga, ON	
α-B7-1	16-10A1, hamster IgG	ATCC, Manassas, VA	
α-B7-2	GL-1, rat IgG_{2a}, κ	Dr. K. Hathcock, National Cancer Institute, Bethesda, MD	Hathcock *et al*, 1994
α-CD2	RM2-5, rat IgG_{2b}, λ	BD Pharmingen, Mississauga, ON	
α-CD3ε	145-2C11, hamster IgG	Dr. J. Bluestone, University of Chicago, Chicago, IL	Leo *et al*, 1987
α-CD3ε-FITC	145-2C11, hamster IgG	Cedarlane Laboratories, Hornby, ON	
α-CD3ε-Fos	Chimeric hamster/mouse $F(ab')_2$	Dr. J Tso, Protein Design Labs, Inc., Mountain View, CA	Yu *et al*, 1996
α-CD4	GK1.5, rat IgG_{2b}	ATCC, Manassas, VA	
α-CD8	3.155, rat IgM	ATCC, Manassas, VA	
α-CD11a-FITC	I21/7, rat IgG2a	Serotec Inc., Raleigh, NC	
α-CD16/CD32	2.4G2, rat IgG_{2b}, κ (Fc Block™)	BD Pharmingen, Mississauga, ON	
α-CD25-FITC	PC61.5.3, rat IgG_1	Cedarlane Laboratories, Hornby, ON	
α-CD28	37.51.1, hamster IgG	Cedarlane Laboratories, Hornby, ON	
α-CD48	HM48-1, hamster IgG	BD Pharmingen, Mississauga, ON	
α-Fas/CD95	Jo2, hamster IgG	BD Pharmingen, Mississauga, ON	
α-LPAM-1 ($\alpha_4\beta_7$ integrin)	DATK32, rat IgG_{2a}, κ	BD Pharmingen, Mississauga, ON	
α-Thy-1	G7, rat IgG_{2c}, κ	BD Pharmingen, Mississauga, ON	
α-Thy-1.2	30-H12, rat IgG_{2b}, κ	BD Pharmingen, Mississauga, ON	

Results

3. Blockade of Thy-1 interferes with the development of MHC-unrestricted killer T cells in response to anti-CD3 Ab

The observation that Ab-mediated triggering of Thy-1 leads to IL-2 production and T cell proliferation has been largely viewed as evidence of the involvement of Thy-1 in the process of T cell activation (Gunter *et al*, 1984; Kroczek *et al*, 1986a). In addition, a number of studies advocate a role for Thy-1 in the development of CTL responses (Kojima *et al*, 2000; Lancki *et al*, 1995b; Ozery *et al*, 1989). However, these studies have mostly used T cell hybridomas or clones, which may have distinct activation requirements, and may not best represent naïve CTLps.

Here, I used a non-stimulatory mAb to Thy-1 (clone 30-H12) to investigate the role of Thy-1 in the context of non-specific CTL induction in response to mitogenic anti-CD3 mAb. Cross-linking of the TCR/CD3 complex by anti-CD3 mAbs is known to bypass the requirement for specific Ag recognition through TCR. One important outcome of this mode of activation is the generation of anti-CD3-activated killer T (AK-T) cells from their "resting" precursors. AK-T cells are armed with cytotoxic effector molecules, and can lyse a broad spectrum of tumor targets in a manner reminiscent of NK cell lytic function. AK-T cells, therefore, provide a suitable model to study CTL "induction" and cytotoxic activity. In addition, the fact that a small, but still significant fraction of CTLs exhibit MHC-unrestricted cytotoxicity (Stotter and Lotze, 1990) lends more credence to this model.

3.1 Blockade of Thy-1 inhibits CD3-driven T cell proliferation and cytotoxicity

Anti-CD3 mAbs trigger T cell proliferation and cytotoxicity against a broad range of tumor cells including P815 mastocytoma cells (Stankova *et al*, 1989). Since Thy-1 has been implicated in the process of T cell activation (Gunter *et al*, 1984; Kroczek *et al*, 1986a), I hypothesized that the blockade of Thy-1 may prevent these responses. First, I confirmed the expression of Thy-1 by the vast majority of cells harvested following a 48-h culture of nylon wool-non-adherent T cells with anti-CD3 mAb (88% Thy-1.2$^+$ by flow cytometry). As illustrated in Figure 3.1A, the presence of a non-stimulatory anti-Thy-1.2 mAb, 30-H12, attenuated anti-CD3-induced T cell proliferation in a clear dose-dependent fashion. T cells stimulated in the presence of similar doses of anti-Thy-1 mAb also failed to lyse P815 cells, and the inhibition of cytotoxicity was consistently more profound in comparison with proliferation (Fig. 3.1B). Anti-CD3-induced responses of fractionated CD4$^+$ or CD8$^+$ T cell populations were equally sensitive to Thy-1 blockade. An 8-μg/ml dose of anti-Thy-1 mAb inhibited CD4$^+$ and CD8$^+$ T cell proliferation by 93% and 90%, respectively (Fig. 3.1C). The CD4$^+$- or CD8$^+$ T cell-mediated lysis of P815 cells was similarly reduced by 92% by the presence of anti-Thy-1 mAb (8 μg/ml) in AK-T cell cultures (Fig. 3.1D). The inhibitory effect of anti-Thy-1 mAb was not simply a reflection of a more general phenomenon that would occur with Abs to any given T cell surface molecule. In one experiment, for instance, a rat anti-mouse LPAM-1 (integrin $\alpha_4\beta_7$ complex) mAb (8 μg/ml) used as a negative control inhibited anti-CD3-induced T cell

proliferation only by 27% (in comparison with a 94% inhibition by anti-Thy-1 mAb). Furthermore, a 1-μg/ml dose of anti-LPAM-1 mAb slightly increased T cell proliferation in response to anti-CD3 mAb.

3.2 T cells stimulated with anti-CD3 in the presence of anti-Thy-1 remain capable of forming conjugates with target cells

Since conjugate formation between CTLs and target cells is an important prerequisite for the delivery of lethal hit to target cells (reviewed by Springer, 1990), I asked whether Thy-1 blockade interferes with AK-T cell cytotoxicity by preventing AK-T cell adherence to target cells. Murine AK-T cells rely on certain sets of adhesion molecules to bind and form stable conjugates with target cells. For example, our group previously demonstrated that the interactions between $\alpha_4\beta_7$ integrin and vascular cell adhesion molecule (VCAM)-1 are important for the adhesion of AK-T cells to syngeneic, MCA-38 colon carcinoma cells (MacKenzie *et al*, 2002), while AK-T cell-mediated lysis of P815 cells depends highly upon LFA-1-ICAM-1 interactions (Kaiser and Hoskin, 1992). Blockade of Thy-1 during AK-T cell development moderately inhibited the expression of LFA-1 (Fig. 3.2A). Surprisingly however, AK-T cell conjugation with P815 cells was consistently increased rather than decreased by the presence of anti-Thy-1 mAb in T cell activation cultures (Fig. 3.2B). My data thus indicate that the failure of T cells stimulated in the presence of anti-Thy-1 mAb to lyse P815 target cells is not a consequence of decreased conjugation with the target cells.

3.3 Blockade of Thy-1 prevents anti-CD3-induced expression of PFN and GzmB

Since AK-T cells are known to employ PFN, GzmB and FasL to lyse susceptible target cells (reviewed by Hoskin *et al*, 1998), I asked whether the failure of AK-T cells generated in the presence of anti-Thy-1 mAb to lyse P815 cells was due to their decreased expression of these cytotoxic effector molecules. The mRNA expression of both PFN and GzmB, which normally peaks at 48 h following stimulation with anti-CD3 Ab (Makrigiannis and Hoskin, 1997), was clearly inhibited by the presence of anti-Thy-1 mAb in culture, with GzmB expression being consistently more sensitive to Thy-1 blockade (Fig. 3.3A). P815 cells express only minute amounts of Fas, and are consequently relatively insensitive to Fas-mediated killing (Williams *et al*, 1997). Nevertheless, I looked at FasL expression by AK-T cells at 4 h post-stimulation since FasL is up-regulated within only a few hours of TCR engagement (Makrigiannis and Hoskin, 1997). Unlike PFN and GzmB, the expression of FasL was not affected by Thy-1 blockade (Fig. 3.3B). Similar results were obtained when FasL mRNA levels were examined in 18- or 48-h cultures (data not shown). All together, my data indicate that the blockade of Thy-1 during AK-T cell induction prevents the arming of CTLps with PFN and GzmB, without impairing FasL up-regulation.

3.4 Thy-1 blockade inhibits the expression of IL-2 and IL-2 receptor α chain in response to anti-CD3 mAb

The expression of PFN and GzmB following TCR ligation is known to be a late event that results at least in part from T cell exposure to cytokines such as

IL-2 (Liu *et al*, 1989; Makrigiannis and Hoskin, 1997). Since cross-linking of Thy-1 with stimulatory mAbs leads to IL-2 production (Gunter *et al*, 1984), I hypothesized that the blockade of Thy-1 prevents the expression of PFN and GzmB through inhibition of IL-2 synthesis. As illustrated in Fig. 3.4A, Ab-mediated blockade of Thy-1 significantly lowered the amount of IL-2 present in 24-h culture supernatants. Interestingly, the observed response to varying concentrations of anti-Thy-1 mAb was consistently contrary to the expected dose response. For example, the highest dose of anti-Thy-1 mAb (8 μg/ml) was the least effective in inhibition of IL-2 production (Fig. 3.4A). However, when I looked at IL-2 expression at mRNA level, lower levels of IL-2 transcripts were detected when higher doses of anti-Thy-1 mAb were present in the culture, as was to be expected (Fig. 3.4B). I speculated that the inconsistency between the mRNA and protein data might reflect a more profound inhibition of IL-2R expression, and as a result less binding and utilization of IL-2 by IL-2R when higher doses of anti-Thy-1 mAb were used. I looked at the expression of the highly inducible IL-2Rα (CD25), which is up-regulated upon T cell activation, and couples with the β and γ_c chains to form the high-affinity IL-2R (reviewed by Gaffen, 2001). Consistent with my hypothesis, anti-CD3-induced up-regulation of IL-2Rα was prevented by anti-Thy-1 mAb in a clear dose-dependent fashion (Fig. 3.4C). Collectively, my results demonstrate that the blockade of Thy-1 results in attenuated expression of both IL-2 and IL-2Rα by T cells in response to anti-CD3 mAb.

3.5 Exogenous IL-2 can partially compensate for Thy-1 blockade during AK-T cell induction

I next examined whether or not exogenous IL-2 was able to reverse the changes caused by Ab-mediated blockade of Thy-1. Recombinant murine IL-2 was added at 100 U/ml at the beginning of T cell cultures performed in the presence or absence of anti-Thy-1 mAb, and GzmB expression and cytotoxicity were examined 48 h following stimulation with anti-CD3 mAb. As expected, exogenous IL-2 enhanced both GzmB expression and cytotoxicity induced by AK-T cells (Fig. 3.5A, 3.5B). IL-2 was also able to almost completely rescue GzmB expression in T cells stimulated with anti-CD3 mAb in the presence of anti-Thy-1 mAb. Interestingly however, AK-T cell-mediated lysis of P815 target cells was only partially restored by exogenous IL-2 (Fig. 3.5), suggesting that the expression of GzmB by itself is not sufficient for killing. Accordingly, T cells activated by IL-2 alone showed no significant cytotoxicity notwithstanding the expression of substantial amounts of GzmB (Fig. 3.5). The addition of other cytotoxicity-inducing cytokines such as IFN-γ and IL-12 alone or in combination with IL-2 into the cultures also failed to fully restore AK-T cell-mediated cytotoxicity. The specific lysis of P815 target cells by AK-T cells generated in the presence of rat IgG, anti-Thy-1 mAb, anti-Thy-1 mAb plus IFN-γ (100 U/ml), anti-Thy-1 mAb plus IL-12 (25 ng/ml), or anti-Thy-1 mAb plus a combination of IL-2, IFN-γ and IL-12 was 56%, 14%, 24%, 19% and 31%, respectively. My data indicate that although blockade of Thy-1 leads to decreased IL-2 synthesis by T cells in response to anti-CD3 Ab, providing T cells with exogenous IL-2 fails to

completely rescue AK-T cell-mediated cytotoxicity from the inhibitory effect of Thy-1 blockade during CTL induction.

3.6 The inhibitory effect of the 30-H12 anti-Thy-1 mAb on CD3-driven T cell proliferation is maintained when anti-Thy-1 mAb is used in excess

Although 30-H12 mAb is extensively used as a non-stimulatory anti-Thy-1 Ab, it was of interest to confirm that the observed inhibitory effects of 30-H12 were not a consequence of possible negative signaling by this mAb. One way to address this question is to use extremely high concentrations of the Ab in order to produce a prozone effect whereby only one Fab arm of the Ab would theoretically bind Thy-1 due to Ab excess (Landsteiner, 1946). Under these circumstances, the inhibitory effects of the anti-Thy-1 mAb would be reversed if the binding of the Ab to T cells through both Fab arms generated a negative signal. Fig. 3.6 demonstrates that the inhibitory effect of 30-H12 mAb on anti-CD3-induced T cell proliferation is not lost when the Ab is used at concentrations as high as 100 μg/ml. This observation suggests that the anti-Thy-1 mAb does not act by generating a negative signal through cross-linking activity, and favors a blocking function for this mAb.

3.7 The 30-H12 anti-Thy-1 mAb does not inhibit T cell activation in response to microbeads coated with anti-CD3 and anti-CD28 mAbs

B cells comprise the majority of non-T cells in nylon wool-non-adherent spleen cell preparations, and are essential to provide FcRs for cross-linking of anti-CD3 mAb as well as costimulatory signals for T cell activation (Okamoto *et al*, 1995). Therefore, I asked whether or not anti-Thy-1-mediated inhibition of T

cell activation in my system also involved B cells. To address this question, I removed B cells from my cell preparations, and used microbeads coated with anti-CD3 and anti-CD28 mAbs as surrogate APCs. Coating of anti-CD3 mAb on microbeads eliminates the need for the presence of non-T cells to cross-link anti-CD3 mAb, while anti-CD28 mAb provides costimulatory signals for T cell activation. Beads coated with anti-CD3 plus anti-CD28 mAb, but not those coated with anti-CD3 alone, can form immunological synapses with T cells, which is considered a prerequisite step in the process of T cell activation (Viola *et al*, 1999).

Immunofluorescence staining for Thy-1 expression showed that Thy-1 is recruited into the immunological synapses formed between T cells and surrogate APCs (Fig 3.7). Although it is not clear whether Thy-1 recruitment into the immunological synapse is an active process or a passive event that simply reflects the aggregation of lipid rafts, the presence and concentration of Thy-1 within the synapse suggests a role for Thy-1 in the context of T cell activation. Interestingly, the presence of the 30-H12 anti-Thy-1 mAb in cultures containing highly purified T cells and anti-CD3/anti-CD28-coated microbeads failed to inhibit T cell proliferation (Fig. 3.8A). Furthermore, T cell proliferation was increased when anti-Thy-1 mAb was immobilized on microbeads together with anti-CD3 and anti-CD28 mAbs prior to incubation with T cells (Fig. 3.8B). Therefore, the 30-H12 mAb has no inhibitory effect on T cell proliferation in a B cell-free system where activation signals are supplied to T cells by Abs coated on microbeads.

3.8 The 30-H12 anti-Thy-1 mAb does not affect T cell proliferation in response to anti-CD3 mAb plus DCs

DCs can be used as highly potent accessory cells along with anti-CD3 mAb to induce T cell proliferation. This is due to the abundant expression of FcγRs as well as costimulatory molecules by DCs (Elbe-Burger *et al*, 2000; Romani *et al*, 1989). I asked whether the inhibitory effect of anti-Thy-1 mAb on CD3-driven T cell responses could also be seen with APCs other than B cells. Fig. 3.9A and 3.9B illustrate that neither anti-CD3-induced nor Ag-specific T cell proliferative responses were blocked by anti-Thy-1 mAb when syngeneic DCs were used instead of B cells as APCs/accessory cells.

3.9 T cell proliferation triggered by anti-CD3 mAb plus LPS blasts is prevented by the 30-H12 anti-Thy-1 mAb

Collectively, the above findings suggested to me that CD3-driven T cell proliferation is not prevented by the anti-Thy-1 mAb when APCs present in culture display pre-existing costimulatory capacity. Our group has previously shown that the costimulatory molecule B7-2 is essential for GzmB expression and AK-T cell induction (Makrigiannis *et al*, 1999). At a resting state, B cells do not constitutively express substantial levels of B7 molecules, and depend highly on dynamic T cell-B cell interactions in order to acquire costimulatory activity (Ranheim and Kipps, 1993). I therefore asked whether or not the anti-Thy-1 mAb acts by preventing the up-regulation of B7 costimulatory molecules on B cells. To address this question, I activated highly purified T cells with anti-CD3 mAb in the presence of LPS blasts, which exhibit substantial expression of B7 costimulatory

molecules in comparison with resting B cells (Makrigiannis *et al*, 1999). Under these conditions, T cell activation was prevented by anti-Thy-1 mAb (Fig. 3.10), indicating that the blockade of Thy-1 can potently inhibit anti-CD3-induced T cell activation even in the presence of pre-existing B cell-mediated costimulatory interactions.

3.10 T cell proliferation in response to PMA plus ionomycin is not affected by the 30-H12 anti-Thy-1 mAb

The observed differences between B cells and other APCs in the context of CD3-driven T cell activation and in terms of sensitivity to the blockade of Thy-1 suggest that the inhibitory effect of the anti-Thy-1 mAb involves initial T cell-B cell interactions. T cell activation in the absence of cell-cell interactions can be induced by treatment with a combination of PMA and ionomycin. The former mimics the action of DAG resulting in the activation of PKC, while the latter increases the intracellular concentration of Ca^{++} (Miller, 1986). Ab-mediated blockade of Thy-1 did not have any inhibitory effect on T cell activation triggered by PMA plus ionomycin (Fig. 3.11), indicating that the inhibitory effect(s) of the anti-Thy-1 mAb involves the proximal events of anti-CD3-induced T cell activation when B cells comprise the main accessory cells in culture. Among several reagents I have tested in my system, PMA was the only one to compensate for Thy-1 blockade and to rescue T cell proliferation in response to anti-CD3 mAb (Fig. 3.12). This suggests that anti-Thy-1 mAb 30-H12 exerts its inhibitory activity upstream of PKC.

Fig. 3.1. Blockade of Thy-1 inhibits anti-CD3-induced T cell proliferation and cytotoxicity. A,B) T cells were stimulated with anti-CD3 mAb in the presence of control IgG or the indicated concentrations of anti-Thy-1 mAb. Cells were pulsed with [^{3}H]TdR during the last 6 h of culture. [^{3}H]TdR incorporation was determined at 48 h as a measure of T cell proliferation. Data are shown as mean cpm ± SD in quadruplicate wells from one representative experiment (A). Cytolytic activity against P815 target cells at a 50:1 effector:target ratio was determined by ^{51}Cr-release assay also after 48 h of culture (B). *** denotes a significant difference with $p<0.001$ compared with control rat IgG. Differences were statistically significant by ANOVA ($p<0.0001$) and Bonferroni multiple comparisons test. C,D) CD4$^+$ and CD8$^+$ T cell subsets were isolated and stimulated with anti-CD3 mAb in the presence of control rat IgG or anti-Thy-1 mAb (8 μg/ml). [^{3}H]TdR incorporation was determined at 48 h as a measure of T cell proliferation (C). Cytolytic activity of CD4$^+$ and CD8$^+$ T cells against P815 target cells was determined after 48 h by ^{51}Cr-release assay (D).

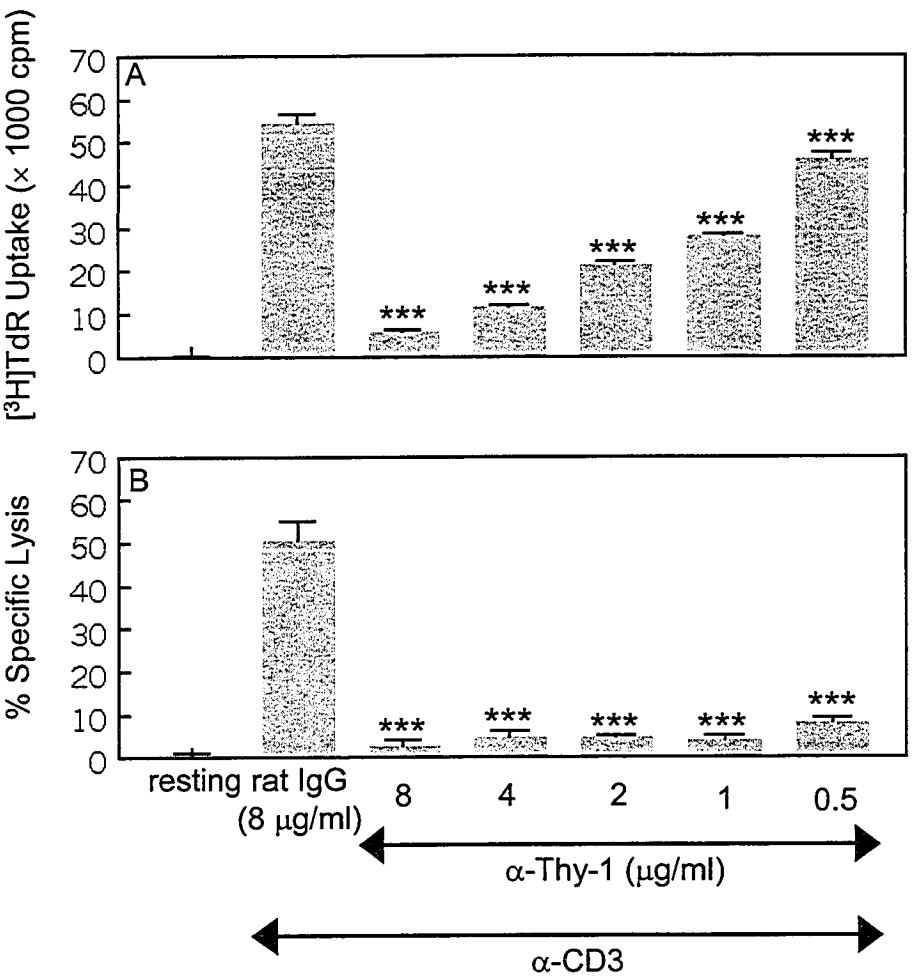

[³H]TdR Uptake (× 1000 cpm)
70
60
50
40
30
20
10
0
A
***
***
***
***
***
% Specific Lysis
70
60
50
40
30
20
10
0
B
***
***
***
***
***
resting rat IgG
(8 μg/ml)
8
4
2
1
0.5
α-Thy-1 (μg/ml)
α-CD3

Figure 3.1(A,B)

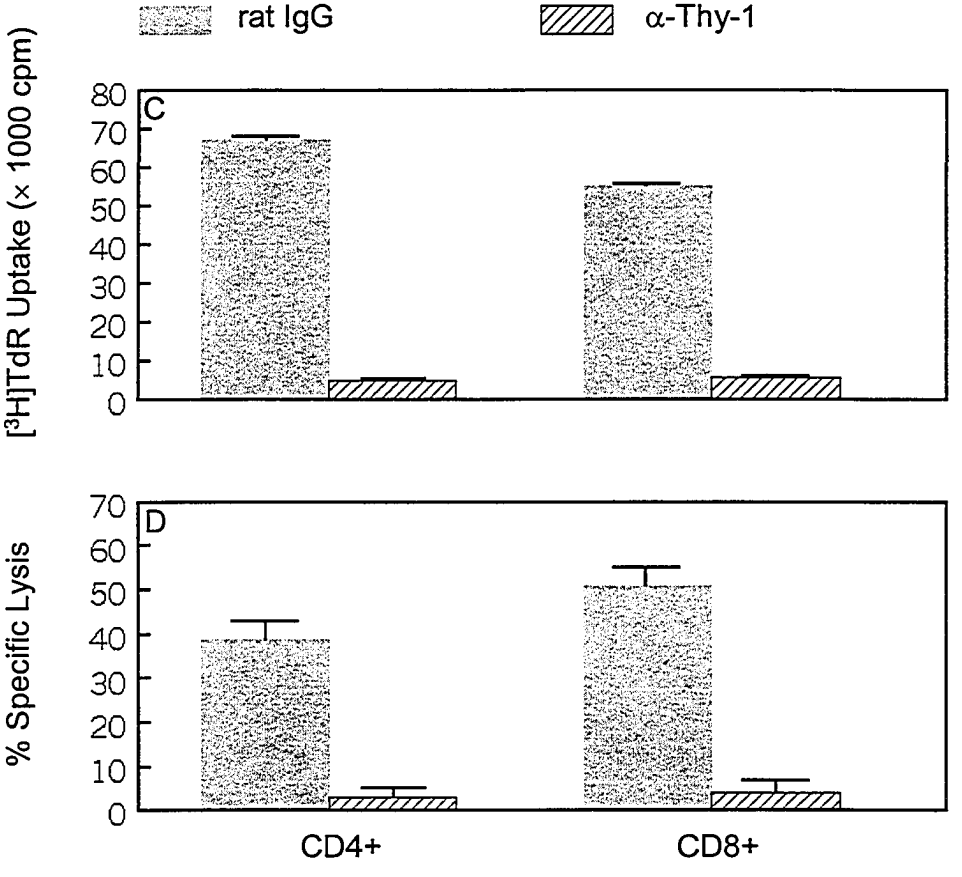

rat IgG
α-Thy-1
[³H]TdR Uptake (× 1000 cpm)
80
70
60
50
40
30
20
10
0
C
% Specific Lysis
70
60
50
40
30
20
10
0
D
CD4+
CD8+

Figure 3.1(C,D)

Fig. 3.2. T cells stimulated in the presence of anti-Thy-1 mAb adhere to target cells in spite of a moderate decrease in LFA-1 expression. A) Resting T cells or T cells stimulated with anti-CD3 mAb in the presence of anti-Thy-1 mAb or control IgG (8 μg/ml) were harvested, washed and stained with FITC-labeled rat anti-mouse LFA-1. Cell surface expression of LFA-1 (open peaks) was assessed by FACS analysis in comparison with background staining with rat IgG_{2a}-FITC isotype control (filled peaks). B) Resting T cells or T cells activated with anti-CD3 in the presence of the indicated concentrations of anti-Thy-1 mAb or IgG control were washed and mixed with neutral red-stained P815 cells followed by examination under a light microscope. . ** denotes a significant difference with $p<0.01$ compared with rat IgG control.

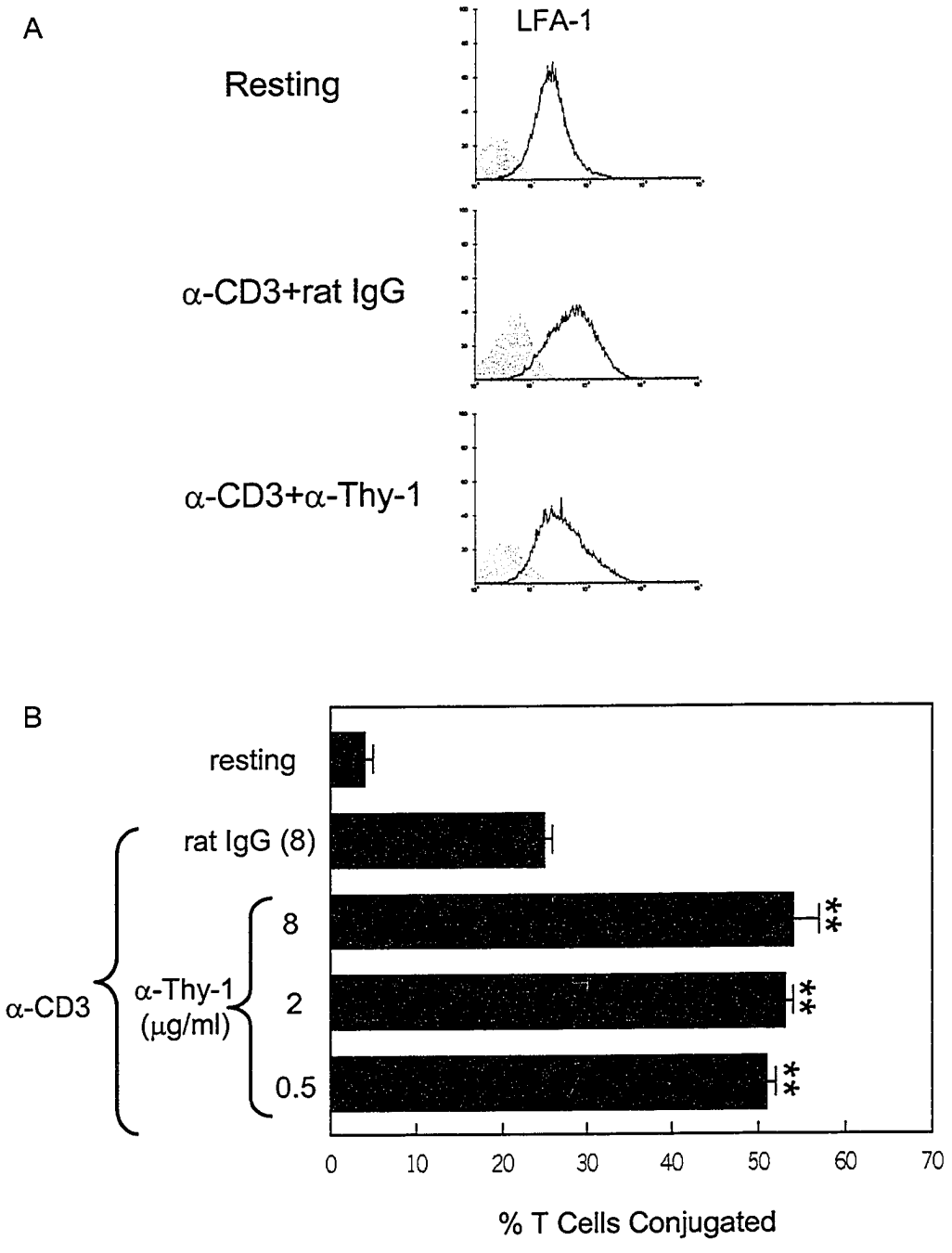

A
LFA-1
Resting
α-CD3+rat IgG
α-CD3+α-Thy-1
B
resting
rat IgG (8)
α-CD3
α-Thy-1 (μg/ml)
8
2
0.5
**
**
**
0
10
20
30
40
50
60
70
% T Cells Conjugated

Figure 3.2

Fig. 3.3. Ab-mediated blockade of Thy-1 decreases PFN and GzmB, but not FasL mRNA levels in AK-T cell cultures. T cells were incubated with anti-CD3 mAb in the presence of rat IgG or the indicated concentrations of anti-Thy-1 mAb. Total RNA was isolated at 48 h (for PFN and GzmB) or 4 h (for FasL) post-stimulation and reverse-transcribed. A semiquantitative PCR with exon-binding, intron-bridging primers specific for PFN, GzmB (A), or FasL (B) was then performed. GAPDH mRNA levels were also determined by RT-PCR at each time point. Amplicons were resolved by gel electrophoresis and visualized by ethidium bromide staining.

Figure 3.3A

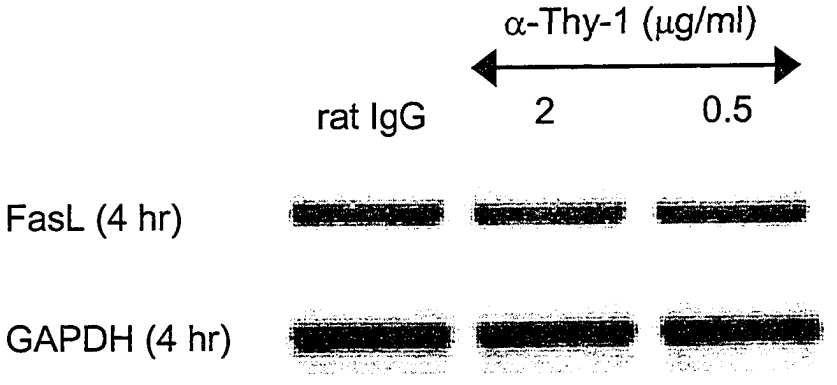


Figure 3.3B

Fig. 3.4. Ab-mediated blockade of Thy-1 leads to decreased IL-2 and IL-2Rα chain expression by anti-CD3-stimulated T cells. A) T cells were left unstimulated or activated with anti-CD3 mAb in the presence of control rat IgG (8 μg/ml) or the indicated concentrations of anti-Thy-1 mAb. Culture supernatants were collected after 24 h and IL-2 levels were measured by ELISA. Results from a representative experiment are expressed as mean ± SD in triplicate wells. Differences were statistically significant by ANOVA ($p<0.0001$) and Bonferroni multiple comparisons test. *** denotes a significant difference with $p<0.001$ compared with rat IgG control; BDL: below detection limit. B) Total RNA was isolated from resting or stimulated T cells at 18 h and reverse-transcribed. A semiquantitative PCR was then performed to determine IL-2 mRNA levels. The amplification of equal amounts of cDNA for different treatment groups was ensured through normalization based on the steady-state expression of GAPDH. Data are from one experiment and are representative of two independent experiments. C) Resting T cells or T cells stimulated with anti-CD3 mAb in the presence of the indicated concentrations of anti-Thy-1 mAb or control rat IgG (8 μg/ml) were harvested at 24 h, washed and stained with FITC-labeled rat anti-mouse IL-2Rα chain (CD25). Cell surface expression of IL-2Rα (open peaks) was assessed by FACS analysis in comparison with background staining with rat IgG_1-FITC isotype control (filled peaks). Data are representative of two independent experiments yielding similar results.

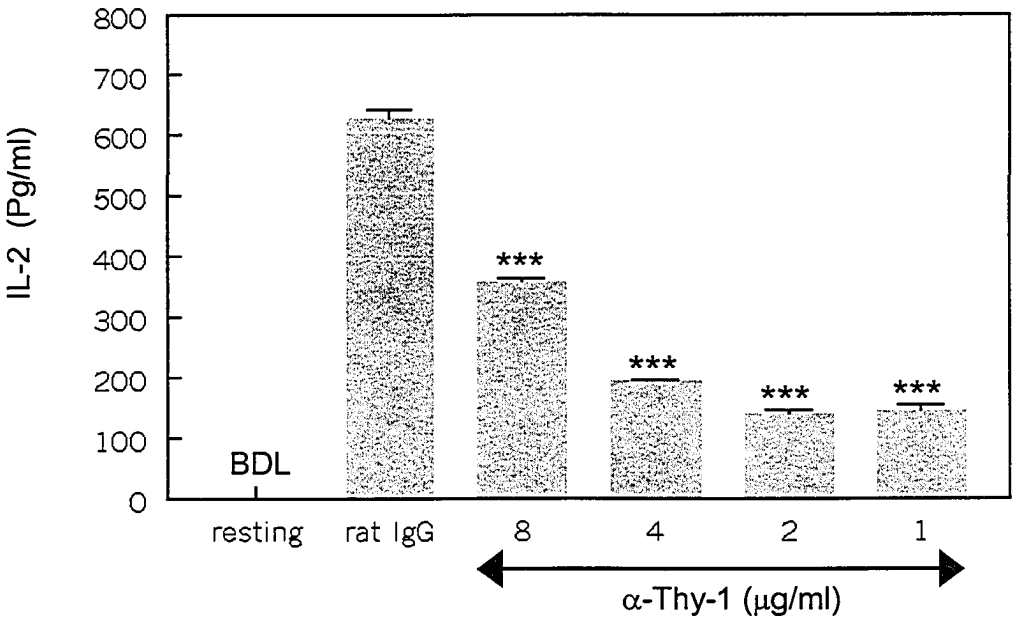

800
700
600
500
400
300
200
100
0
IL-2 (Pg/ml)
BDL
***
***
***
***
resting
rat IgG
8
4
2
1
α-Thy-1 (μg/ml)

Figure 3.4A

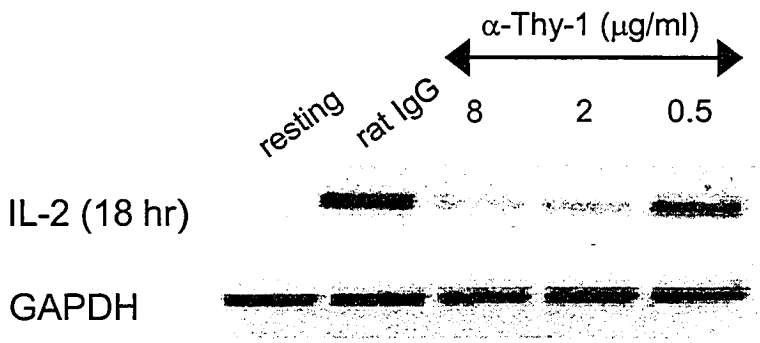


Figure 3.4B

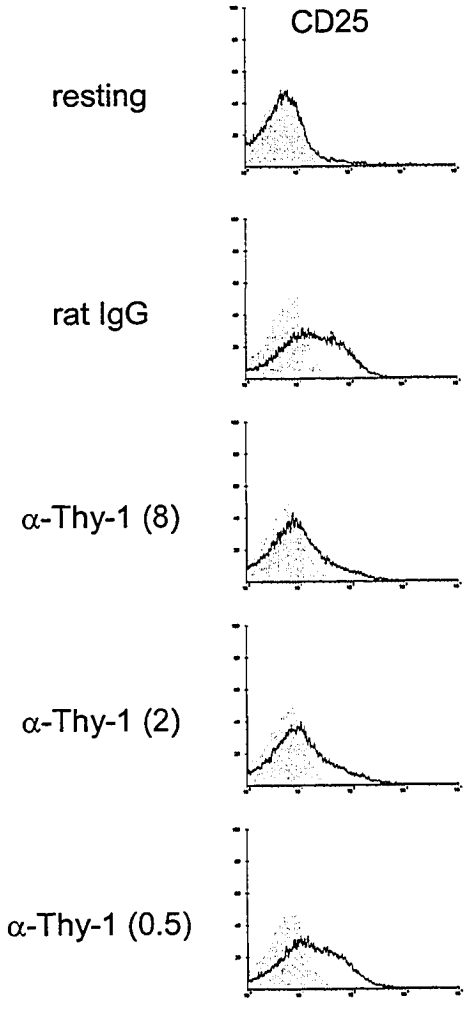


Figure 3.4C

Fig. 3.5. Exogenous IL-2 partially reverses the effect of Ab-mediated blockade of Thy-1 during AK-T cell induction. A) T cells were incubated with recombinant murine IL-2 (100 U/ml) or with anti-CD3 mAb in combination with rat IgG (8 μg/ml), rat IgG plus IL-2, anti-Thy-1 mAb (8 μg/ml), or anti-Thy-1 mAb plus IL-2. After 48 h, T cells were harvested, washed and used at an effector:target ratio of 50:1 against P815 mastocytoma cells in a ^{51}Cr release assay. ** and *** denote a significant difference with $p<0.001$ and $p<0.0001$, respectively. B) GzmB mRNA expression was assessed in 48-h cultures using a semiquantitative RT-PCR method. Amplicons were resolved by gel electrophoresis and visualized by ethidium bromide staining. The images were then scanned and inverted (using Adobe Photoshop 6.0 software), and GzmB expression in different treatment groups was compared by densitometric analysis relative to the steady-state expression of GAPDH.

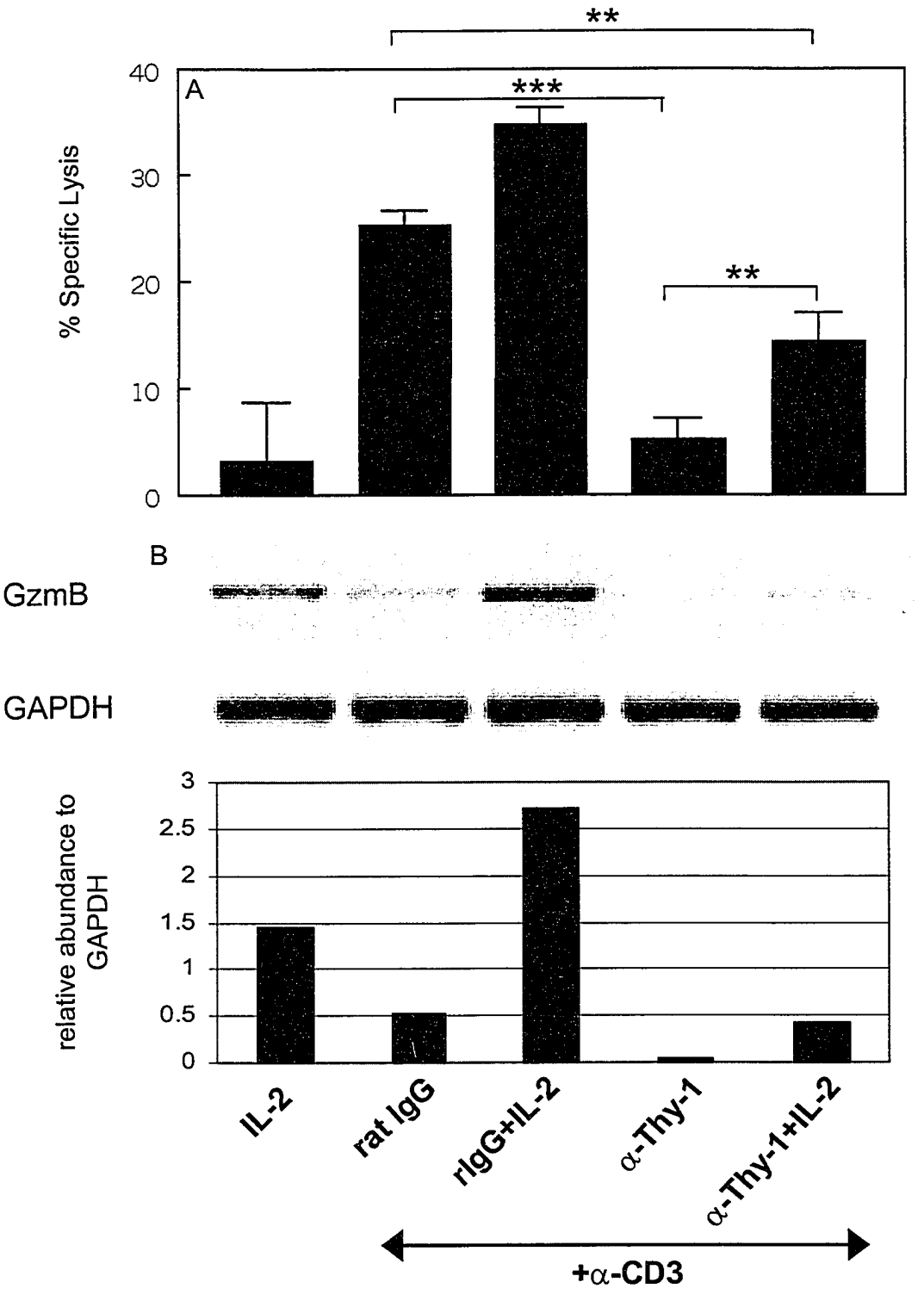

**
A
***
40
30
20
10
0
% Specific Lysis
**
B
GzmB
GAPDH
relative abundance to GAPDH
3
2.5
2
1.5
1
0.5
0
IL-2
rat IgG
rIgG+IL-2
α-Thy-1
α-Thy-1+IL-2
+α-CD3

Figure 3.5

Fig. 3.6. The inhibitory effect of anti-Thy-1 mAb on CD3-driven T cell proliferation is maintained in high concentrations of anti-Thy-1 mAb. T cells were stimulated by anti-CD3 mAb in the presence of anti-Thy-1 mAb or rat IgG at 5, 50 or 100 μg/ml. [^{3}H]TdR incorporation was determined after 48 h as a measure of T cell proliferation.

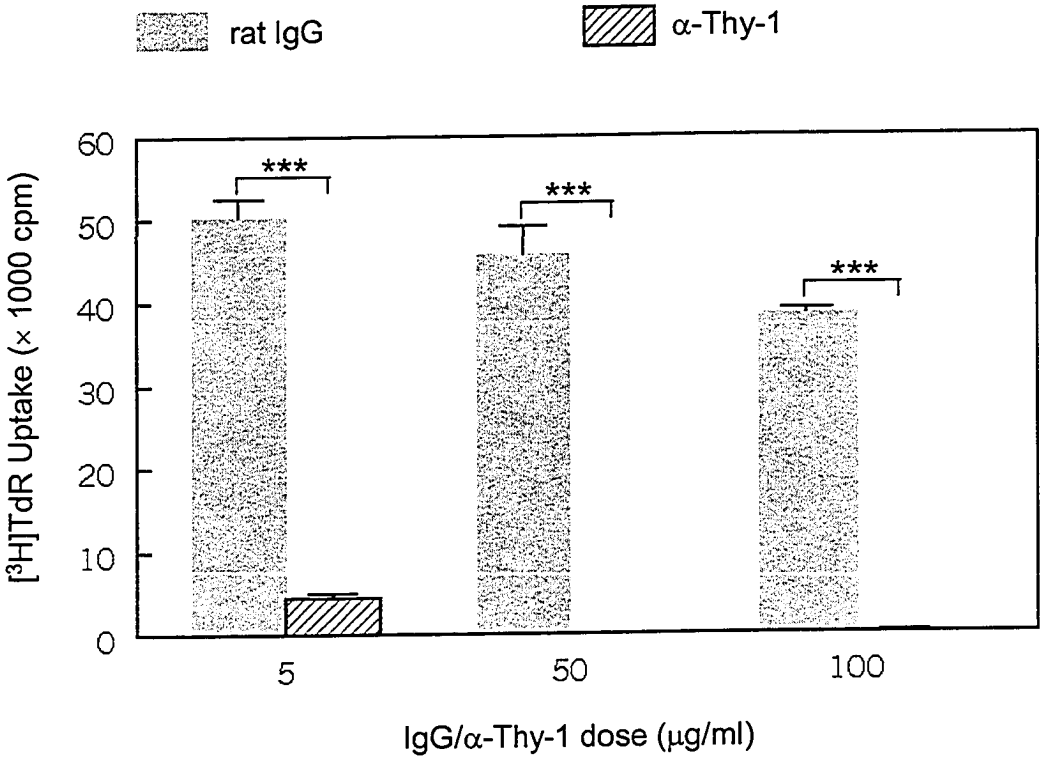
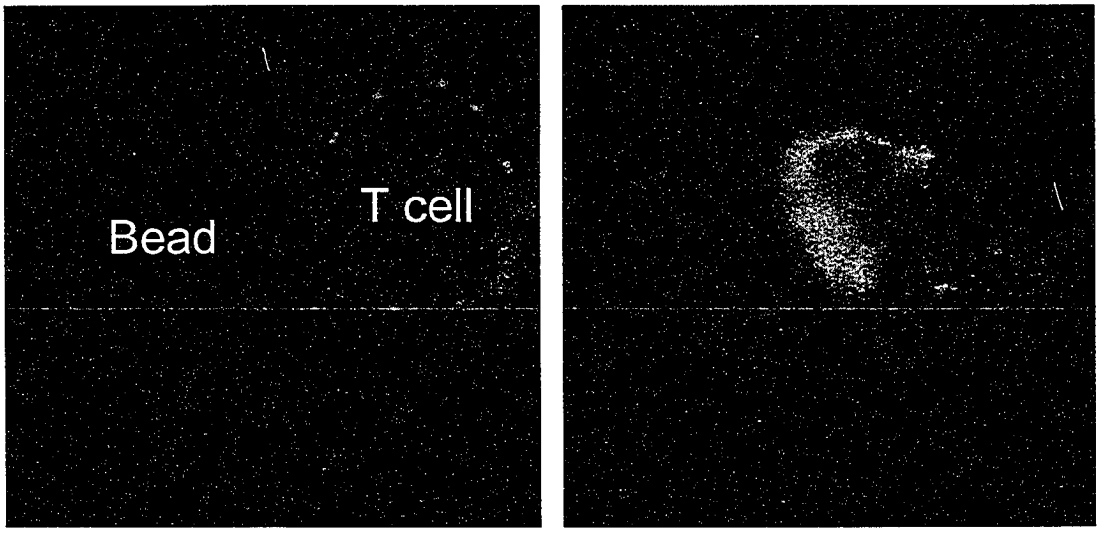

rat IgG
α-Thy-1
[3H]TdR Uptake (× 1000 cpm)
60
50
40
30
20
10
0
***
***
***
5
50
100
IgG/α-Thy-1 dose (μg/ml)

Figure 3.6

Fig. 3.7. Thy-1 is present within the immunological synapse formed between T cells and surrogate APCs. Highly purified T cells and microbeads pre-coated with anti-CD3 mAb (1 μg) or a combination of anti-CD3 (1 μg) and anti-CD28 (10 μg) mAbs were combined at a 1:1 ratio and gently mixed for 20 min at 37°C. An aliquot of the mixtures was subsequently placed on poly-L-lysine-coated slides, dried and fixed. Slides were subsequently stained for Thy-1 using rat anti-mouse Thy-1.2 mAb as the primary Ab and FITC-conjugated mouse anti-rat IgG $F(ab')_2$ as secondary reagent. T cell:APC conjugates were then examined for Thy-1 staining under an ultraviolet microscope.



α-CD3 α-CD3 + α-CD28

Antibodies coated on beads

Figure 3.7

Fig. 3.8. Anti-Thy-1 mAb fails to inhibit T cell proliferation in response to a combination of anti-CD3 and anti-CD28 mAbs coated on beads. T cells were combined with microbeads pre-coated with anti-CD3 (1 μg) and anti-CD28 (10 μg) mAbs at a 1:1 ratio. Anti-Thy-1 mAb was either added in soluble form at 8 μg/ml into the culture (A) or immobilized on microbeads (8 μg) together with anti-CD3 and anti-CD28 mAbs (B). [^{3}H]TdR incorporation was determined at 48 h as a measure of T cell proliferation.

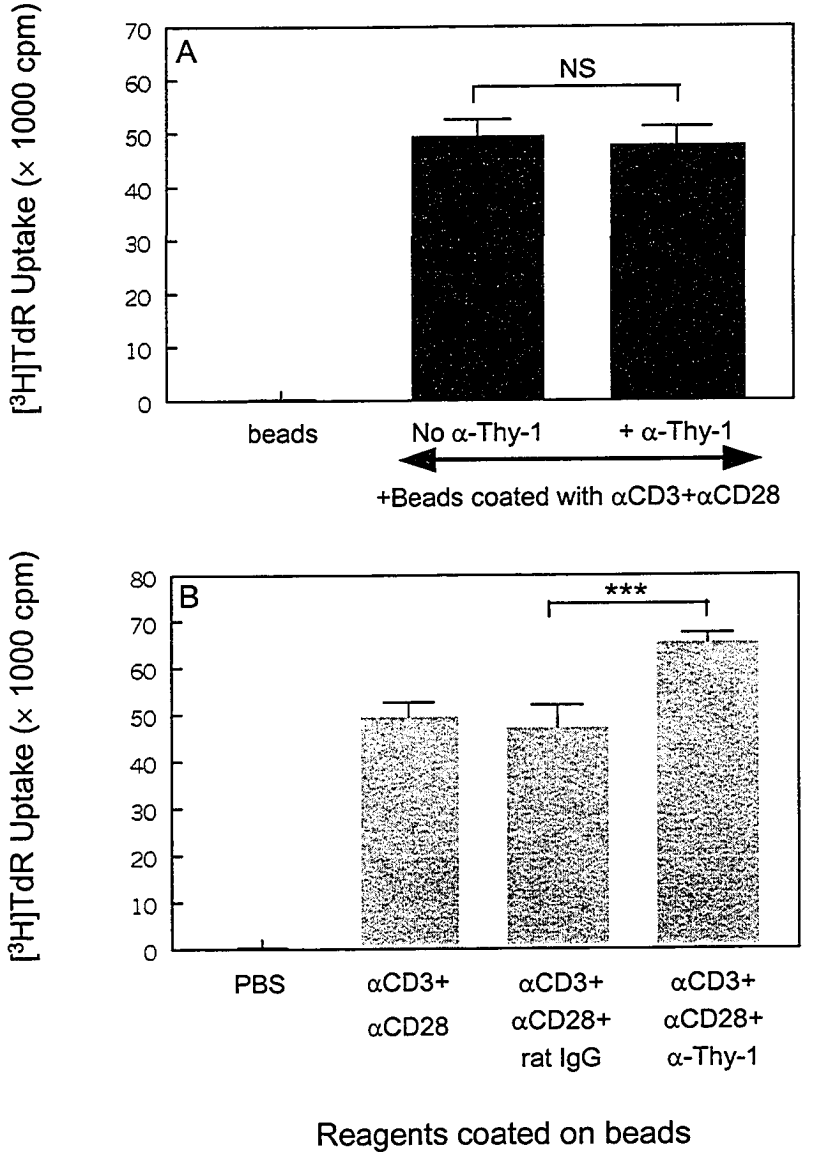

[³H]TdR Uptake (× 1000 cpm)
A
NS
70
60
50
40
30
20
10
0
beads
No α-Thy-1
+ α-Thy-1
+Beads coated with αCD3+αCD28
[³H]TdR Uptake (× 1000 cpm)
B
***
80
70
60
50
40
30
20
10
0
PBS
αCD3+ αCD28
αCD3+ αCD28+ rat IgG
αCD3+ αCD28+ α-Thy-1
Reagents coated on beads

Figure 3.8

Fig. 3.9. The 30-H12 anti-Thy-1 mAb does not block anti-CD3-induced or OVA-specific T cell proliferation in the presence of DCs. A) Highly purified, unfractionated T cells and syngeneic bone marrow-derived DCs were combined at a 25:1 (T:DC) ratio and incubated with anti-CD3 mAb for 72 h in the absence or presence of anti-Thy-1 mAb or control rat IgG (8 μg/ml). [^{3}H]TdR incorporation was then determined as a measure of T cell proliferation. B) Highly purified CD4$^+$ T cells expressing TCR transgene DO11.10 were combined with syngeneic bone marrow-derived DCs at a 25:1 ratio. The cognate peptide, $OVA_{323-339}$, was used at 300 nM to activate T cells in the absence or presence of anti-Thy-1 mAb or control rat IgG (8 μg/ml). [^{3}H]TdR incorporation was determined after 72 h as a measure of T cell proliferation. The control peptide, $OVA_{324-334}$, did not induce any T cell proliferation when combined with T cells and DCs.

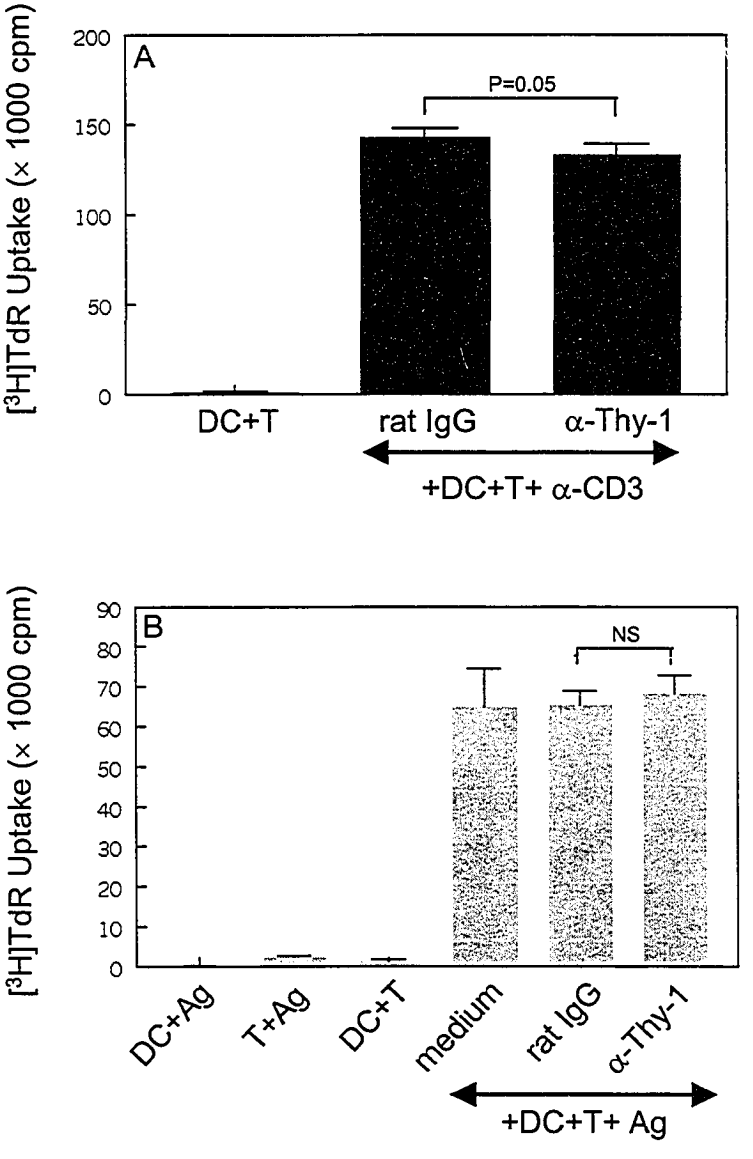

A
[³H]TdR Uptake (× 1000 cpm)
200
150
100
50
0
P=0.05
DC+T
rat IgG
α-Thy-1
+DC+T+ α-CD3
B
[³H]TdR Uptake (× 1000 cpm)
90
80
70
60
50
40
30
20
10
0
NS
DC+Ag
T+Ag
DC+T
medium
rat IgG
α-Thy-1
+DC+T+ Ag

Figure 3.9

Fig. 3.10. Anti-Thy-1 mAb 30-H12 inhibits T cell proliferation in response to anti-CD3 mAb plus LPS blasts. B cells were isolated and incubated with LPS (5 μg/ml) for 72 h. LPS-activated B lymphoblasts thus generated were harvested, thoroughly washed and used as accessory cells in our T cell activation protocol. 2×10^5 highly purified T cells were combined with the indicated numbers of LPS blasts together with anti-CD3 mAb in each well of a U-bottom microtiter plate. Anti-Thy-1 mAb or control rat IgG was also present at 8 μg/ml during T cell activation. [^{3}H]TdR incorporation was then determined at 48 h as a measure of T cell proliferation.

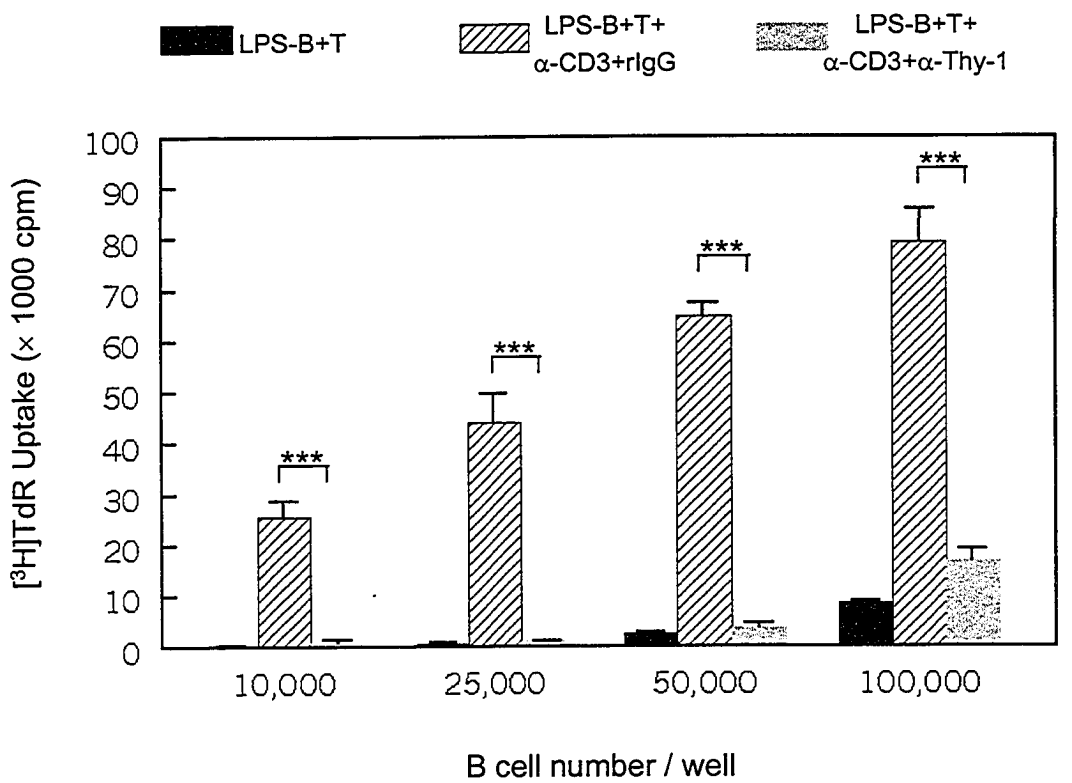

LPS-B+T
LPS-B+T+
α-CD3+rIgG
LPS-B+T+
α-CD3+α-Thy-1
[3H]TdR Uptake (× 1000 cpm)
100
90
80
70
60
50
40
30
20
10
0
***
***
***
***
10,000
25,000
50,000
100,000
B cell number / well

Figure 3.10

Fig. 3.11. Anti-Thy-1 mAb 30-H12 fails to inhibit T cell proliferation induced by PMA plus ionomycin. T cells were left unstimulated or activated with PMA (15 ng/ml) plus ionomycin (500 ng/ml) in the absence or presence of anti-Thy-1 mAb or control rat IgG (8 μg/ml). Cultures were incubated at 37°C in dark and T cell proliferation was determined by [^{3}H]TdR incorporation after 48 h.

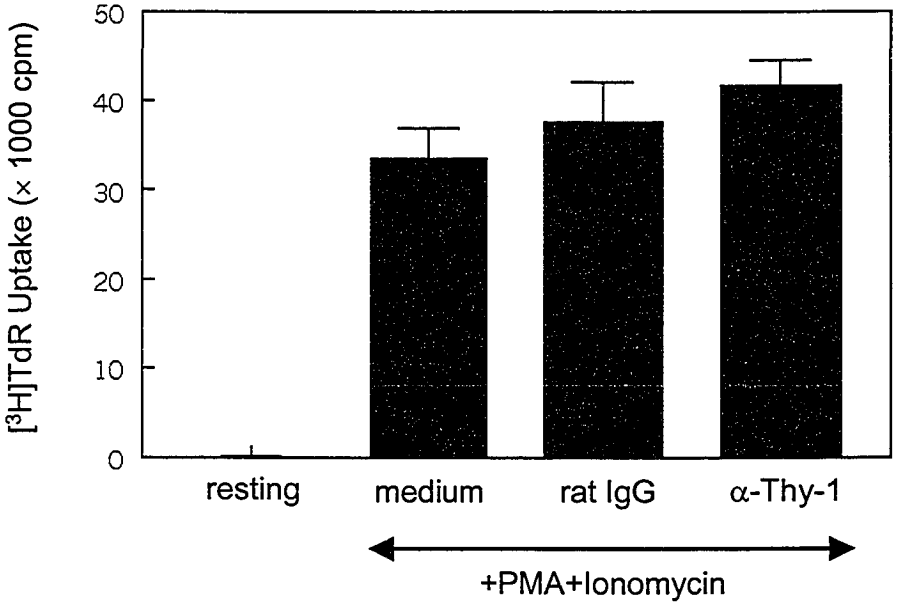

[3H]TdR Uptake (× 1000 cpm)
50
40
30
20
10
0
resting
medium
rat IgG
α-Thy-1
+PMA+Ionomycin

Figure 3.11

Fig. 3.12. PMA reverses the inhibitory effect of anti-Thy-1 mAb on CD3-driven T cell proliferation. T cells were stimulated with anti-CD3 mAb in combination with control rat IgG (8 μg/ml), rat IgG plus PMA (15 ng/ml), anti-Thy-1 (8 μg/ml), or anti-Thy-1 plus PMA. Cultures were incubated at 37°C and T cell proliferation was determined by [^{3}H]TdR incorporation after 48 h.

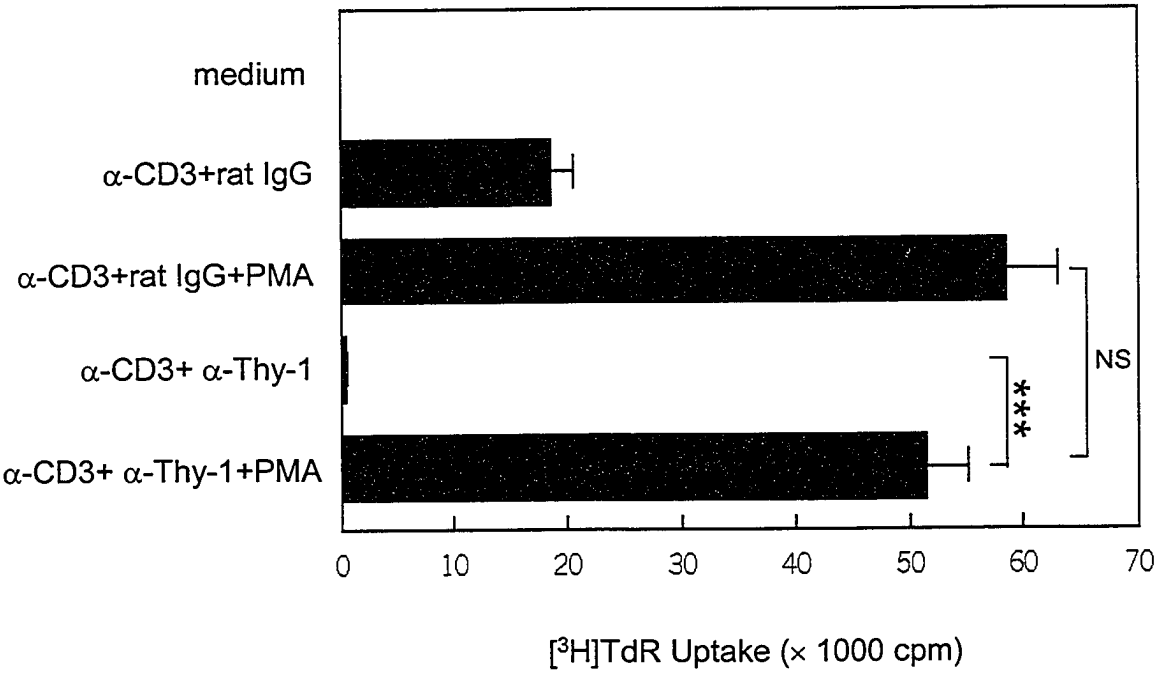

medium
α-CD3+rat IgG
α-CD3+rat IgG+PMA
α-CD3+ α-Thy-1
α-CD3+ α-Thy-1+PMA
NS
***
0
10
20
30
40
50
60
70
[3H]TdR Uptake (× 1000 cpm)

Figure 3.12

4. Selective pharmacological inhibitors reveal differences between Thy-1- and T cell receptor-mediated signal transduction in T lymphocytes

Clustering of Thy-1 by certain mAbs results in T cell proliferation and IL-2 production (Gunter *et al*, 1984; Kroczek *et al*, 1986a). Although there is abundant evidence that Thy-1 is capable of transducing an activation signal (Killeen, 1997; Kroczek *et al*, 1986b), it is puzzling how Thy-1, which does not even possess a classical transmembrane portion or a cytoplasmic tail, could act as a signaling molecule for T cell activation.

A possible interplay between the TCR/CD3 complex and Thy-1 has been suspected since a requirement for the co-expression of an intact, functional TCR/CD3 complex in Thy-1-associated T cell activation has been reported in several studies using TCR/CD3-deficient or -altered T cells (Gunter *et al*, 1987; Sato *et al*, 1990; Schmitt-Verhulst *et al*, 1987). Reciprocally, T cells deficient in GPI-anchored proteins, of which Thy-1 is a prototype, exhibit impaired early events in TCR-mediated signal transduction including tyrosine phosphorylation of TCR-ζ chain and ZAP-70 and activation of TCR-associated *Src* kinases *Fyn* and *Lck* (Leyton *et al*, 1999; Romagnoli and Bron, 1997; Yeh *et al*, 1988). However, these observations are contrary to the finding that murine T cells pretreated with phosphatidylinositol-specific phospholipase C to remove GPI moieties do not show a reduced proliferative response to anti-CD3 mAb (Presky *et al*, 1990). Even more contradictory are the results of studies with Thy-1 KO mice where a negative regulatory function for Thy-1 was suggested based on the hyper-responsiveness of Thy-1$^{-/-}$ thymocytes to TCR triggering (Hueber *et al*, 1997),

whereas peripheral T cells from Thy-1$^{-/-}$ mice exhibit a diminished Ca^{++} influx and reduced PTK activity following TCR stimulation (Beissert *et al*, 1998).

The controversy surrounding the relationship between Thy-1 and the TCR/CD3 complex in T cell activation prompted us to compare the role of several key signal transduction elements in T cell signaling through Thy-1 and the TCR/CD3 complex. I used a panel of selective pharmacological inhibitors to determine the importance of different intracellular biochemical components/steps of the T cell activation pathway initiated by cross-linking of Thy-1 by a mitogenic Thy-1-specific mAb (clone G7) relative to signaling triggered through the TCR/CD3 complex.

4.1 T cell proliferation in response to Thy-1 triggering

A 1:20 dilution of anti-CD3 hybridoma supernatant was determined to be the optimal dose of anti-CD3 mAb to induce T cell proliferation measured at 48 h (Fig. 4.1A). Stimulation of spleen-derived resting T lymphocytes with anti-Thy-1 mAb (clone G7) also led to a dose-dependent proliferative response that was maximal at 6 μg/ml of mAb (Fig. 4.1B). Increasing the concentration of either mAb beyond the optimal dose not only failed to further increase the proliferative response of T cells, but led to less vigorous proliferation. Anti-Thy-1-induced DNA synthesis was measured at 72 h of culture since prior kinetic studies have determined that peak T cell proliferative responses to Thy-1 triggering occur at this time point (Gunter et al, 1984). I next compared T cell proliferative responses triggered by anti-Thy-1 mAb with those induced by optimal concentrations of mitogenic anti-CD3 mAb (1:20 dilution of hybridoma

supernatant) or ConA (5 μg/ml). Anti-Thy-1 mAb, while the least potent of the three stimuli, was, nevertheless, capable in my hands of yielding a strong stimulation index of 209, compared to stimulation indices of 453 and 791 for anti-CD3 mAb and ConA, respectively.

4.2 Effect of the inhibition of PTKs on Thy-1 and TCR signal transduction

Protein tyrosine phosphorylation regulated by PTKs and protein tyrosine phosphatases is among the earliest signal transduction events occurring during T cell activation via the TCR (Neel, 1997; Weiss and Littman, 1994). In order to compare the involvement of PTKs in Thy-1 and TCR-mediated signal transduction pathways, I employed herbimycin A, an agent that irreversibly degrades *Src*-family PTKs (June *et al*, 1990). Herbimycin A similarly inhibited anti-CD3 and anti-Thy-1-induced T cell proliferation in a dose-dependent fashion (Fig. 4.2), indicating a requirement for PTKs in both signaling pathways.

4.3 Effect of PI-3K inhibition on Thy-1 and TCR signaling

Activation of PI-3K is an important step in T cell activation following the engagement of the TCR (Ward *et al*, 1996). To determine whether PI-3K is also activated subsequent to Thy-1 cross-linking by G7 mAb, I employed the selective PI-3K inhibitor LY294002 (Vlahos *et al*, 1994). As shown in Fig. 4.3, anti-Thy-1-induced T cell proliferation was inhibited in the presence of LY294002, as was anti-CD3-induced T cell proliferation.

4.4 Effect of PKC activation or inhibition on Thy-1-triggered T cell proliferation

Stimulation of T cells via the TCR is known to cause translocation of PLCγ1 to the plasma membrane where it functions to hydrolyze PIP_2 to DAG and IP_3. DAG activates PKC, while IP_3 formation leads to Ca^{++} release from intracellular stores (Weiss and Littman, 1994). Artificial PKC activation by phorbol esters is also known to augment anti-Thy-1-induced T cell proliferation (Kroczek *et al*, 1986a). In our system, T cell proliferation in response to G7 mAb was similarly amplified in a synergistic manner by concomitant treatment with PMA (Fig. 4.4). Increased proliferation paralleled a synergistic increase in IL-2 bioactivity in T cell cultures, as judged by the ability of culture supernatants to support the growth of the IL-2-dependent cell line, CTLL-2 (Fig. 4.5A). This was not due to a direct effect of the G7 anti-Thy-1 mAb on CTLL-2 cells as adding G7 to CTLL-2 cells neither induced CTLL-2 proliferation nor modulated CTLL-2 response to exogenous IL-2 (Fig. 4.5B). I confirmed these results by measuring the IL-2 content of culture supernatants by ELISA (Table 4.1) and by assessing IL-2 mRNA transcription via RT-PCR (Fig. 4.6). The observed synergism between the anti-Thy-1 mAb and PMA in terms of IL-2 mRNA transcription was so impressive that even a 1:20 dilution of cDNA obtained from T cells treated with anti-Thy-1 mAb plus PMA yielded abundant PCR product corresponding to IL-2 (Fig. 4.6).

Next, I used calphostin C, a highly selective PKC inhibitor (Kobayashi *et al*, 1989), to compare TCR- and Thy-1-associated signaling pathways in terms of

PKC involvement. Both anti-CD3- and anti-Thy-1-induced T cell proliferative responses were inhibited in the presence of calphostin C (Fig. 4.7), indicating a requirement for PKC participation in both signaling pathways. However, the Thy-1 signaling pathway was consistently more sensitive than TCR signaling to PKC inhibition.

4.5 Effect of CsA on Thy-1- and TCR-associated T cell proliferative responses

When complexed with cyclophilins, CsA inhibits calcineurin, a key protein phosphatase for T cell activation (Clipstone and Crabtree, 1992). As shown in Fig. 4.8, T cell proliferation induced by anti-Thy-1 or anti-CD3 mAb was inhibited to a similar extent by CsA, which is consistent with the involvement of calcineurin in both signaling pathways.

4.6 Effects of Inhibition of MAPKs on anti-Thy-1- and anti-CD3-induced T cell proliferation

The mammalian MAPK family members, *i.e.* extracellular signal-regulated kinases 1 and 2 (ERK1/ERK2), c-Jun N-terminal kinase (JNK, also known as stress-activated protein kinase), and p38 MAPK, are known to participate in T cell activation (DeSilva *et al*, 1998). ERK1/ERK2 are activated by the upstream kinase MEK1, which is inhibitable by PD98059 (Dudley *et al*, 1995). I observed inhibition of anti-CD3-induced T cell proliferation by the MEK1 inhibitor PD98059. Interestingly, Thy-1-driven T cell proliferation was even more sensitive than anti-CD3-induced T cell proliferation to MEK1 inhibition (Fig. 4.9). To determine the role of p38 MAPK in Thy-1- and TCR-driven T cell activation, I used the highly

selective p38 inhibitor SB203580 (Lee *et al*, 1999). The data shown in Fig. 4.10A indicate that anti-CD3-induced T cell proliferation is partially suppressed in the presence of SB203580. However, to my surprise, p38 inhibition augmented T cell proliferation in response to anti-Thy-1 mAb. This result was consistently obtained in four independent experiments (Fig. 4.10B) and was confirmed in a separate series of experiments in which T cells were activated with anti-Thy-1 mAb in the presence of SK&F 86002, another inhibitor of p38 (Pouliot *et al*, 1997)(Fig. 4.11).

Fig. 4.1. Ab-mediated triggering of TCR or Thy-1 leads to T cell proliferation in a dose-dependent fashion. T cells were stimulated with different dilutions of anti-CD3 hybridoma supernatant (clone 145-2C11) or with the indicated concentrations of purified anti-Thy-1 mAb (clone G7) for 48 and 72 h, respectively. [^{3}H]TdR incorporation by T cells was then determined as a measure of T cell proliferation. Results from a representative experiment are shown.

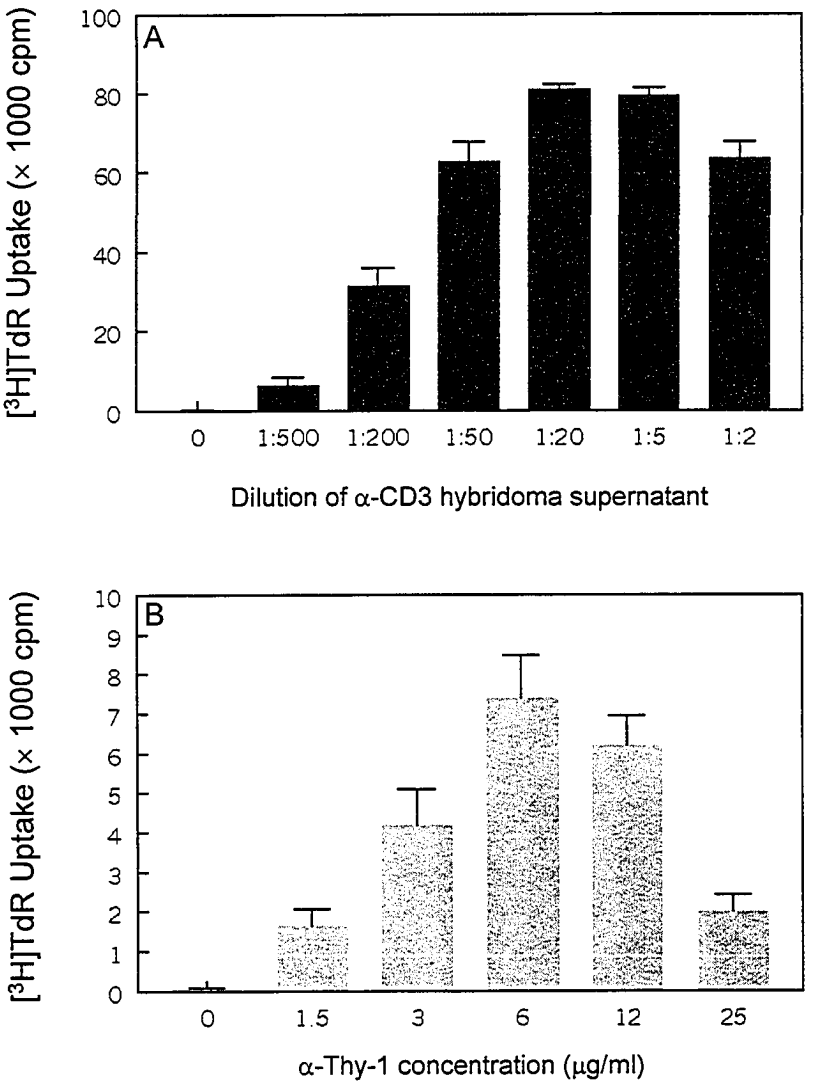

A
[³H]TdR Uptake (× 1000 cpm)
100
80
60
40
20
0
0
1:500
1:200
1:50
1:20
1:5
1:2
Dilution of α-CD3 hybridoma supernatant
B
[³H]TdR Uptake (× 1000 cpm)
10
9
8
7
6
5
4
3
2
1
0
0
1.5
3
6
12
25
α-Thy-1 concentration (μg/ml)

Figure 4.1

Fig. 4.2. Herbimycin A inhibits both anti-CD3- and anti-Thy-1-induced T cell proliferation. T cells were stimulated with or without anti-CD3 mAb (1:20 dilution of culture supernatant) or anti-Thy-1 mAb (6 μg/ml) in the absence or presence of the indicated concentrations of herbimycin A (HerbA). [^{3}H]TdR incorporation by T cells was measured after 48 h of culture for anti-CD3-activated T cells and after 72 h of culture for anti-Thy-1-activated T cells. Differences were extremely significant by ANOVA ($p<0.0001$) and Bonferroni multiple comparisons test (* denotes $p<0.05$, *** denotes $p<0.001$ compared with the DMSO vehicle control).

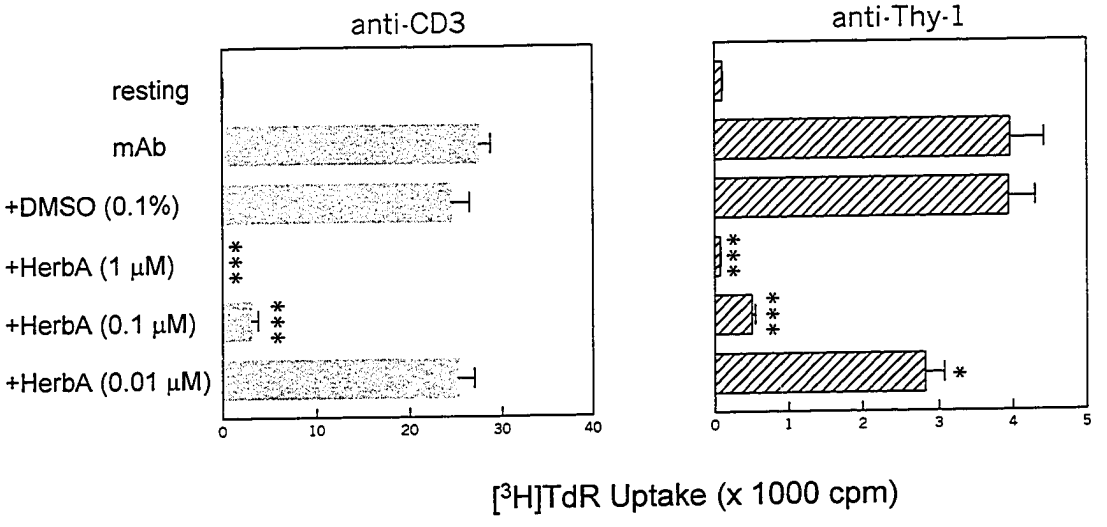

anti-CD3
anti-Thy-1
resting
mAb
+DMSO (0.1%)
+HerbA (1 μM)
+HerbA (0.1 μM)
+HerbA (0.01 μM)
0
10
20
30
40
0
1
2
3
4
5
[3H]TdR Uptake (x 1000 cpm)

Figure 4.2

Fig. 4.3. LY294002 inhibits both anti-CD3- and anti-Thy-1-induced T cell proliferation. T cells were stimulated with or without anti-CD3 or anti-Thy-1 mAb in the absence or presence of the indicated concentrations of LY294002 (LY). [^{3}H]TdR incorporation by T cells was measured after 48 h of culture for anti-CD3-activated T cells and after 72 h of culture for anti-Thy-1-activated T cells. Differences were extremely significant using ANOVA ($p<0.0001$) and Bonferroni multiple comparisons test (* denotes $p<0.05$, *** denotes $p<0.001$ compared with the DMSO vehicle control).

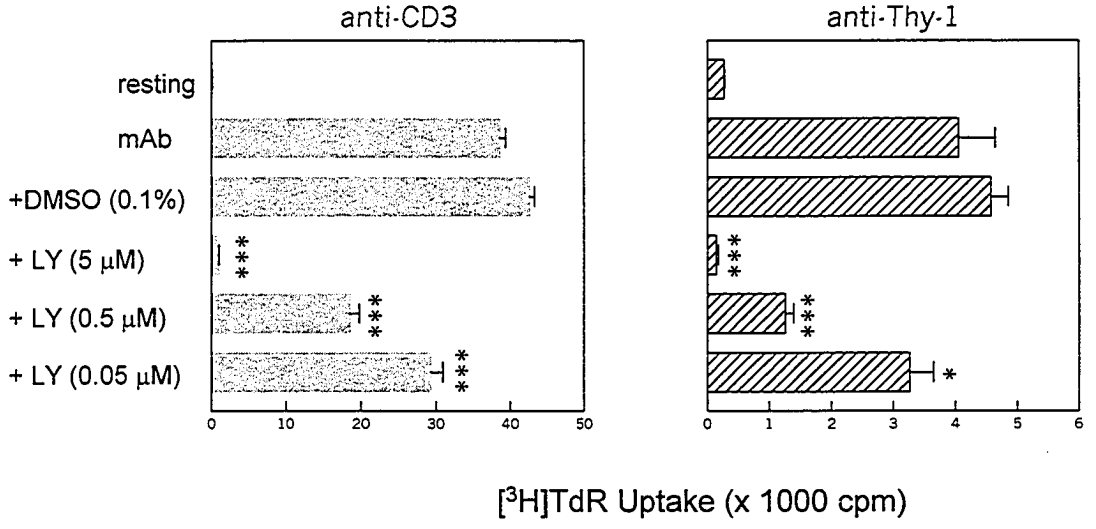

anti-CD3
anti-Thy-1
resting
mAb
+DMSO (0.1%)
+ LY (5 μM)
+ LY (0.5 μM)
+ LY (0.05 μM)
0
10
20
30
40
50
0
1
2
3
4
5
6
[3H]TdR Uptake (x 1000 cpm)

Figure 4.3

Fig. 4.4. A combination of anti-Thy-1 mAb and PKC activator PMA induces strong T cell proliferation. T cells were incubated with anti-Thy-1 mAb (6 μg/ml), PMA (10 ng/ml), or anti-Thy-1 mAb plus PMA. [^{3}H]TdR incorporation by T cells was determined at 48 h as a measure of T cell proliferation. [^{3}H]TdR incorporation by T cells treated with a combination of rat IgG control (6 μg/ml) and ethanol (the vehicle for PMA) was 286 ± 75 cpm.

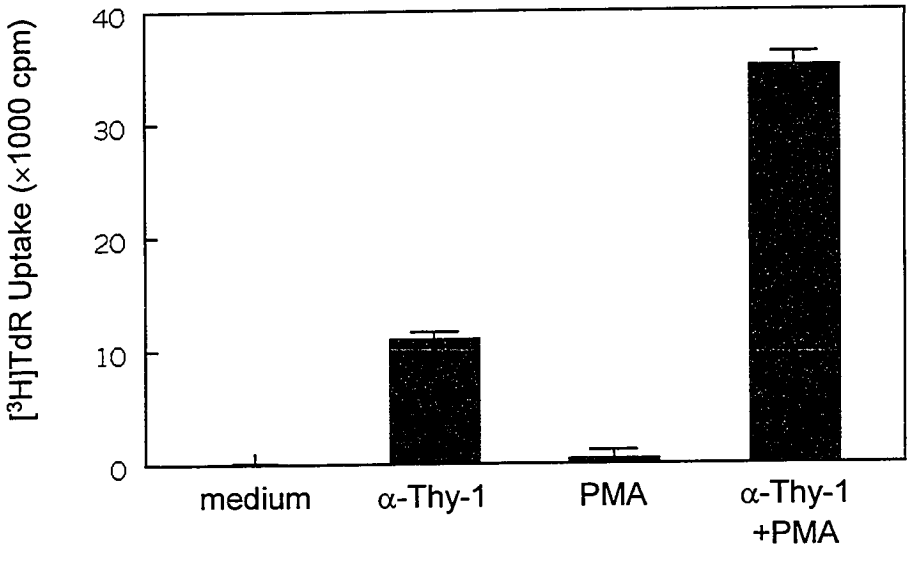
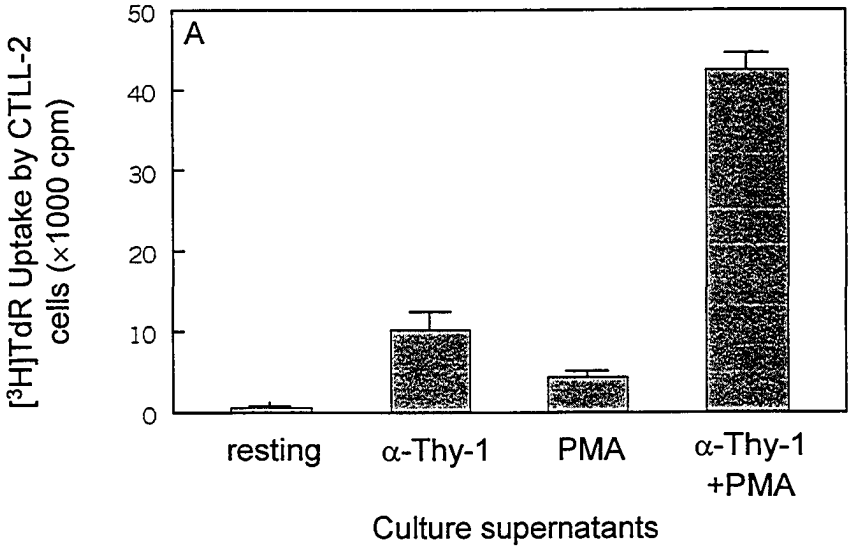


Figure 4.4

Fig. 4.5. A) PKC activator PMA augments Thy-1-driven IL-2 production by T cells in a synergistic manner. Culture supernatants were collected from T cells following a 24-h incubation with medium, anti-Thy-1 mAb (6 μg/ml), PMA (10 ng/ml) or a combination of anti-Thy-1 mAb and PMA. Culture supernatants were added to CTLL-2 cells and [^{3}H]TdR uptake by CTLL-2 cells was determined after 24 h as an indirect measure of IL-2 bioactivity in T cell cultures. B) Anti-Thy-1 mAb does not directly induce CTLL-2 cell proliferation. CTLL-2 cells were cultured with or without recombinant mouse IL-2 (10 U/ml) in the absence or presence of anti-Thy-1 mAb (6 μg/ml). CTLL-2 cell proliferation was then determined by measuring [^{3}H]TdR incorporation after 24 h.


A
[³H]TdR Uptake by CTLL-2 cells (×1000 cpm)
50
40
30
20
10
0
resting
α-Thy-1
PMA
α-Thy-1 +PMA
Culture supernatants

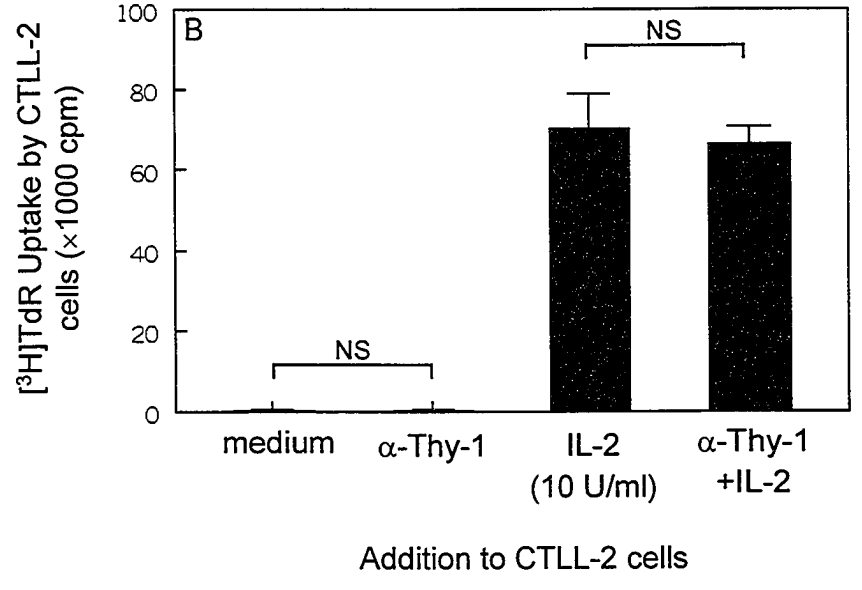

B
[³H]TdR Uptake by CTLL-2 cells (×1000 cpm)
100
80
60
40
20
0
NS
NS
medium
α-Thy-1
IL-2 (10 U/ml)
α-Thy-1 +IL-2
Addition to CTLL-2 cells

Figure 4.5

Table 4.1. Synergism between anti-Thy-1 mAb and PMA in inducing IL-2 synthesis by T cells

	IL-2 synthesis (pg/ml)			
Experiment	Resting	α-Thy-1	PMA	α-Thy-1+PMA
1	BDL	490 ± 12	BDL	25,413 ± 6,215
2	BDL	632 ± 32	1 ± 1	25,451 ± 125

Culture supernatants were collected from T cells following a 24-h incubation with medium, anti-Thy-1 mAb (6 μg/ml), PMA (10 ng/ml) or a combination of anti-Thy-1 mAb and PMA. The IL-2 content of culture supernatants was immediately quantified by sandwich ELISA. Data are expressed as mean ± SD in triplicate wells from each experiment. BDL: below detection limit.

Fig. 4.6. Anti-Thy-1 mAb and PKC activator PMA act synergistically to up-regulate IL-2 mRNA levels. Total RNA was isolated from resting T cells or T cells incubated with the indicated Abs/reagents for 18 h. Approximately 1 μg RNA was reverse-transcribed followed by a semiquantitative PCR to determine IL-2 mRNA levels. The amplification of equal amounts of cDNA for different treatment groups was ensured through normalization based on the steady-state expression of GAPDH. A 1:20 dilution of cDNA obtained from T cells stimulated with anti-Thy-1 mAb plus PMA was also subjected to PCR simultaneously.

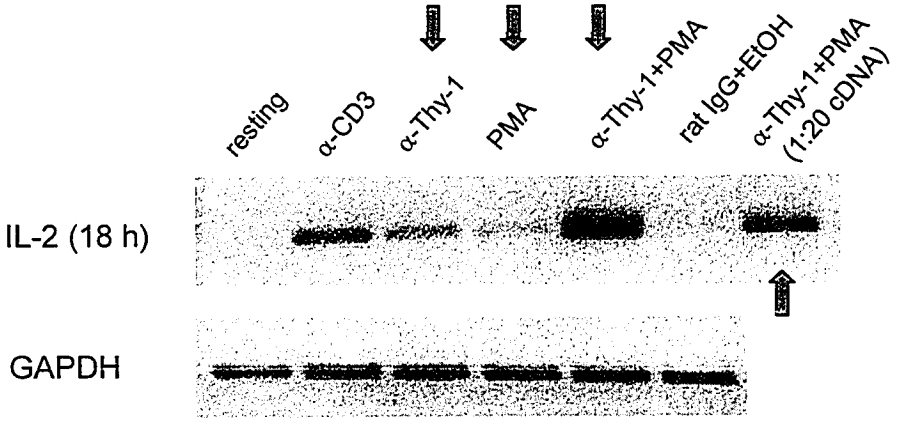

resting
α-CD3
α-Thy-1
PMA
α-Thy-1+PMA
rat IgG+EtOH
α-Thy-1+PMA (1:20 cDNA)
IL-2 (18 h)
GAPDH

Figure 4.6

Fig. 4.7. Calphostin C inhibits both anti-CD3- and anti-Thy-1-induced T cell proliferation. T cells were stimulated with anti-CD3 or anti-Thy-1 mAb in the absence or presence of the indicated concentrations of calphostin C (CalC). [^{3}H]TdR incorporation by T cells was measured after 48 h of culture for anti-CD3-activated T cells and after 72 h of culture for anti-Thy-1-activated T cells. Differences were extremely significant by ANOVA (p<0.0001) and Bonferroni multiple comparisons test (* denotes p<0.05, *** denotes p<0.001 compared with the DMSO vehicle control).

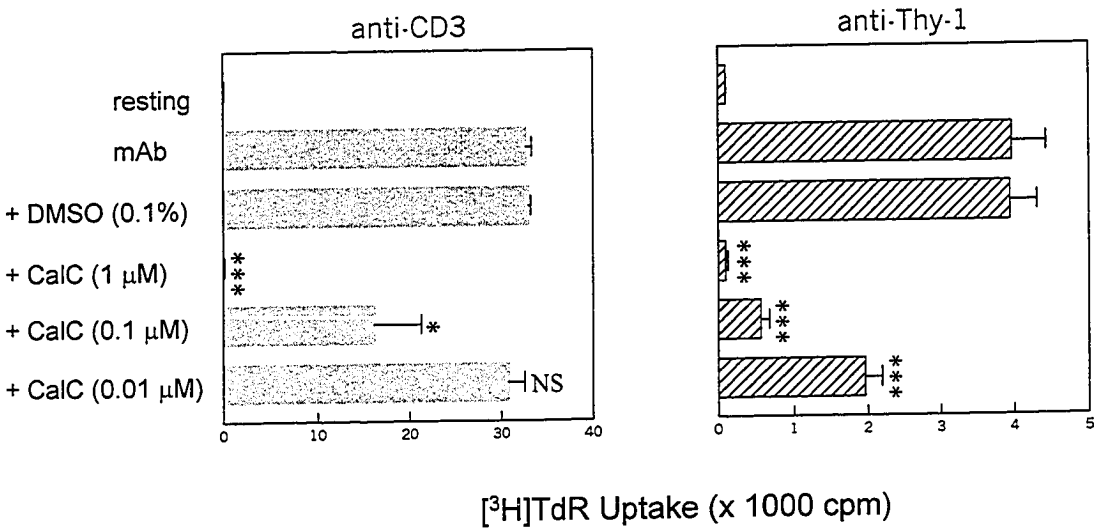

anti-CD3
anti-Thy-1
resting
mAb
+ DMSO (0.1%)
+ CalC (1 μM)
+ CalC (0.1 μM)
+ CalC (0.01 μM)
NS
0
10
20
30
40
0
1
2
3
4
5
[3H]TdR Uptake (x 1000 cpm)

Figure 4.7

Fig. 4.8. CsA inhibits both anti-CD3- and anti-Thy-1-induced T cell proliferation. T cells were stimulated with or without anti-CD3 or anti-Thy-1 mAb in the absence or presence of the indicated concentrations of CsA. [^{3}H]TdR incorporation by T cells was measured after 48 h of culture for anti-CD3-activated T cells and after 72 h of culture for anti-Thy-1-activated T cells. Differences were extremely significant by ANOVA (p<0.0001) and Bonferroni multiple comparisons test (*** denotes p<0.001 compared with control). Treatment with ethanol (the vehicle for CsA) did not affect [^{3}H]TdR incorporation by T cells stimulated with either mAb.

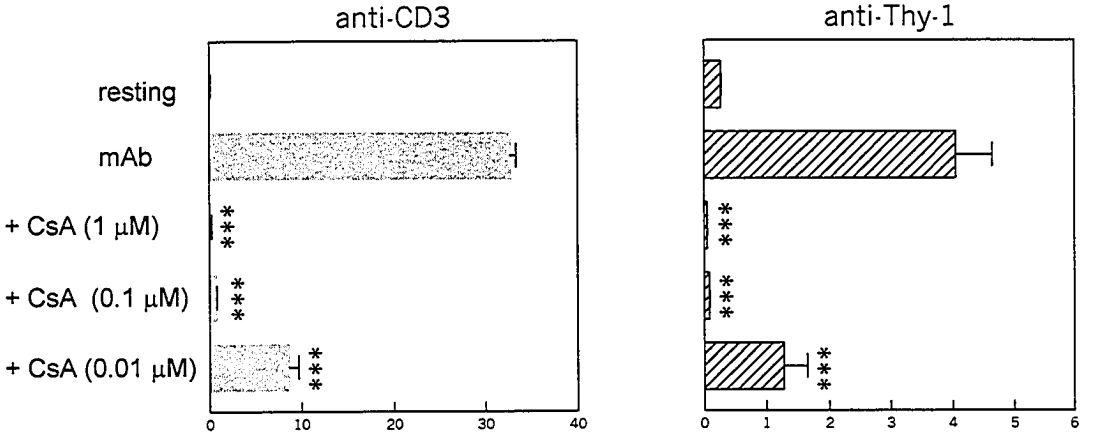

anti-CD3
anti-Thy-1
resting
mAb
+ CsA (1 μM)
+ CsA (0.1 μM)
+ CsA (0.01 μM)
***
0
10
20
30
40
0
1
2
3
4
5
6
[3H]TdR Uptake (x 1000 cpm)

Figure 4.8

Fig. 4.9. PD98059 inhibits T cell proliferation induced by anti-CD3 or anti-Thy-1 mAbs. T cells were stimulated with or without anti-CD3 or anti-Thy-1 mAb in the absence or presence of the indicated concentrations of PD98059 (PD). [^{3}H]TdR incorporation by T cells was measured after 48 h of culture for anti-CD3-activated T cells and after 72 h of culture for anti-Thy-1-activated T cells. Differences were statistically significant by ANOVA (p=0.0003 and p<0.0001 in anti-CD3- and anti-Thy-1-activated cultures, respectively) and Bonferroni multiple comparisons test (*** denotes p<0.001, ** denotes p<0.01 compared with the DMSO vehicle control).

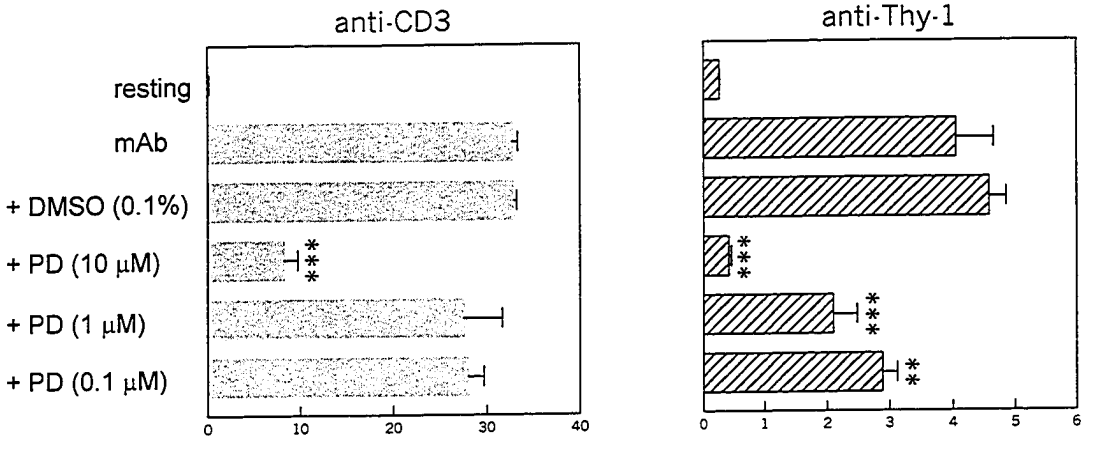
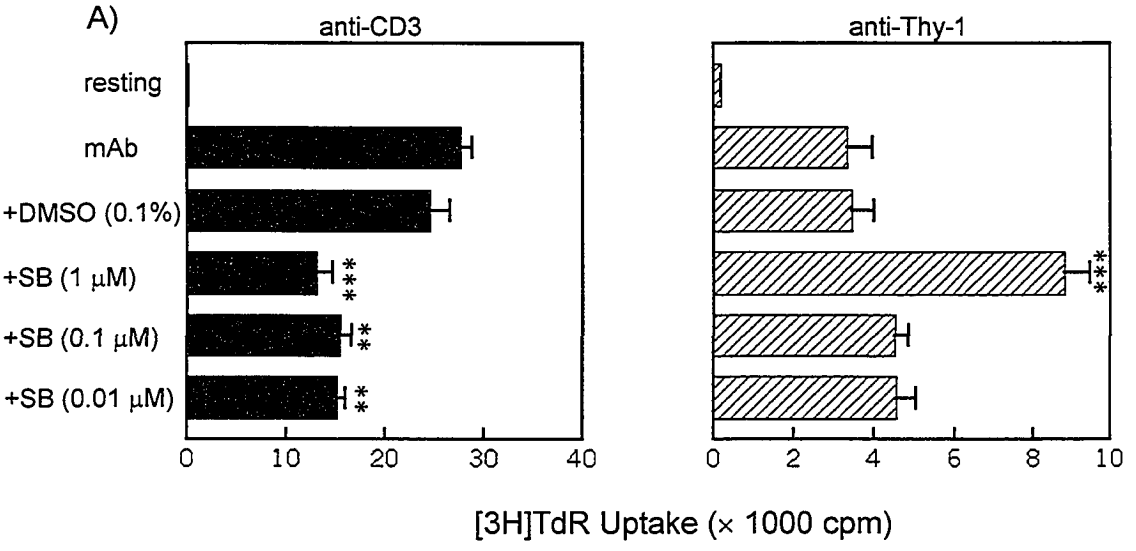

anti-CD3
anti-Thy-1
resting
mAb
+ DMSO (0.1%)
+ PD (10 μM)
+ PD (1 μM)
+ PD (0.1 μM)
0
10
20
30
40
0
1
2
3
4
5
6
[3H]TdR Uptake (x 1000 cpm)

Figure 4.9

Fig. 4.10. SB203580 has differential effects on anti-CD3- and anti-Thy-1-induced T cell proliferation. A) T cells were stimulated with or without anti-CD3 or anti-Thy-1 mAb in the absence or presence of the indicated concentrations of SB203580 (SB). [^{3}H]TdR incorporation by T cells was measured after 48 h of culture for anti-CD3-activated T cells and after 72 h of culture for anti-Thy-1-activated T cells. Data are from one experiment representative of three independent experiments yielding similar results. Differences were statistically significant by ANOVA (p=0.0008 and p<0.0001 in anti-CD3 and anti-Thy-1-activated cultures, respectively) and Bonferroni multiple comparisons test (*** denotes p<0.001, ** denotes p<0.01, * denotes p<0.05 compared with the DMSO vehicle control). B) Combined results from four independent experiments using anti-Thy-1 mAb as the T cell stimulus are depicted (* denotes p<0.05 compared with unstimulated T cell cultures).


A)
anti-CD3
anti-Thy-1
resting
mAb
+DMSO (0.1%)
+SB (1 μM)
+SB (0.1 μM)
+SB (0.01 μM)
0
10
20
30
40
0
2
4
6
8
10
[3H]TdR Uptake (× 1000 cpm)

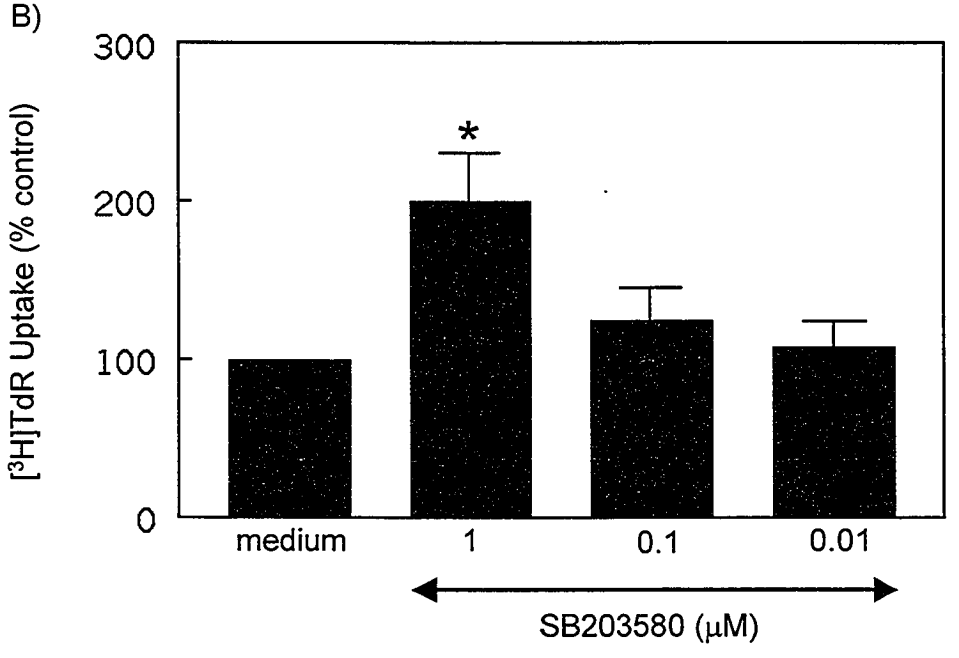

B)
[3H]TdR Uptake (% control)
300
200
100
0
medium
1
0.1
0.01
SB203580 (μM)

Figure 4.10

Fig. 4.11. Inhibition of p38 by SK&F 86002 augments Thy-1-driven T cell proliferation. T cells were left unstimulated or activated with anti-Thy-1 mAb in the absence or presence of the indicated concentrations of SK&F 86002. [^{3}H]TdR incorporation by T cells was measured after 72 h of culture. Data are shown as mean cpm ± SD in triplicate wells from one representative experiment (n=2). Differences were extremely significant by ANOVA (p<0.0001) and Bonferroni multiple comparisons test (*, **, and *** denote p<0.05, p<0.01, and p<0.001, respectively in comparison with the DMSO vehicle control).

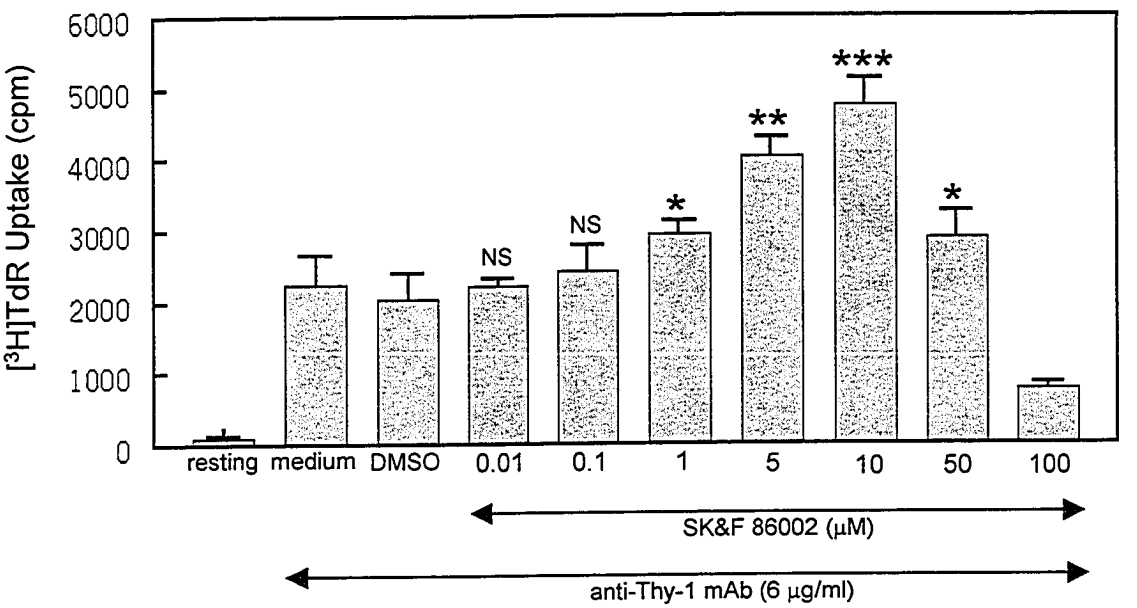

[3H]TdR Uptake (cpm)
6000
5000
4000
3000
2000
1000
0
resting
medium
DMSO
0.01
0.1
1
5
10
50
100
NS
NS
*
**
***
*
SK&F 86002 (μM)
anti-Thy-1 mAb (6 μg/ml)

Figure 4.11

5. Thy-1 triggering generates an incomplete form of signal 1 for T cell activation in the context of costimulation provided by DCs

Although a physiological ligand for Thy-1 has yet to be identified, cross-linking of Thy-1 by certain mAbs results in IL-2 production and T cell proliferation (Gunter *et al*, 1984; Kroczek *et al*, 1986), suggesting that Thy-1 may be a potential source of signal 1 for T cell activation. Several other roles have been proposed for Thy-1 in the process of T cell activation, ranging from negative regulation (Hueber *et al*, 1997; Killeen 1997) to accessory function (Johnson *et al*, 1993). However, whether Thy-1 can substitute for signal 1 in the context of optimal costimulation is not yet known.

DCs are the most potent APCs owing to their expression of high levels of class II MHC as well as costimulatory molecules (reviewed by Banchereau *et al*, 2000). T cell-dependent immune responses initiated by DCs depend highly on the DC-mediated provision of B7-1 and/or B7-2 costimulatory signals. The specialized ability of DCs to bind and form immunological synapses with resting T cells allows DCs to prime naïve T cells both *in vivo* and *in vitro*, and distinguishes DCs from other professional APCs (Al-Alwan *et al*, 2001; Norbury *et al*, 2002). Given the importance and efficiency of DCs in costimulating T cell responses, I asked whether T cell activation via Thy-1 as a potential source of signal 1 could be enhanced by a strong, DC-generated signal 2. The data presented in this chapter indicate that Thy-1 triggering can in fact substitute for signal 1 for driving T cell proliferation and IL-2 synthesis when DCs are present to provide CD28-dependent costimulation. However, in contrast to signaling

through the TCR, Thy-1-mediated signal 1 failed to induce cytotoxic effector function, even in the presence of strong costimulation. This reflects a hitherto unrecognized fundamental difference between Thy-1- and the TCR-associated T cell signaling pathways.

5.1 DCs augment Thy-1-driven T cell proliferation and IL-2 production

The requirement for concurrent stimulation with phorbol ester in order for Thy-1 signaling to elicit IL-2 synthesis and substantial T cell proliferation in the absence of APCs (Gunter *et al*, 1984; Kroczek *et al*, 1986a) suggests a need for costimulation. Since DCs are a potent source of costimulatory molecules (Banchereau *et al*, 2000), I stimulated mouse T cells with a mitogenic anti-Thy-1 mAb (clone G7) in the presence of mature, syngeneic DCs. The T cells responded by vigorously proliferating and producing substantial amounts of IL-2 in comparison to T cells activated with anti-Thy-1 mAb in the absence of DCs (Fig. 5.1A and 5.1B, respectively). The presence of IL-2 bioactivity in cultures was judged by the ability of culture supernatants to support the growth of the IL-2-dependent CTLL-2 cells. The enhanced Thy-1-driven responses were clearly dependent on the number of DCs present in the cultures (Fig. 5.1C). In contrast, even high numbers of B cells failed to appreciably enhance T cell activation through Thy-1 triggering, indicating a somewhat unique role for DCs in the activation of T cells through Thy-1. DCs did not potentiate measurable T cell proliferation in the presence of irrelevant IgG of the same isotype, a non-stimulatory mAb to Thy-1 (clone 30-H12), or mAbs to other T cell surface molecules such as class I MHC, CD2 or CD28 (data not shown). This indicates

that the DC enhancement of Thy-1-driven T cell proliferation is not simply a general phenomenon that might occur in response to the presence of IgG or any T cell-specific mAb in the T cell cultures.

5.2 DC enhancement of Thy-1-stimulated T cell proliferation is not dependent on Fcγ receptors

Since T cell activation by mitogenic Abs normally requires cross-linking of Abs by FcγRs expressed by accessory cells (Smith *et al*, 1986), I next examined bone marrow-derived DCs for FcγR expression. As depicted in Fig. 5.2, freshly isolated DCs showed substantial immunoreactivity with 2.4G2 mAb (Fc Block^{TM}), which is routinely used to detect FcγRII (CD32)/FcγRIII (CD16) (Ravetch *et al*, 1986; Unkeless, 1979). I then used FcRγ KO mice that lack FcγRI (CD64) and FcγRIII (Takai *et al*, 1994), as well as Ab-mediated blockade of FcγRII and FcγRIII to determine whether or not DC FcγRs play a role in anti-Thy-1-induced T cell activation. As a control, T cells were stimulated in parallel by anti-CD3 mAb since the interactions between anti-CD3 Fc portion and FcγRs expressed by accessory cells including DCs are known to be essential for T cell activation in response to anti-CD3 mAb (Elbe-Burger *et al*, 2000; Romani *et al*, 1989). This was confirmed by the ability of anti-CD3 mAb whole molecule, but not anti-CD3-Fos [functionally identical to anti-CD3 F(ab')$_2$], to induce T cell proliferation in the presence of DCs (Fig. 5.3A). Accordingly, artificial cross-linking of anti-CD3-Fos by coating this molecule on microbeads enabled anti-CD3-Fos to induce substantial T cell proliferation of similar magnitude to that induced by anti-CD3 whole molecule (Fig. 5.3B). Fig. 5.4 demonstrates that anti-CD3-induced T cell

proliferation was marginally attenuated (23% inhibition) by the absence of FcγRI and FcγRIII on FcRγ$^{-/-}$ DCs, while the Ab-mediated blockade of FcγRII/III nearly completely inhibited anti-CD3-induced T cell proliferation (92% inhibition). Moreover, the use of Fc Block^{TM} with DCs from KO mice abolished anti-CD3-induced T cell proliferation. In contrast, the absence and/or blockade of FcγRs did not affect T cell proliferation triggered by anti-Thy-1 mAb. I did not detect any differences between DCs from FcRγ$^{-/-}$ mice and DCs isolated from their WT littermates in terms of cell yields and gross morphology in cultures. Furthermore, the KO DCs were as potent as WT DCs in presenting an ovalbumin peptide to T cells rendered transgenic for the corresponding TCR, and initiating an Ag-specific T cell response (Fig. 5.5). Collectively, my data demonstrate that although mouse bone marrow-derived DCs express FcγRs, FcγRs are not needed for DC-mediated enhancement of T cell proliferation induced by Thy-1 triggering.

5.3 Costimulation through CD28 enhances T cell activation triggered by Thy-1-driven signal 1

Although B7 family members are important costimulators of TCR-driven T cell proliferation (Lanier *et al*, 1995), little is known about the role of CD28/B7 interactions during Thy-1-mediated T cell activation. I, therefore, asked whether or not DC enhancement of Thy-1-driven T cell activation is a consequence of costimulation provided by DCs. As illustrated in Fig. 5.6, anti-CD3- and anti-Thy-1-induced T cell proliferative responses were similarly inhibited by the inclusion of blocking Abs specific to B7-1 (31% and 33% inhibition, respectively) or B7-2 (34% and 45% inhibition, respectively). Blocking both B7-1 and B7-2 resulted in a

more pronounced inhibition of T cell proliferation triggered by anti-CD3 (87% inhibition) or anti-Thy-1 (90% inhibition) mAb, emphasizing the importance of both costimulatory interactions in driving these responses. These observations also led me to hypothesize that the inability of resting B cells to costimulate Thy-1-triggered T cell proliferation may be due to low expression of B7 costimulatory molecules by B cells. When I used LPS blasts displaying up-regulated levels of B7 molecules (Makrigiannis *et al*, 1999) as accessory cells, anti-Thy-1-induced T cell proliferation was significantly enhanced (Fig. 5.7), which is consistent with the above hypothesis.

To confirm that Thy-1 is capable of providing a signal 1 substitute for T cell activation, I coated microbeads with anti-Thy-1 and/or anti-CD28 mAbs and used these microbeads as surrogate APCs to provide T cells with signal 1 and/or signal 2, respectively. The presence of anti-CD28 mAb on beads was adequate to replace CD28/B7 interactions and dramatically enhanced T cell proliferation when combined with anti-Thy-1 mAb (Fig. 5.8A). The presence of varying concentrations of anti-Thy-1 mAb together with a constant amount of anti-CD28 mAb on beads caused T cell proliferation in a dose-dependent manner (Fig. 5.8B). Taken together, these data demonstrate that Thy-1 signaling can substitute for signal 1, and leads to robust T cell activation upon coupling with a strong signal 2.

5.4 T cells stimulated with anti-Thy-1 in the presence of DCs do not exhibit cytotoxic effector function

CD28-B7 costimulatory interactions are known to play an important role in the generation of Ag-specific (Sigal *et al*, 1998), allogeneic (Gajewski, 1996) and anti-CD3-induced (Makrigiannis *et al*, 1999) CTL responses. The data presented here and my unpublished observations indicate that Thy-1 triggering alone or in the presence of weak costimulation by B cells and macrophages does not give rise to nonspecific cytotoxicity against target cells. Since DCs potently enhanced Thy-1-driven T cell proliferation and IL-2 synthesis in a costimulation-dependent manner, it was of interest to determine whether or not DCs could also contribute to the generation of a cytotoxic phenotype following Thy-1 triggering. Unlike anti-CD3-activated T cells, T cells stimulated with anti-Thy-1 in the presence of DCs failed to kill P815 mastocytoma cells (Fig. 5.9A). Because P815 cells express only minute amounts of Fas, and are resistant to Fas-mediated killing (Williams *et al*, 1997), I used Fas-transfected P815 cells (P815-Fas) to rule out the possibility that anti-Thy-1-stimulated T cells may utilize FasL rather than PFN and GzmB to induce cell death in target cells. As shown in Fig. 5.9B, P815-Fas cells were lysed by an agonistic anti-Fas mAb (clone Jo2), and were also more susceptible than their wild-type counterparts to cell death induced by anti-CD3-activated T cells. In contrast, anti-Thy-1-activated T cells did not lyse P815-Fas cells, leading me to conclude that Thy-1-stimulation does not result in the generation of nonspecific cytotoxic effector function that is dependent on either cytolytic pathway. Extending the incubation time to a maximum of 7 days also

failed to generate detectable cytotoxicity by Thy-1-stimulated T cells against P815 cells (Table 5.1). This was not a cell line-specific phenomenon since Thy-1-stimulated T cells also failed to kill other tumor targets such as EL-4 T lymphoma and A20 B lymphoma cells (4% and 3% specific lysis, respectively). In contrast, anti-CD3-activated T cells were able to kill EL-4 and A20 target cells (11% and 67% specific lysis, respectively). The failure of anti-Thy-1-stimulated T cells to destroy P815 cells was in spite of their ability to efficiently conjugate with P815 target cells (Fig. 5.10A), and also to express substantial amounts of mRNA transcripts coding for PFN, GzmB and FasL (Fig. 5.10B, 5.10C). Collectively, my data indicate that T cells that have received a signal 1 substitute through Thy-1 triggering in combination with a strong, DC-generated signal 2 express cytotoxic effector molecules and adhere to target cells, but do not acquire cytolytic function.

5.5 TCR signaling "redirects" Thy-1-stimulated T cells to lyse target cells

I next used a redirected lysis assay with anti-CD3 mAb to determine whether the cytotoxic machinery of Thy-1-stimulated T cells was in fact functional. Fig. 5.11A illustrates that the TCR/CD3 complex was still present on the surface of anti-Thy-1-activated T cells at the end of the culture period. In contrast, anti-CD3-activated T cells exhibited little immunoreactivity with anti-CD3ε-PE, most likely because of TCR/CD3 complex internalization following T cell activation (Schaffar *et al*, 1988; Valitutti *et al*, 1996b). Thy-1-stimulated T cells showed significant cytotoxic capability against FcR-bearing P815 cells when anti-CD3 mAb was present during the effector phase of killing (Fig. 5.11B). A20 B

lymphoma cells that are also FcR^+ were similarly lysed by anti-Thy-1-activated T cells when anti-CD3 mAb was present in the killing assay (38% specific lysis at a 50:1 E:T ratio). In contrast, mitogenic anti-Thy-1 mAb failed to cause the redirected lysis of FcR^+ target cells by Thy-1-stimulated T cells (Fig. 5.11B). Several different mAbs to other T cell surface molecules including CD28 also failed to trigger redirected lysis of target cells by anti-Thy-1-activated T cells. This demonstrates a unique requirement for TCR signaling in the induction of a fully functional cytotoxic phenotype, and suggests that Thy-1 is not able to provide a complete form of signal 1 for T cell activation.

Figure 5.1. DCs selectively enhance Thy-1-driven T cell responses. A) T cells were incubated with or without anti-Thy-1 mAb (6 μg/ml) in the absence or presence of DCs for 72 hr. T cells were pulsed with [^{3}H]TdR 6 h before the culture was harvested. [^{3}H]TdR incorporation was then determined as a measure of T cell proliferation. B) Supernatants from 24-h T cell cultures (or increasing doses of recombinant murine IL-2 as a positive control) were added to IL-2-dependent CTLL-2 cells that were then cultured for 24 h. CTLL-2 cells were pulsed with [^{3}H]TdR 8 hr before the culture was harvested. [^{3}H]TdR incorporation by CTLL-2 cells was then quantified as an indicator of IL-2 bioactivity in the supernatants. C) The indicated numbers of DCs or B cells were incubated with 2 × 10^5 T cells and anti-Thy-1 mAb for 72 h and T cell proliferation was determined by [^{3}H]TdR incorporation. *** denotes a significant difference with $p<0.001$ compared with control cultures receiving no DCs [Differences were extremely significant by ANOVA ($p<0.0001$) and Bonferroni multiple comparisons test]. Data are representative of three (A) or two (B, C) independent experiments yielding similar results.

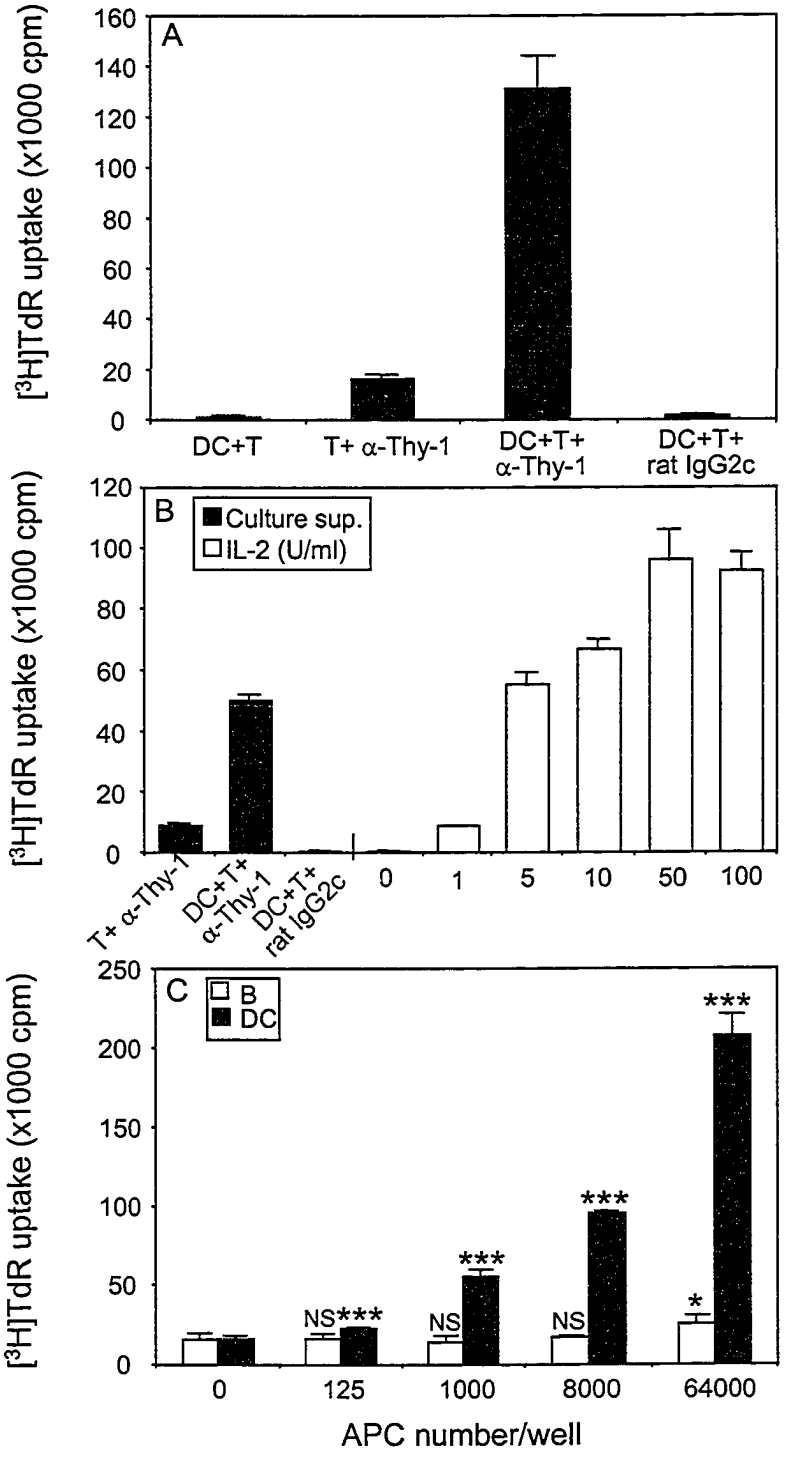

A
[3H]TdR uptake (x1000 cpm)
160
140
120
100
80
60
40
20
0
DC+T
T+ α-Thy-1
DC+T+ α-Thy-1
DC+T+ rat IgG2c
B
Culture sup.
IL-2 (U/ml)
[3H]TdR uptake (x1000 cpm)
120
100
80
60
40
20
0
T+ α-Thy-1
DC+T+ α-Thy-1
DC+T+ rat IgG2c
0
1
5
10
50
100
C
B
DC
[3H]TdR uptake (x1000 cpm)
250
200
150
100
50
0
NS
***
NS
***
NS
***
*
***
0
125
1000
8000
64000
APC number/well

Figure 5.1

Figure 5.2. Bone marrow-derived DCs express FcγRII and/or FcγRIII. Cell surface expression of FcγRs by mouse bone marrow-derived DCs was assessed by flow cytometric analysis after a two-step staining with a rat anti-mouse FcγRIII (CD16)/ FcγRsII (CD32) mAb (clone 2.4G2) and FITC-conjugated mouse anti-rat IgG $F(ab')_2$ (open peak), in comparison with background staining using no primary Ab (filled peak).

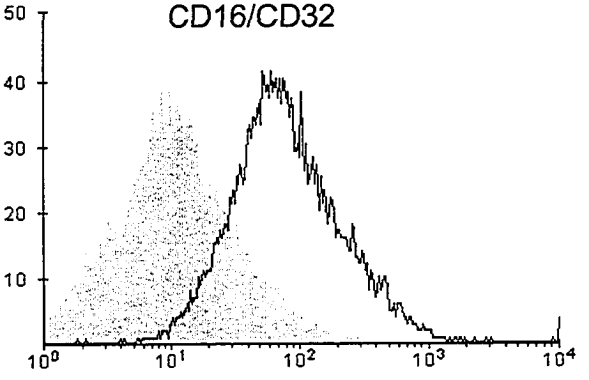


Figure 5.2

Fig. 5.3. Anti-CD3-induced T cell proliferation requires Ab cross-linking that can be provided by DC FcRs or through immobilization on plastic microbeads. A) T cells were combined with bone marrow-derived DCs at a T:DC ratio of 25:1 in the presence of anti-CD3 mAb whole molecule (1:20 dilution of culture supernatant) or purified anti-CD3-Fos chimeric protein (5 μg/ml) used as anti-CD3 $F(ab')_2$. Cultures were pulsed with [^{3}H]TdR during the final 6 h of a 72-h incubation period after which [^{3}H]TdR incorporation was determined as a measure of T cell proliferation. B) T cells were combined at a 1:1 ratio with microbeads pre-coated with the indicated Abs and incubated for 72 h. Cultures were pulsed with [^{3}H]TdR during the final 6 h and [^{3}H]TdR incorporation was determined as a measure of T cell proliferation. Anti-CD3 mAb, anti-CD3-Fos, anti-CD28 mAb and hamster IgG were used at 1, 1, 10 and 10 μg, respectively to coat microbeads prior to use in our T cell activation protocol.

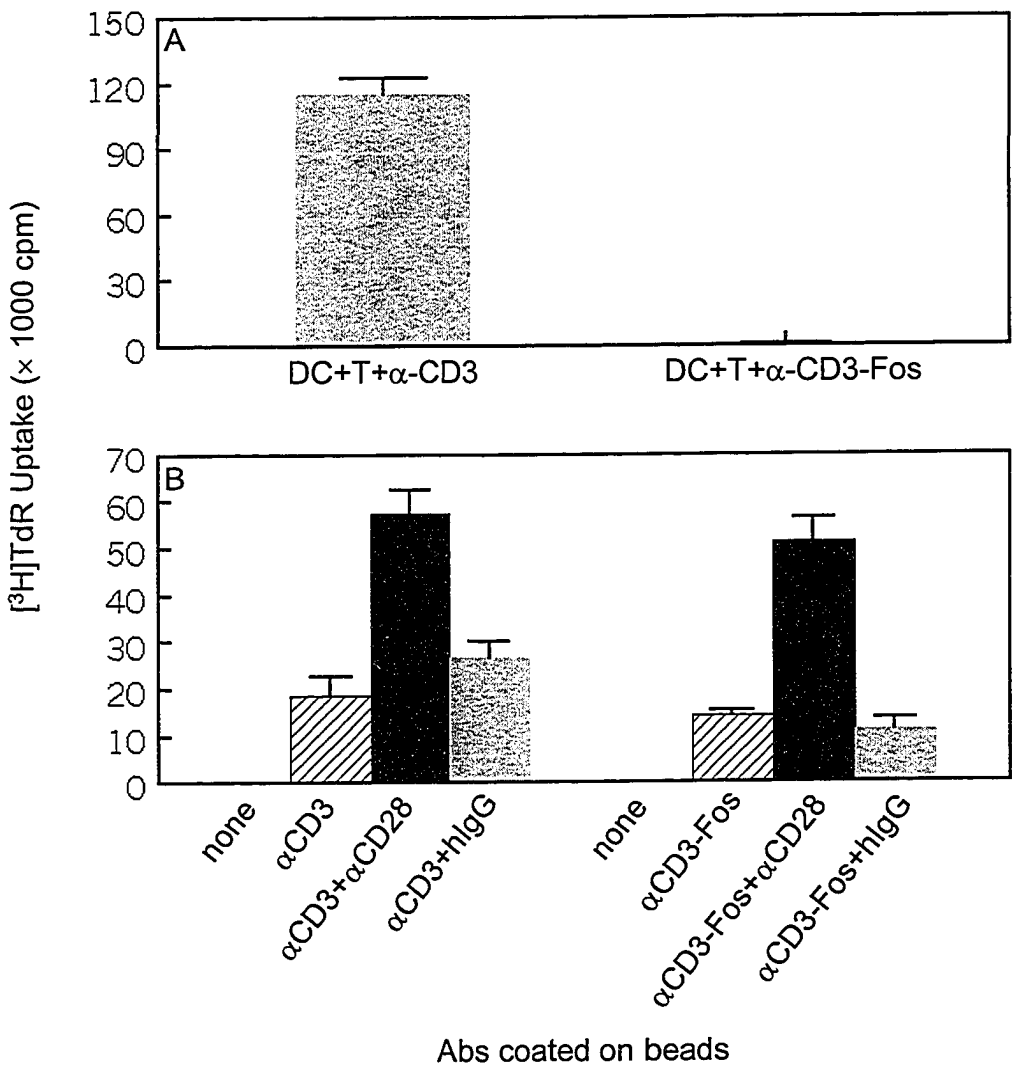

150
120
90
60
30
0
A
DC+T+α-CD3
DC+T+α-CD3-Fos
70
60
50
40
30
20
10
0
B
[³H]TdR Uptake (× 1000 cpm)
none
αCD3
αCD3+αCD28
αCD3+hIgG
none
αCD3-Fos
αCD3-Fos+αCD28
αCD3-Fos+hIgG
Abs coated on beads

Figure 5.3

Fig. 5.4. FcγRs expressed by bone marrow-derived DCs do not contribute to the DC enhancement of Thy-1-driven T cell activation. T cells were stimulated with anti-Thy-1 mAb plus DCs obtained from wild-type (WT) or FcRγ knockout (KO) mice in the absence or presence of 2.4G2 mAb (Fc block). Parallel cultures of T cells were stimulated with anti-CD3 mAb as a positive control. T cell proliferation was then measured by [^{3}H]TdR incorporation.

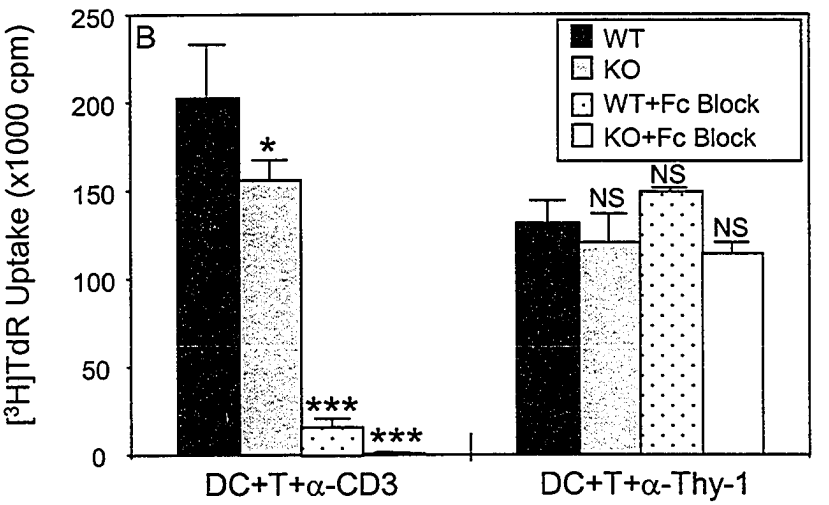

B
[³H]TdR Uptake (x1000 cpm)
250
200
150
100
50
0
WT
KO
WT+Fc Block
KO+Fc Block
*
***
***
NS
NS
NS
DC+T+α-CD3
DC+T+α-Thy-1

Figure 5.4

Fig. 5.5. FcRγ-deficient DCs are capable of driving Ag-specific T cell proliferation. Resting $CD4^+$ T cells expressing I-A^d-restricted TCR transgene DO11.10 were combined with syngeneic DCs obtained from wild-type ($FcR^{+/+}$) or FcRγ knockout ($FcR^{-/-}$) mice and pulsed with a cognate ($OVA_{323-339}$) or control ($OVA_{324-334}$) peptide. T cell proliferation was measured after 72 h by [^{3}H]TdR incorporation. Data are representative of two independent experiments yielding similar results.

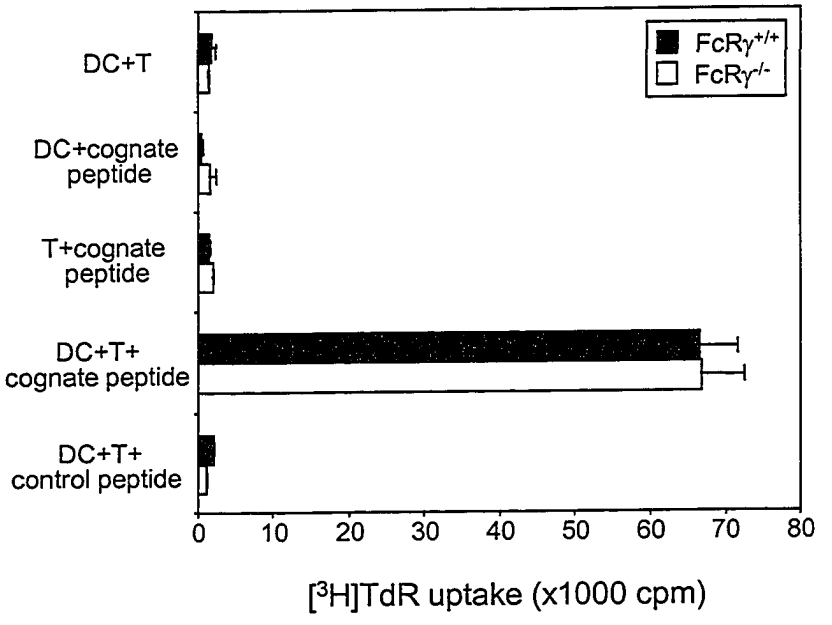

FcRγ+/+
FcRγ-/-
DC+T
DC+cognate peptide
T+cognate peptide
DC+T+ cognate peptide
DC+T+ control peptide
0
10
20
30
40
50
60
70
80
[3H]TdR uptake (x1000 cpm)

Figure 5.5

Fig. 5.6. DC enhancement of Thy-1-driven T cell proliferation is dependent on B7 costimulatory molecules. T cells were stimulated with anti-Thy-1 mAb plus syngeneic DCs in the absence or presence of anti-B7-1 and/or anti-B7-2 mAb (1:10 hybridoma supernatants) or irrelevant IgGs. Parallel cultures of T cells stimulated with anti-CD3 mAb served as a positive control. T cell proliferation was then determined by [^{3}H]TdR incorporation.

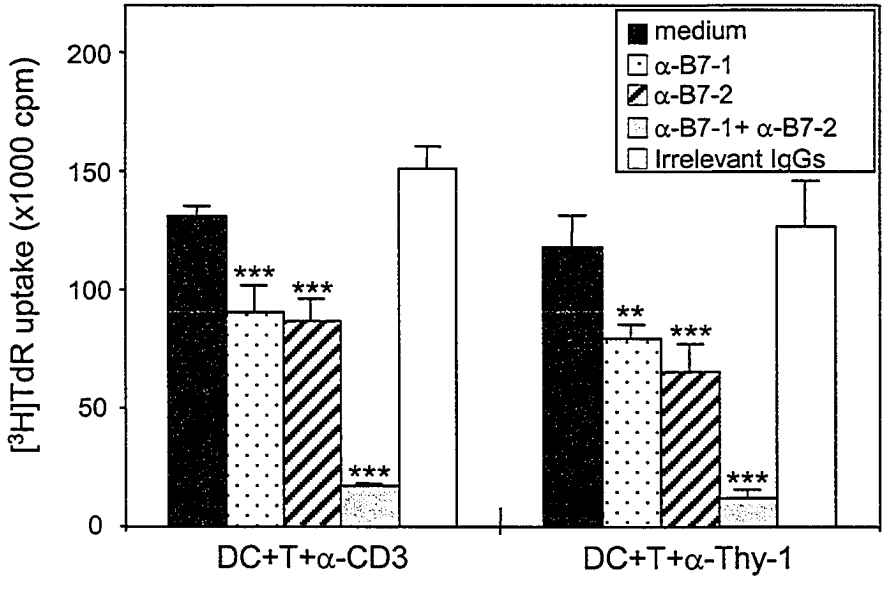

medium
α-B7-1
α-B7-2
α-B7-1+ α-B7-2
Irrelevant IgGs
[3H]TdR uptake (x1000 cpm)
200
150
100
50
0
***
***
***
**
***
***
DC+T+α-CD3
DC+T+α-Thy-1

Figure 5.6

Fig. 5.7. Anti-Thy-1-Induced T cell proliferation is enhanced by LPS blasts. B cells were isolated and incubated with LPS (5 μg/ml) for 72 h. LPS-activated B lymphoblasts thus generated were harvested, thoroughly washed and used as accessory cells in our T cell activation protocol. 2×10^5 highly purified T cells were combined with the indicated numbers of LPS blasts together with anti-Thy-1 mAb or rat IgG_{2c} control (6 μg/ml) in wells of a U-bottom microtiter plate. [^{3}H]TdR incorporation was then determined at 48 h as a measure of T cell proliferation. ** and *** denote significant differences with $p<0.01$ and $p<0.001$, respectively, in comparison with control cultures containing no LPS blasts. Differences were extremely significant by ANOVA ($p<0.0001$) and Bonferroni multiple comparisons test.

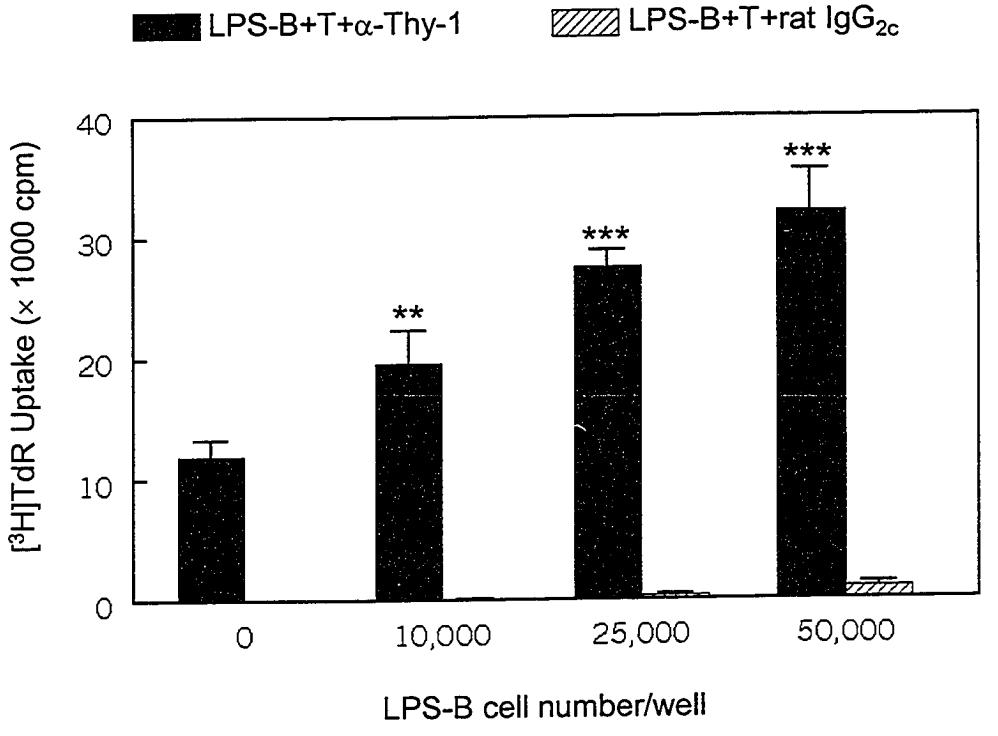

LPS-B+T+α-Thy-1
LPS-B+T+rat IgG2c
[3H]TdR Uptake (× 1000 cpm)
40
30
20
10
0
**
***
***
0
10,000
25,000
50,000
LPS-B cell number/well

Figure 5.7

Fig. 5.8. Microbeads coated with a combination of anti-Thy-1 and anti-CD28 mAbs act as surrogate APCs to activate T cells. A) T cells were mixed with anti-Thy-1 and/or anti-CD28 mAb-coated microbeads and T cell proliferation was measured 48 h post-stimulation by [^{3}H]TdR incorporation. B) T cells were stimulated with microbeads coated with a constant concentration (10 μg) of anti-CD28 and the indicated doses of anti-Thy-1 mAb. T cell proliferation was determined by [^{3}H]TdR incorporation after 48 h of culture.

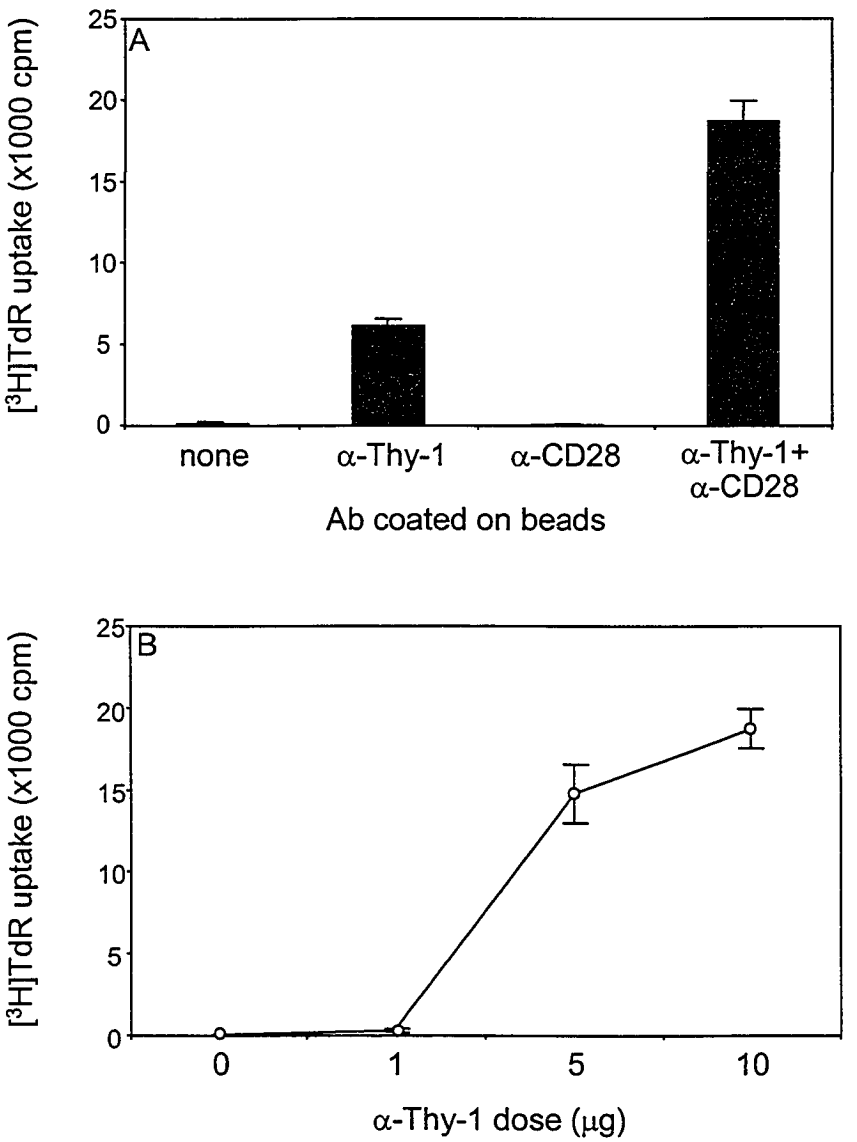

A
[3H]TdR uptake (x1000 cpm)
25
20
15
10
5
0
none
α-Thy-1
α-CD28
α-Thy-1+
α-CD28
Ab coated on beads
B
[3H]TdR uptake (x1000 cpm)
25
20
15
10
5
0
0
1
5
10
α-Thy-1 dose (μg)

Figure 5.8

Fig. 5.9. Thy-1 stimulation in the presence of DCs does not generate nonspecific CTL. A) T cells activated with anti-Thy-1 or anti-CD3 mAb (as a positive control) in the presence of DCs were combined at the indicated effector:target ratios with ^{51}Cr-labeled P815 mastocytoma cells. After 4 h, a 100-µl aliquot of the culture supernatants was harvested from each well and specific ^{51}Cr-release from the target cells was calculated. Standard deviations in triplicate wells were always less than 10 % of the means, and error bars have been omitted for the purpose of clarity. B) T cells activated with anti-Thy-1- or anti-CD3 mAb (as a positive control) in the presence of DCs were combined with P815 or P815-Fas cells at a 50:1 effector:target ratio and specific ^{51}Cr release from the target cells was used to determine % specific lysis. An agonistic anti-Fas mAb was used as an additional positive control to induce cell death in Fas-positive P815 cells.

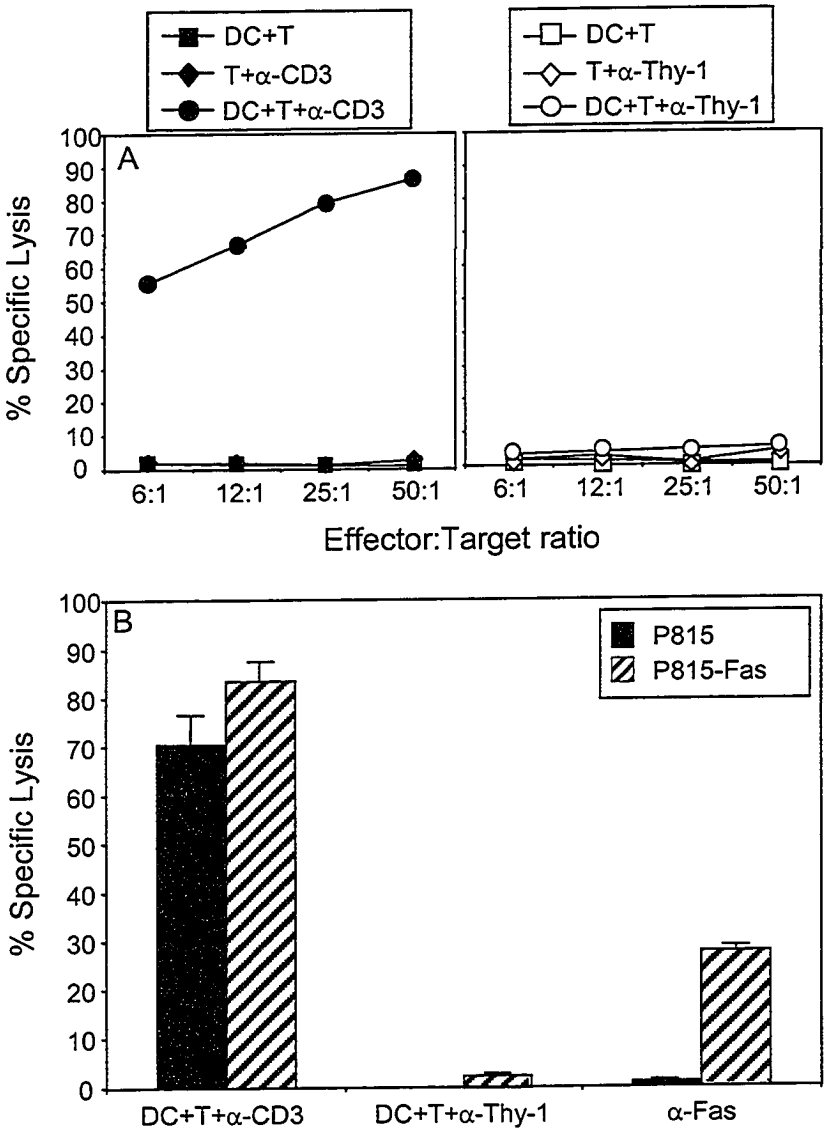

DC+T
T+α-CD3
DC+T+α-CD3
DC+T
T+α-Thy-1
DC+T+α-Thy-1
A
% Specific Lysis
100
90
80
70
60
50
40
30
20
10
0
6:1
12:1
25:1
50:1
6:1
12:1
25:1
50:1
Effector:Target ratio
B
P815
P815-Fas
% Specific Lysis
DC+T+α-CD3
DC+T+α-Thy-1
α-Fas

Figure 5.9

Table 5.1. Time course of cytolytic activity of anti-CD3- and anti-Thy-1-activated T cells

	Anti-CD3-activated T Cells		***Anti-Thy-1-activated T Cells***	
Day	**Specific killing (%)**	**Viability (%)**	***Specific killing (%)***	***Viability (%)***
1	36.4 ± 2.3	ND	*0.2 ± 0.3*	*ND*
3	51.2 ± 3.6	ND	*0.8 ± 0.7*	*ND*
5	56.4 ± 2.7	70	*0*	*60*
7	76.2 ± 2.3	35	*0.1 ± 0.2*	*25*

T cells were mixed with DCs at a 25:1 T:DC ratio and incubated for 1, 3, 5 or 7 days in the presence of anti-CD3 or anti-Thy-1 mAb. Stimulated T cells were harvested on the indicated days, extensively washed and examined for viability using trypan blue dye exclusion. For the assessment of MHC-unrestricted cytotoxicity, stimulated T cells were combined with ^{51}Cr-labeled P815 target cells at a 50:1 effector:target ratio and specific lysis of P815 cells was calculated after 4 h. ND: not determined.

Fig. 5.10. T cells stimulated with anti-Thy-1 mAb in the presence of DCs adhere to target cells, and express cytotoxic effector molecules. A) T cells activated with anti-Thy-1- or anti-CD3 mAb in the presence of DCs were incubated in a 1:1 ratio with neutral red dye-stained P815 cells. Conjugates were then enumerated under a light microscope. The percentage of T cells conjugated with P815 cells (± SD) from triplicate tubes was calculated. B and C) Total RNA was isolated from T cells activated with anti-Thy-1- or anti-CD3 mAb in the presence of DCs after 72 h of culture for detection of PFN and GzmB mRNA transcripts, or after 4 h for detection of FasL mRNA transcripts. Cytotoxic effector molecule mRNA levels were determined by RT-PCR in comparison with the steady-state expression of GAPDH. Data are representative of two independent experiments yielding similar results.

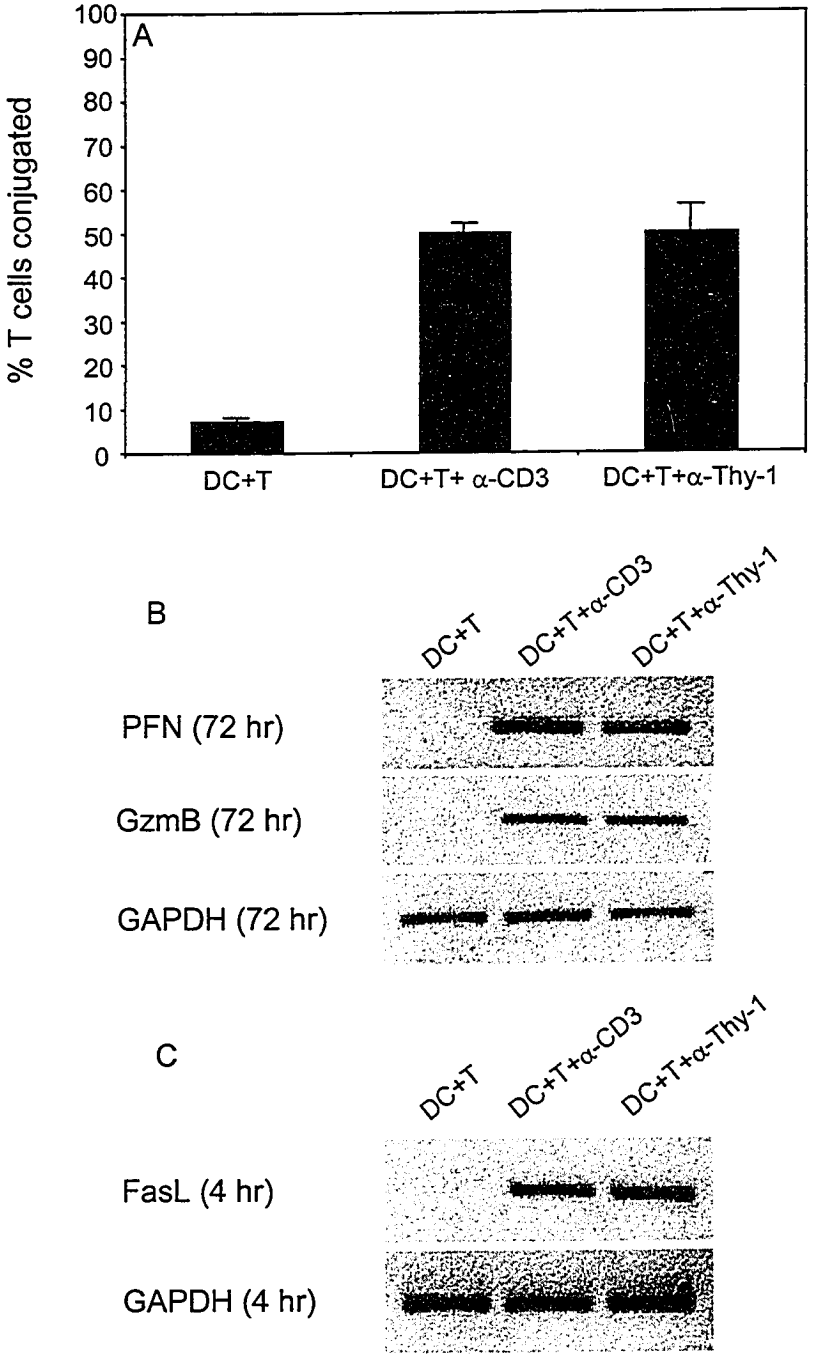

A
% T cells conjugated
100
90
80
70
60
50
40
30
20
10
0
DC+T
DC+T+ α-CD3
DC+T+α-Thy-1
B
DC+T
DC+T+α-CD3
DC+T+α-Thy-1
PFN (72 hr)
GzmB (72 hr)
GAPDH (72 hr)
C
DC+T
DC+T+α-CD3
DC+T+α-Thy-1
FasL (4 hr)
GAPDH (4 hr)

Figure 5.10

Fig. 5.11. Thy-1-stimulated T cells are capable of cytotoxic effector function following TCR but not Thy-1 triggering in a re-directed lysis assay. A) Cell surface expression of the TCR/CD3 complex was determined by flow cytometric analysis at 72 h post-stimulation with anti-CD3 mAb or anti-Thy-1 mAb in the presence of DCs. PE-labeled anti-CD3ε mAb was used to stain the TCR/CD3 complex (open peaks) in comparison to background staining (filled peaks) with PE-labeled hamster IgG. B) T cells were stimulated with anti-Thy-1 or anti-CD3 (as a positive control) mAbs in the presence of DCs. Stimulated T cells were harvested at 72 h, washed and combined with FcR^+ P815 target cells at a 50:1 ratio in the presence or absence of anti-CD3 mAb, anti-Thy-1 mAb or isotype controls. ^{51}Cr release from target cells was determined after 4 h to determine % specific lysis.

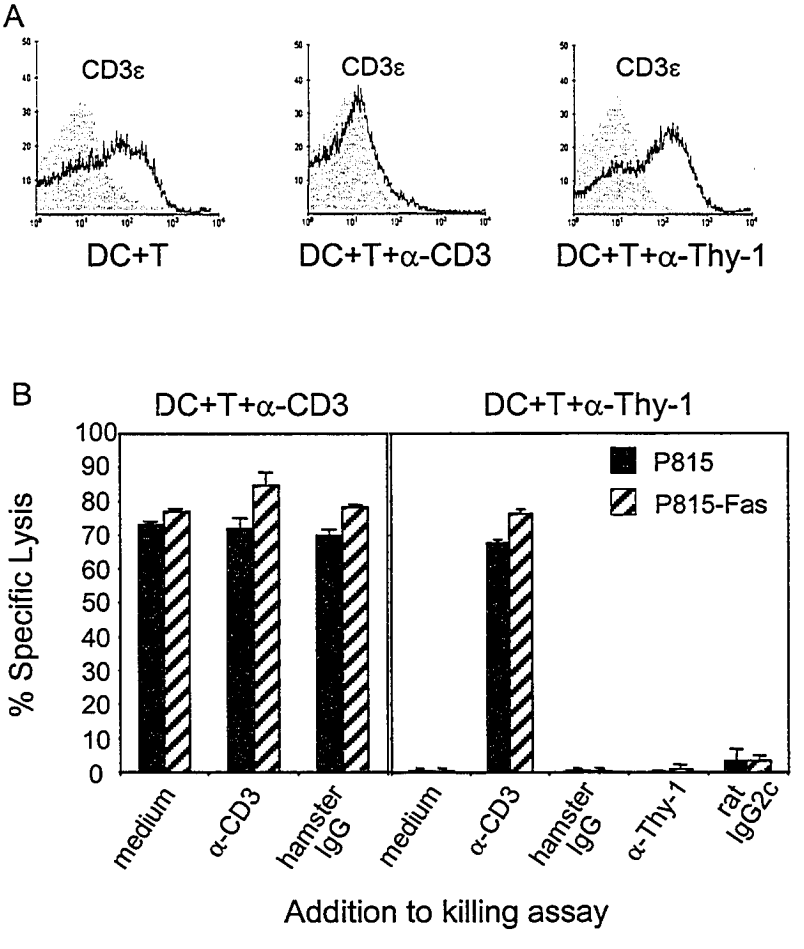

A
CD3ε
CD3ε
CD3ε
DC+T
DC+T+α-CD3
DC+T+α-Thy-1
B
DC+T+α-CD3
DC+T+α-Thy-1
P815
P815-Fas
% Specific Lysis
100
90
80
70
60
50
40
30
20
10
0
medium
α-CD3
hamster IgG
medium
α-CD3
hamster IgG
α-Thy-1
rat IgG2c
Addition to killing assay

Figure 5.11

6. Discussion

6.1 Thy-1 plays a role in non-specific CTL induction in response to anti-CD3 mAb

Although a role for Thy-1 has been suggested in enhancing cytotoxic capabilities of CTL clones (Lancki *et al*, 1995b; Kojima *et al*, 2000), whether signaling through Thy-1 is involved in the generation of CTLs from naïve CTLps is not clearly understood. I therefore examined a possible role for Thy-1 during AK-T cell generation in response to anti-CD3 mAb as a model for CTL induction.

I observed that the presence of a non-stimulatory anti-Thy-1 mAb (clone 30-H12) in AK-T cell cultures, even at low concentrations, abolished the induction of cytotoxicity against P815 tumor cells. This was not due to the failure of T cells activated in the presence of anti-Thy-1 mAb to adhere to P815 target cells, as these T cells exhibited unexpectedly a greater degree of binding to P815 cells. I have ruled out the remote possibility that Ab-mediated blockade of Thy-1 somehow up-regulates the expression of LFA-1, which would tend to facilitate LFA-1-ICAM-1 interactions needed for AK-T cell conjugation to P815 cells (Kaiser and Hoskin, 1992). In fact, the expression of LFA-1 on anti-CD3-stimulated T cells was moderately decreased by the presence of anti-Thy-1 mAb in culture. The most likely explanation for greater binding of these T cells to P815 cells is that the T cell-bound anti-Thy-1 mAb binds through its Fc portion to P815 cells that are known to express FcRs (van de Griend *et al*, 1987), thereby acting as a bridge to physically bring together the T cells and the P815 target cells.

I next looked at the expression of cytotoxic effector molecules by AK-T cells generated in the presence of anti-Thy-1 mAb. Cytotoxic lymphocytes employ two major pathways to induce cell death in target cells, namely granule exocytosis (using PFN and GZMs) and the death receptor pathway executed mainly through Fas (reviewed by Berke, 1995). A minor pathway has also been described, which involves membrane-bound or secreted forms of TNF-α, and is responsible for relatively slower lysis of target cells (Ratner and Clark, 1993). The cytotoxic machinery of AK-T cells includes FasL, PFN and GzmB (reviewed by Hoskin *et al*, 1998). Stimulation of mouse T cells by anti-CD3 mAb has also been shown to result in secretion of biologically active TNF-α (Stankova *et al*, 1989), although the possible contribution of AK-T cell-derived TNF-α to the lysis of susceptible target cells is not clear. Cytotoxicity in our system is primarily attributable to the PFN/GzmB pathway since P815 target cells are relatively insensitive to Fas- (Williams *et al*, 1997) or TNF-α-mediated killing (Ju *et al*, 1990).

The presence of anti-Thy-1 mAb during AK-T cell induction led to diminished or ablated expression of PFN and GzmB mRNA transcripts, while FasL mRNA levels remained unchanged. The importance of these findings is several-fold. First, I have shown for the first time a link between Thy-1 signaling and the expression of PFN and GzmB. My data are not in keeping with the results of Kojima *et al* (2000) who reported a preferential activation of FasL-*versus* PFN/GZM-mediated killing as a result of Thy-1 triggering. However, Kojima *et al* used a stimulatory anti-Thy-1 mAb (clone G7) in a redirected lysis

assay using CTL clones, while I have used a non-stimulatory anti-Thy-1 mAb (clone 30-H12) to address the role of Thy-1 during the induction phase of CTL responses. T cell clones are likely to have distinct activation requirements in comparison with naïve CTLps, and may not accurately reflect the changes that occur during CTL induction. Secondly, the G7 mAb targets a non-polymorphic epitope of Thy-1 (Gunter *et al*, 1984), whereas the 30-H12 mAb used in this study recognizes a specific epitope found only on Thy-1.2 alloantigen (Ledbetter *et al*, 1980). Differences in epitope specificity between these two mAbs may therefore be at least partially responsible for the observed differences. My finding that anti-CD3-induced up-regulation of FasL was not affected by the presence of the 30-H12 mAb also indicates that anti-Thy-1 mAb does not cause a global inhibition of gene transcription. Furthermore, since FasL up-regulation results from signaling through TCR (Anel *et al*, 1994; Vignaux *et al*, 1995), certain aspects of TCR signaling seem not to be affected by the presence of anti-Thy-1 mAb. This is consistent with reports based on my own work and others that fundamental differences exist between TCR- and Thy-1-associated signal transduction pathways (Lancki *et al*, 1995b; Haeryfar and Hoskin, 2001). These differences will be discussed in greater detail later.

In contrast with FasL that is induced within few hours of TCR ligation, the up-regulation of PFN and GzmB expression is a later event that is in part secondary to T cell exposure to cytotoxicity-inducing cytokines such as IL-2 (Liu *et al*, 1989; Makrigiannis and Hoskin, 1997). My data demonstrate diminished expression of both IL-2 and IL-2Rα as a result of Thy-1 blockade. Therefore,

attenuated signaling through IL-2Rα may explain why the up-regulation of PFN and GzmB was prevented by the blockade of Thy-1. Interestingly, exogenous IL-2 added at the beginning of the culture only partially restored AK-T cell-mediated cytotoxicity, in spite of bringing GzmB mRNA levels back to the control levels, indicating that GzmB expression by itself is not sufficient to induce lethal function. This is supported by the observation that T cells activated with IL-2 alone showed no lytic capability in our system even though they exhibited higher expression of GzmB in comparison with AK-T cells. I have not ruled out the possibility that exogenous IL-2 may fail to restore PFN expression by AK-T cells generated in the presence of anti-Thy-1 mAb, which may be the reason for the failure of exogenous IL-2 to completely rescue AK-T cell-mediated killing under these conditions. This possibility seems likely in light of previous studies indicating that PFN-deficient CTLs, in which GZM expression is intact, fail to kill certain (*i.e.*, Fas-negative) target cells (Clark *et al*, 1995; Kagi *et al*, 1994).

My data indicate that the addition of exogenous IL-2 in combination with IFN-γ and IL-12, which are also important for the development of CTL responses (Chouaib *et al*, 1994; Fitzpatrick *et al*, 1996), also failed to compensate for Thy-1 blockade during AK-T cell induction. Collectively, these data point to a more fundamental, somewhat irreversible impairment(s) of AK-T cell cytotoxicity caused by the blockade of Thy-1 during CTL induction.

Whether T cells do not receive a survival signal or are rendered anergic as a result of the blockade of Thy-1 is not clear at this point. The majority of T cells appeared viable at the end of cultures containing anti-Thy-1 mAb. However,

this was judged only by trypan blue exclusion. Whether or not more subtle morphological changes characteristic of apoptosis might be caused by the blockade of Thy-1, that could be detected by more sensitive methods remains to be tested. Marmor *at al* (1999) reported an inhibition of T cell growth, as well as profound changes in T cell size, shape and adherence by the 30-H12 anti-Thy-1 mAb when plate-coated anti-TCR mAb was used to activate primary $CD8^+$ lymph node T cells. However, in order for such morphological changes to occur, the anti-Thy-1 mAb needed to be co-immobilized with anti-TCR mAb on plates, as anti-Thy-1 mAb added in soluble form did not have the same effect. In contrast, in my hands, the same anti-Thy-1 mAb (30-H12) exerted its inhibitory effects in the absence of noticeable morphological changes when used in soluble form. These discrepancies most probably stem from the way in which anti-Thy-1 mAb was presented to T cells (*i.e.*, soluble *vs.* immobilized) along with anti-TCR/CD3 Abs. Also contrary to the findings of Marmor *et al* (1999), primary T cell proliferation in response to a combination of anti-CD3 and anti-CD28 mAbs coated on cell-sized microbeads was enhanced by anti-Thy-1 mAb 30-H12 co-immobilized on beads.

Marmor and Julius (2001) reported that immobilized Abs to GPI-anchored proteins including Thy-1, which are highly enriched within lipid rafts, perturb the IL-2-mediated hetero-trimerization of the high affinity IL-2R through sequestration of IL-2Rα within lipid rafts. Although this may account for the inhibitory effect of anti-Thy-1 mAb on IL-2-induced growth of IL-2-dependent T cell clones or "pre-activated" T cells that already express IL-2Rα, additional mechanisms may

underlie such inhibitory effects on primary T cell growth in response to TCR/CD3 ligation. For instance, these investigators did not elaborate on whether or not anti-Thy-1 mAb prevents the up-regulation of IL-2Rα as a result of TCR ligation, which I have shown in our system. Moreover, while I demonstrated diminished IL-2 biosynthesis in the presence of soluble anti-Thy-1 mAb, Marmor *et al* (1999) did not find lower IL-2 production when immobilized anti-Thy-1 mAb was used together with anti-TCR mAb. These differences again highlight the importance of the mode by which anti-Thy-1 mAb is presented to T cells.

The 30-H12 mAb is routinely used as a "non-stimulatory" Ab to Thy-1. The inhibitory effects of this Ab in our system appear not to be due to a Thy-1-associated negative signal affecting T cell activation. This is suggested by my observation that excessively high doses of this anti-Thy-1 Ab remain capable of inhibiting T cell proliferation in response to TCR/CD3 ligation. Also in support of this notion is the finding that the same anti-Thy-1 mAb failed to inhibit T cell proliferation induced by a combination of anti-CD3 and anti-CD28 mAbs coated on microbeads. The observation that T cell proliferation in response to PMA plus ionomycin was not affected by the presence of anti-Thy-1 mAb also seems to suggest that the anti-Thy-1 mAb does not deliver a negative signal to T cells, at least involving distal events of T cell activation cascade. Among several reagents I have tested in our system, PMA was the only one to compensate for Thy-1 blockade and to rescue T cell proliferation in response to anti-CD3 mAb. This suggests that the anti-Thy-1-mediated inhibitory effect occurs upstream of PKC activation.

I demonstrated that neither anti-CD3-induced nor OVA-specific T cell responses are affected by the 30-H12 anti-Thy-1 mAb when DCs are used as APCs. Unlike B cells, DCs constitutively express high levels of B7 costimulatory molecules (Banchereau *et al*, 2000). In addition, the 30-H12 mAb failed to inhibit T cell proliferation in response to anti-CD3 mAb coated on microbeads when anti-CD28 Ab was also present on the same beads as an artificial source of signal 2. Since resting B cells do not express substantial levels of costimulatory molecules, and depend on T-B interactions to do so (Ranheim and Kipps, 1993), one may wonder whether the 30-H12 anti-Thy-1 mAb acts to inhibit the up-regulation of costimulatory molecules on B cells in our T cell activation model. This hypothesis seems justifiable in light of a previous study by Wu *et al* (1995) that reported an inhibition of B7-2 up-regulation on spleen-derived APCs by another anti-Thy-1 mAb (21F10) in the context of anti-CD3-induced T cell activation. However, this hypothesis can, at the same time, be challenged by the observation that the 21F10 anti-Thy-1 mAb could also inhibit anti-CD3-induced $CD4^+$ T cell proliferation when either LPS-activated B cells or B7-2-transfected Chinese hamster ovary cells were used as accessory cells in the same study. When I used LPS-activated B lymphoblasts with high levels of B7 expression as accessory cells in our system, the 30-H12 anti-Thy-1 mAb strongly inhibited anti-CD3-induced T cell proliferation, suggesting that anti-Thy-1 mAb 30-H12 does not prevent CD3-driven T cell proliferation by interfering with the induction of costimulatory activity in B cells.

Based on the data presented in chapter 3, I propose that the 30-H12 anti-Thy-1 mAb may block the interactions between Thy-1 and its putative ligand on B cells, which seem to be important for the development of T cell proliferative and cytotoxic responses. I predict that Fab fragments of the 30-H12 anti-Thy-1 mAb will be as efficient in preventing these responses. The fact that anti-CD3-induced T cell proliferation in the presence of resting or activated B cells, but not DCs, is inhibited by anti-Thy-1 mAb suggests that B cells, but not DCs, express the putative ligand for Thy-1. I will later discuss how the hypothetical absence of a Thy-1 ligand from DCs may prevent massive T cell proliferation, which would otherwise ensue *in vivo* with potentially hazardous consequences.

In summary, my data demonstrate that a non-stimulatory mAb to Thy-1 (clone 30-H12) prevents the induction of AK-T cell tumoricidal activity by interfering with the arming of AK-T cell precursors with PFN and GzmB. This was in turn due in part to an attenuated cytokine response. The inhibitory effect of the anti-Thy-1 mAb was seemingly neither a consequence of a negative signal delivered as a result of Thy-1 cross-linking to T cells nor through interference with the induction of costimulatory activity on accessory B cells. Rather, I suggest that this inhibitory effect is due to a possible blockade of Thy-1 interactions with its putative ligand on B cells.

I have provided the first evidence that Thy-1 participates in the induction phase of CTL responses giving rise to killer T cells from resting precursors. AK-T cells display MHC-unrestricted lytic activity towards a broad range of target cells

and have tremendous potential for immunotherapy of cancer. Whether or how Thy-1 signaling affects CTL responses *in vivo* remains to be explored.

6.2 Thy-1- and TCR-associated signal transduction pathways in murine T cells are not identical

I employed several different pharmacological inhibitors of signaling intermediates to compare and contrast downstream requirements for TCR- and Thy-1-associated signaling cascades in murine T lymphocytes. Dose response studies with both anti-CD3 and anti-Thy-1 mAbs were performed to determine the optimal concentrations of these stimulatory mAbs for use in my experiments. In my hands, maximal T cell proliferative responses were obtained with a 6-μg/ml dose of anti-Thy-1 mAb and a 1:20 dilution of anti-CD3 hybridoma supernatant. Increasing the concentration of either mAb beyond the optimal dose led to less vigorous T cell proliferation likely due to a prozone effect. Moreover, cross-linking anti-CD3 or anti-Thy-1 mAb with an appropriate secondary antibody failed to enhance T cell proliferation (data not shown), indicating the presence of sufficient accessory cells in my T cell-enriched preparations for effective mAb-crosslinking. Taken together, these observations indicate that my data reflects the pharmacological sensitivities of fully activated T cell populations.

T cell proliferation initiated by either anti-CD3 or anti-Thy-1 mAb was similarly inhibited by the PTK inhibitor herbimycin A, indicating a requirement for PTK in both pathways. Although neither TCR nor Thy-1 exhibits intrinsic PTK activity, both seem to activate the cytoplasmic *Src* family PTK *Lck* and *Fyn* (Ilangumaran *et al*, 2000; Lancki *et al*, 1995b). The expression of *Fyn*, however,

appears to be more selectively required for Thy-1 signaling since Thy-1-mediated functional activation, unlike stimulation via TCR, is reportedly impaired in *Fyn* $^{-/-}$ T cell clones (Lancki *et al*, 1995b). *Lck* and *Fyn* are found in specific immunoprecipitates of GPI-anchored proteins such as Thy-1 (Stefanova *et al*, 1991; Thomas and Samelson, 1992). Interestingly, *Lck* and *Fyn*, which co-precipitate with activated TCR complexes show less kinase activity in the GPI-deficient mutants than in wild-type cells (Romagnoli and Bron, 1997). This observation is consistent with a role for GPI-anchored molecules such as Thy-1 in the enhancement of TCR signal transduction, but does not rule out Thy-1 signaling being independent of the TCR.

During T cell activation via the TCR, coupling and/or activation of PI-3K precede the activation of PKC, MAPKs, and $p70^{S6k}$ (Ward *et al*, 1996). My data indicate that Thy-1 signaling pathway of T lymphocytes, like the TCR signaling pathway, is coupled to PI-3K. Interestingly, PI-3K activation is also reported to occur following Ab-mediated cross-linking of CD7 (Ward *et al*, 1995), a human T cell surface molecule that shares several characteristics with mouse Thy-1, and that has been proposed to be a functional homologue of mouse Thy-1 (Schanberg *et al*, 1991).

Anti-Thy-1 mAbs reportedly induce a rise in cytoplasmic Ca^{++} in peripheral T lymphocytes and T cell hybridomas (Kroczek *et al*, 1986a; Kroczek *et al*, 1986b), suggesting second messenger production in response to Thy-1 signaling. Both anti-CD3- and anti-Thy-1-induced T cell proliferative responses were inhibited in the presence of the PKC inhibitor calphostin C, indicating a

requirement for PKC participation in both pathways, although the latter pathway is apparently more sensitive to PKC inhibition. Furthermore, the PKC activator PMA synergistically augmented anti-Thy-1-mediated T cell proliferation and IL-2 production, suggesting that the reason Thy-1 signaling is more sensitive than TCR signaling to PKC inhibition is that Thy-1 triggering, by itself, does not strongly induce PKC activation in T lymphocytes. During normal Ag-specific T cell activation when Thy-1 may act as an accessory molecule for T cell stimulation, optimal PKC activation is most likely provided through participation of the TCR/CD3 complex and additional T cell surface molecules. Pont (1987) has suggested that DAG, which is responsible for anchoring of Thy-1 to the plasma membrane, might be involved in Thy-1 intracellular signaling via PKC stimulation. On the other hand, Thy-1 expression by T cells may also be regulated by PKC-dependent pathways since Thy-1 expression on human dermal microvascular endothelial cells is up-regulated by phorbol-12,13-dibutyrate, and this up-regulation can be prevented by the PKC inhibitor RO31-8220 (Mason *et al*, 1996). Inhibition of PKC activity in T cells may, therefore, further diminish the anti-Thy-1-induced proliferative response by negatively affecting Thy-1 expression.

The Ca^{++}/calmodulin-regulated protein phosphatase calcineurin is responsible for dephosphorylating the cytoplasmic component of the transcription factor NFAT, allowing for translocation of NFAT to the nucleus where it induces transcription of cytokine genes (*e.g.* IL-2) involved in T cell activation (Weiss and Littman, 1994). A role for calcineurin could, therefore, be assumed in Thy-1

signaling since T cells stimulated with anti-Thy-1 mAb synthesize IL-2 (Gunter *et al*, 1984). My finding that the calcineurin inhibitor cyclosporine A inhibits Thy-1-driven T cell proliferation confirms the participation of calcineurin in Thy-1-associated signal transduction.

To examine the roles of MAPKs in Thy-1 signaling as compared to the TCR-associated pathway, I employed selective pharmacological inhibitors of MAPK family members. MEK1 inhibition has recently been shown to suppress human T lymphocyte proliferation induced by TCR triggering, an effect which is reversed by the addition of cytokine-containing conditioned media (Dumont *et al*, 1998). I also observed inhibition of anti-CD3-induced mouse T cell proliferation by the MEK1 inhibitor PD98059. Interestingly, anti-Thy-1-induced T cell proliferation was even more sensitive to MEK1 inhibition with PD98059, suggesting that the activation status of MEK1 may differ between Thy-1 and TCR-associated signaling pathways. To determine the role of p38 MAPK in Thy-1- and TCR-triggered T cell activation, I activated T cells by corresponding mAbs in the presence of SB203580, a highly selective inhibitor of p38. My finding that anti-CD3-induced T cell proliferation was partially suppressed in the presence of SB203580 is in good agreement with the results of Zhang *et al* (Zhang *et al*, 1999). Surprisingly, T cell proliferation in response to anti-Thy-1 mAb was enhanced, rather than inhibited, in the presence of SB203580. The known isoforms of p38 MAPK are p38α, β, γ and δ (English *et al*, 1999). The α and β isoforms of p38 are inactivated by SB203580, while p38α and p38δ comprise the major p38 isoforms in lymphoid tissues (Hsu *et al*, 1999). Furthermore, p38α

activation by anti-CD3 mAb has previously been reported in the EL4 thymoma cell line and in T cell hybridomas (Hsu *et al*, 1999). Therefore, it is most likely that p38α is the isoform involved in the differential regulation of Thy-1- and TCR-induced T cell activation. Augmentation of anti-Thy-1-induced T cell proliferation by p38 inhibition is a novel observation that suggests a negative regulatory role for p38 in Thy-1 signaling. Such a role for p38 has also been suggested in the context of angiotensin II signal transduction in vascular smooth muscle cells (Kusuhara *et al*, 1998). The differential regulation of TCR- and Thy-1-associated T cell signaling by p38 is not consistent with the hypothesis that Thy-1 signaling takes place solely in association with the TCR/CD3 complex. Rather, at least some aspects of the Thy-1 signal transduction pathway are fundamentally different from TCR signal transduction.

6.3 Thy-1 triggering provides a signal 1 substitute for select features of T cell activation

Although T cell activation occurs normally in response to TCR triggering, signaling through Thy-1 has been suggested also to result in T cell activation. Thy-1 cross-linking with certain mAbs leads to T cell proliferation and IL-2 production (Gunter *et al*, 1984; Kroczek *et al*, 1986a) that are considered to be hallmarks of T cell activation. However, the exact function of Thy-1 in the context of T cell activation has remained puzzling and highly controversial. The results presented in chapter 5 show for the first time that Thy-1 triggering can substitute for signal 1 for T cell activation, which, in combination with a strong signal 2 (delivered by B7 family members on DCs, or by anti-CD28 mAb coated on

microbeads), leads to robust T cell proliferation and IL-2 synthesis. Thy-1-driven signal 1 is, however, insufficient to trigger cytotoxic effector function, pointing to a fundamental difference between conventional, TCR-mediated signal 1 and the form of signal 1 that is generated by Thy-1 triggering.

I initially discovered that DCs dramatically enhance T cell proliferation and IL-2 synthesis in response to Thy-1 triggering. To delineate the mechanism(s) accounting for this phenomenon, I first examined the possibility that DCs enhance Thy-1-driven responses by cross-linking the anti-Thy-1 mAb via their FcγRs. DC expression of FcγRs is known to depend on the DC subtype and state of maturation, the method of cell preparation, and the species from which the DCs are obtained (Amigorena, 2002; Elbe-Burger *et al*, 2000; Fanger *et al*, 1997). Murine DC lines and primary DCs of the Langerhans cell type reportedly potentiate anti-TCR/CD3-mediated T cell proliferation in an FcR-dependent manner (Elbe-Burger *et al*, 2000; Romani *et al*, 1989). My results indicate that murine bone marrow-derived DCs also express functional FcγRs, and confirm that DC FcγRs are required for anti-CD3-driven T cell proliferation.

DCs from FcRγ$^{-/-}$ mice were somewhat less efficient in potentiating anti-CD3-induced T cell proliferation. This is probably not caused by an unrelated defect in other components of DC stimulatory/costimulatory machinery in these mice as FcRγ-deficient DCs were equally capable of presenting an antigenic peptide to TCR-transgenic CD4^{+} T cells, leading to T cell activation. This is consistent with a previous report indicating the normal ability of FcRγ$^{-/-}$ mice in

inducing an Ag-specific T cell response in a similar system in which irradiated splenocytes were used as APCs (Hamano *et al*, 2000).

FcR$\gamma^{-/-}$ mice are devoid of FcγRI and FcγRIII, which are regarded as "activation" FcγRs characterized by the presence of an ITAM motif in the cytoplasmic tail of their associated γ chain. As a result, these mice provide a valuable experimental tool to study effector responses associated with activation FcγRs, such as macrophage phagocytosis, Ab-dependent cell-mediated cytotoxicity, neutrophil activation and degranulation, and release of inflammatory mediators. FcR$\gamma^{-/-}$ mice do, however, express the ITIM-bearing FcγRIIB, which does not directly contribute to the previously mentioned effector functions (reviewed by Ravetch and Bolland, 2001). Nevertheless, FcγRIIB binds to and cross-links IgG molecules as evidenced by the ability of murine B cells which only express FcγRIIB (Ravetch and Bolland, 2001) to promote anti-CD3-induced T cell activation in an FcR-dependent fashion (Tamada *et al*, 1995). Therefore, using FcR$\gamma^{-/-}$ mice alone is not sufficient to eliminate FcγR-mediated cross-linking of IgG antibodies. In fact, my observation that anti-CD3-induced T cell proliferation was largely retained in the presence of FcR$\gamma^{-/-}$ DCs suggests that FcγRIIB is the major FcγR used by bone marrow-derived DCs to cross-link anti-CD3 mAb. This is further supported by the very dramatic, although not complete, inhibition of proliferation by the blockade of FcγRII/III. Also as expected, when FcRγ-deficient DCs were used in combination with Fc Block, anti-CD3-mediated T cell proliferation was totally abrogated, emphasizing the very critical role of DC

FcγRs in this process. Interestingly and in sharp contrast with anti-CD3-induced T cell proliferation, Thy-1-driven proliferation was not inhibited by the absence or blockade of FcγRs. Moreover, a combination of $FcR\gamma^{-/-}$ DCs and Fc Block did not affect anti-Thy-1-induced T cell proliferation, which rules out the possibility that FcγRs display redundancy in cross-linking anti-Thy-1 mAb. It is likely that the G7 anti-Thy-1 mAb by itself cross-links Thy-1 without any requirement for FcγRs. This may be due to the heavy expression of Thy-1 on T cell surface and/or a reflection of a physicochemical characteristic of this mAb *per se*. Collectively, my data indicate that even though bone marrow-derived DCs bear at least FcγRs I and II, DC contribution to Thy-1-stimulated T cell proliferation is not a consequence of FcγR-dependent cross-linking of anti-Thy-1 mAb.

Ruling out a possible role for FcγRs in DC enhancement of Thy-1-driven T cell proliferation, I hypothesized that DCs augment Thy-1-mediated responses by providing costimulation through B7 interactions with CD28. Bone marrow-derived DCs display abundant levels of both B7-1 and B7-2 (Lutz *et al*, 1999), which participate in the costimulation of T cell responses to stimuli such as ConA and anti-TCR αβ mAb (Elbe-Burger *et al*, 2000). Anti-Thy-1-induced T cell proliferation similarly depended on both B7-1- and B7-2-mediated costimulation. My results are in stark contrast with an earlier report that relatively inefficient $CD4^+$ T-cell proliferation and IL-2 synthesis occur in response to the G7 anti-Thy-1 mAb in a xenogeneic system that used B7-transfected Chinese hamster ovary (CHO) cells as accessory cells (Reiser *et al*, 1992). However, since B7-2 was not

identified at the time, the failure of B7-1-transfected CHO cells to optimally costimulate Thy-1-driven responses could be at least in part due to a lack of CD28-B7-2 interactions. In any case, my use of syngeneic DCs, which naturally express ample B7-1 and B7-2 together with other potentially important costimulatory molecules, provides a more physiologically relevant system for T cell activation. The requirement for B7 costimulation in Thy-1-stimulated responses may also explain why resting B cells fail to augment T cell proliferation in response to anti-Thy-1 mAb since the B7 expression by resting B cells is modest in comparison with DCs. Indeed, I found that LPS-activated B cells in which the expression of B7-1 and B7-2 is substantially up-regulated (Makrigiannis *et al*, 1999) were able to efficiently costimulate Thy-1-driven T cell responses. My overall observations are consistent with the hypothesis that Thy-1 triggering can substitute for signal 1 for T cell activation. I have confirmed my findings by using anti-Thy-1 and anti-CD28 mAb-coated microbeads as surrogate APCs and showed that the combination of immobilized anti-Thy-1 and anti-CD28 mAbs could provide signal 1 and signal 2, respectively, for T cell activation. T cell proliferation under these conditions follows the same pattern as that induced by microbeads coated with anti-CD3 and anti-CD28 mAbs.

A critical outcome of T cell activation in the context of appropriate costimulation is the differentiation of CTL precursors into armed effector cells, which can detect and eliminate target cells via two major cytolytic pathways (reviewed by Berke, 1995). The granule-dependent pathway relies on the expression and function of PFN and serine proteases such as GzmB, while the

death receptor pathway employs FasL to cross-link Fas and activate the death program in susceptible target cells. Several studies have pointed to a role for Thy-1 in the generation of CTL responses. Pre-incubation with the G7 anti-Thy-1 mAb was reported by Ozery *et al* (1989) to enhance the cytolytic activity of several memory-like CTL hybridomas generated *in vivo* or in MLR. The same anti-Thy-1 mAb was also reported to boost the cytolytic function of OVA-specific $CD4^+$ Th1 clones, as well as alloreactive $CD8^+$ T-cell clones (Lancki *et al*, 1995a). In contrast, I was unable to demonstrate cytotoxic activity by anti-Thy-1-activated T cells, whereas anti-CD3-activated T cells exhibited potent nonspecific cytotoxicity against P815 target cells. However, it should be noted that T cell hybridomas or clones have already been exposed to Ag (signal 1) at some point and are therefore considered to be "primed". As a result, such cells may not accurately represent naïve T lymphocytes, which are likely to have different activation requirements and may have a greater inherent ability to acquire MHC-unrestricted cytotoxic activity.

I demonstrated that both proliferative and IL-2 responses of resting T cells to Thy-1 triggering are greatly increased by the DC contribution of costimulatory B7 molecules. Since signal 2 delivered through B7-CD28 interactions is known to be important in the generation of CTL responses (Lanier *et al*, 1995; Makrigiannis *et al*, 1999), I analyzed T cell cytotoxic responses following stimulation with anti-Thy-1 mAb in the presence of DCs. My data indicate that Thy-1 triggering of primary T cells in the presence of DCs leads to the acquisition of cytotoxic effector molecules (FasL, PFN and GzmB). The up-regulation of PFN and GzmB

gene expression as a result of Thy-1 triggering conflicts with a recent report that the G7 anti-Thy-1 mAb selectively activates the FasL-, but not granule-mediated cytotoxicity by CTL clones (Kojima *et al*, 2000). Moreover, Kojima *et al* were able to induce target cell lysis in a redirected manner with anti-Thy-1 mAb whereas, in my hands, anti-Thy-1 mAb failed to function in a redirected lysis assay with FcR^+ target cells. The discrepancies between my findings and those of Kojima *et al* may reflect distinct activation requirements for resting T cells versus CTL clones. Furthermore, the changes observed by Kojima *et al* may not necessarily represent events taking shape during the induction phase of a CTL response. In this regard, up-regulation of FasL expression and that of PFN and GzmB do not follow the same kinetics. FasL induction takes place within hours of TCR ligation whereas up-regulation of PFN and GzmB gene expression is a later event that is at least in part secondary to the production of cytokines such as IL-2 (Liu *et al*, 1989; Makrigiannis *et al*, 1997). Therefore, short-term exposure to anti-Thy-1 mAb in a redirected assay may not be sufficient to induce PFN and GzmB expression, while a longer period of T cell incubation with anti-Thy-1 mAb and DCs may allow for sufficient costimulation and exposure to cytotoxicity-inducing cytokines to induce the granule-dependent pathway of cytolysis.

Interestingly, Thy-1-stimulated T cells in our system were not able to lyse target cells, in spite of expressing cytotoxic effector molecules. This was not due to a defect in the recognition/adhesion phase of cytolysis since anti-Thy-1-activated T cells efficiently formed conjugates with the target cells. Moreover, when I added anti-CD3 mAb into a redirected killing assay containing FcR^+ target

cells, anti-Thy-1-stimulated $CD3^+$ T cells exhibited very potent killing activity. Surprisingly, the addition of anti-Thy-1 mAb into the re-directed lysis assay failed to have a similar effect. These findings, on one hand, rule out the unlikely failure of Thy-1-stimulated T cells to express cytotoxic effector molecules at protein level, and on the other hand, point to a requirement for TCR/CD3 triggering in the development of a fully functional cytotoxic phenotype. One possible reason as to why anti-Thy-1-activated T cells are not lytic is the absence of TCR internalization following Thy-1 triggering. Internalization of the TCR/CD3 complex normally takes place following stimulation with specific Ag or anti-CD3 mAb (Schaffar *et al*, 1989; Valitutti *et al*, 1996b), and may therefore be a critical step in the intracellular events that eventually give rise to fully functional CTLs. This is supported by the observation that, unlike anti-Thy-1-stimulated T cells, anti-CD3-activated T cells exhibit potent MHC-unrestricted cytotoxicity against a broad spectrum of tumor cells (Stankova *et al*, 1989).

My results are particularly important considering the speculation that signaling through Thy-1 occurs via a structural and/or functional association of Thy-1 with the TCR (Gunter *et al*, 1987; Schmitt-Verhulst *et al*, 1987). The failure of Thy-1-stimulated T cells to kill provides new evidence for existence of fundamental differences between the TCR- and Thy-1-mediated pathways of T cell activation. Whether such differences are a consequence of distinct intracellular signaling cascades associated with the two pathways is not clear, although differential requirements for *Fyn* PTK and p38 MAPK in cytotoxic and

proliferative responses associated with the two pathways have been reported (Lancki *et al*, 1995b; Haeryfar and Hoskin, 2001).

In summary, I have shown that Thy-1 triggering provides an incomplete form of signal 1, which can be coupled with a strong signal 2 provided by DCs to induce T cell proliferation and IL-2 synthesis. This provides a useful model system in which to study the consequences of T cell activation in the absence of traditional TCR signaling. In addition, my data reveal that T cell expansion in the absence of conventional signal 1 is not associated with the development of cytotoxic effector function. This may constitute a mechanism for the maintenance of T cell homeostasis without the risk of developing promiscuous cytotoxicity.

6.4 Future directions and concluding remarks

A compelling body of evidence suggests a role for Thy-1 in the process of T cell activation. Although Thy-1 has been a subject of intense investigation for the past four decades, the exact function(s) of Thy-1 during T cell activation has remained puzzling and highly controversial. This study was undertaken to address several unresolved issues concerning the role of Thy-1 in the context of T cell proliferative and cytotoxic responses induced in the absence or presence of classical TCR signaling and/or principal costimulatory interactions.

I found that the blockade of Thy-1 by a non-stimulatory anti-Thy-1 mAb (clone 30-H12) prevented the induction of non-specific CTLs in response to anti-CD3 mAb. The inhibition of cytotoxic effector function as a result of the blockade of Thy-1 paralleled an inhibition/ablation of PFN and GzmB expression, but not FasL expression, which was at least in part due to lower IL-2 synthesis by anti-

CD3-activated T cells cultured in the presence of anti-Thy-1 mAb. These results extend the existing literature by demonstrating that signaling through Thy-1 is important not only for the effector phase of cell-mediated cytotoxicity as has previously been described for CTL clones (Kojima *et al*, 2000; Lancki *et al*, 1995b; Ozery *et al*, 1989), but also during the induction phase of a CTL response. The inhibitory effect of anti-Thy-1 mAb 30-H12 appears not to be due to negative signaling, but likely stems from the blockade of Thy-1 interactions with its putative ligand. My results suggest that the Thy-1 ligand exists on B cells, but not on DCs. Future studies using Fab fragments of anti-Thy-1 mAb 30-H12 will be needed to confirm the blocking property of this mAb.

The fact that Thy-1 lacks a cytoplasmic tail, and is tethered to the cell membrane only through a GPI anchor, has led many to speculate that signaling through Thy-1 may occur via an as yet unidentified structural or functional association between Thy-1 and the TCR. Such a hypothetical interdependence between Thy-1 and the TCR leads to the assumption that Thy-1- and TCR-associated signaling pathways are identical. However, I took advantage of several pharmacological inhibitors of intracellular signaling molecules to demonstrate the existence of both similarities and novel differences between the two signaling pathways. Most interestingly, inhibition of MAPK p38 by two different inhibitors led to an augmentation of Thy-1-driven T cell proliferation, whereas p38 inhibitors attenuated anti-CD3-induced T cell proliferation. This finding has led me to suggest that p38 differentially regulates Thy-1- and TCR-associated T cell activation pathways. While the activation and kinase activity of

p38 may constitute an essential component of the TCR-coupled signaling cascade, signal transduction in response to Thy-1 triggering may be negatively regulated by p38. During the course of T cell activation when both TCR and Thy-1 are presumably triggered as a result of interacting with specific Ag and a putative Thy-1 ligand, respectively, possible cross-talk between the TCR- and Thy-1-associated signaling pathways may be provided via p38. It would therefore be interesting to examine the extent of p38 activation and the status of p38 kinase activity following TCR and Thy-1 triggering either separately or simultaneously. It would also be worthwhile to investigate possible inter-connection between p38 and other members of the MAPK family in the context of Thy-1- and TCR-mediated T cell activation.

Another point of difference between the TCR- and Thy-1-associated signaling pathways that is also worthy of further examination lies at the level of PKC activation. I demonstrated that Thy-1-driven T cell proliferation was more sensitive than TCR signaling to the PKC inhibitor calphostin C, which makes one wonder whether different PKC isoforms participate in the two pathways. Selective inhibitors of different PKC isoforms are now available and can be employed to test this hypothesis. Furthermore, I noted a synergistic augmentation of Thy-1-driven T cell proliferation and IL-2 production by the PKC activator PMA. This suggests that the reason Thy-1 signaling is more sensitive than TCR signaling to PKC inhibition is that triggering of Thy-1, by itself, fails to induce "strong" PKC activation, which might also be responsible for the low magnitude of Thy-1-driven T cell responses in the absence of PMA. The results presented in chapter 5

demonstrate that Thy-1-driven T cell proliferation was also synergistically augmented by concomitant CD28-mediated costimulation in a manner strikingly similar to that induced by PMA (see figures 4.4 and 5.8A). I propose, based on these observations, that signaling through CD28 enhances Thy-1-triggered T cell proliferation at least in part by inducing the activation of certain or all isoforms of PKC. This hypothesis is supported by previous reports of functional coupling of PKCθ to the CD28 costimulatory pathway (Coudronniere *et al*, 2000; Huang *et al*, 2002). PKCθ is selectively expressed in skeletal muscle and T cells, and unlike other PKC isoforms, is recruited to the central core of the immunological synapse where it functions in concert with other molecules to generate signals essential for productive T cell activation and IL-2 production (reviewed by Isakov and Altman, 2002).

I found that Thy-1 triggering in combination with CD28-mediated costimulation (provided by DCs or immobilized anti-CD28 mAb) leads to robust T cell proliferation, IL-2 synthesis, and the acquisition of cytotoxic effector molecules, but not cytotoxic effector function. The importance of this discovery is several-fold. First, I propose for the first time that triggering of Thy-1 can generate a signal that substitutes for signal 1 with regard to select features of T cell activation. Secondly, the insufficiency of Thy-1 triggering, even in the presence of strong costimulation, to induce lytic effector function may reflect a regulatory step to avoid promiscuous cytotoxicity when T cells are activated in the absence of a classical TCR-generated signal 1. Thirdly, the ability or failure of signaling through the TCR and Thy-1, respectively, to induce fully functional killer

cells can also be viewed as yet another fundamental difference between the two pathways of T cell activation, which has not previously been reported.

Hypothetically, robust T cell proliferation and IL-2 synthesis as a result of Thy-1 triggering in the context of a DC-generated costimulatory signal could have harmful consequences. In other words, if DCs expressed the putative ligand for Thy-1, Thy-1 signaling together with pre-existing, strong costimulation could lead to constant T cell proliferation of a high magnitude in the absence of an Ag-specific signal. This may result, at least theoretically, in a "cytokine release syndrome" similar to what is encountered in allograft recipients in the aftermath of the *in vivo* administration of OKT3 mAbs (Chatenoud, 1993), and/or autoimmune reactions secondary to polyclonal T cell activation. Since I, like others, have used a stimulatory anti-Thy-1 mAb (clone G7) to induce T cell proliferation, one cannot rule out the possibility that the interactions between Thy-1 and its actual ligand would not generate a similar signal. If this is the case, one could question the relevance of many studies on Thy-1 using stimulatory mAbs. However, it is heartening to know that in several circumstances where the actual ligands for other GPI-anchored proteins have been characterized, their cognate ligation results in responses that can be mimicked by those using mAbs. Perhaps, the best examples are CD48 and CD59 whose interactions with CD2 and complement component C8, respectively, mediate similar effects as mAbs specific for the former two molecules (Moran and Miceli, 1998; Murray and Robbins, 1998). It is also possible that Thy-1-Thy-1 ligand interactions induce only modest T cell responses in the absence of foreign Ag, contributing, for

example, to homeostatic T cell proliferation. Finally, it is possible that DCs do not express the putative ligand for Thy-1. This notion is supported by the findings presented in chapter 3, which indicate that the blockade of Thy-1 fails to inhibit both Ag-specific and anti-CD3-induced T cell proliferation in the presence of DCs as accessory cells (see figure 3.9).

My finding that a combination of anti-Thy-1 mAb and syngeneic DCs can efficiently activate T cells is also important from a methodological point of view. Delivery of costimulation through DC B7 molecules eliminates the need for the use of PMA along with anti-Thy-1 mAb in order to generate substantial T cell activation in experimental protocols. Future studies will address whether DCs are also capable of enhancing T cell signaling in response to other GPI-anchored proteins. It will also be interesting to isolate naïve and memory T cell populations from resting T cell pools and examine the responses of these T cells to anti-Thy-1 mAb in the presence or absence of CD28-mediated costimulation. Naïve T cells, but not memory T cells, are highly dependent on CD28 costimulation in order to respond to TCR/CD3 ligation (Croft and Dubey, 1997). Based on the results presented in chapter 5, I predict that memory T cells, unlike naïve T cells, do not strictly require CD28 costimulation in order to respond to Thy-1 triggering. If proven to be true, this will provide an extension of the above-mentioned paradigm of costimulation requirements of naïve versus memory T cells.

Finally, it is noteworthy that the differences in terms of mitogenicity between anti-Thy-1 mAbs used in my studies cannot be attributed to their ability to bind FcRs. Anti-Thy-1 mAb 30-H12 has no mitogenicity, in spite the likelihood

that it binds to FcRs (see discussion for figure 3.2B). In contrast, stimulatory anti-Thy-1 mAb G7 is mitogenic and "self-aggregating" (Fujita et al, 1997) without requiring FcRs for its cross-linking. This is also evidenced by the ability of this mAb in my hands to induce strong T cell proliferation and IL-2 synthesis in the presence of FcγR-deficient DCs plus Fc block (see figure 5.4). The functional differences between these two mAbs may be a reflection of physicochemical properties and/or epitope specificity of these two mAbs. Anti-Thy-1 mAb 30-H12 recognizes a specific epitope found only on Thy-1.2 alloantigen (Ledbetter *et al*, 1980), while the G7 mAb targets a non-polymorphic epitope of Thy-1 (Gunter *et al*, 1984). Differences in terms of epitope specificities may reconcile some seemingly conflicting results obtained using these two mAbs in my studies. For example, while the blockade of Thy-1 by the 30-H12 mAb affected the expression of PFN and GzmB, but not FasL, cross-linking of Thy-1 by the G7 mAb led to up-regulation of all three effector molecules. Therefore, distinct signaling pathways may be triggered depending on which Thy-1 epitope(s) are recognized by anti-Thy-1 mAbs or the putative ligand for Thy-1.

In summary, my results re-emphasize the important role of Thy-1 in the process of T cell activation, and suggest a novel role for Thy-1 as a potential source of signal 1 in the context of strong costimulation. Moreover, I have provided new evidence that the signal transduction pathways governing TCR- and Thy-1-driven T cell responses are not identical. I expect that more complete understanding of stimulatory and costimulatory pathways of T cell activation in a not-too-distant future together with technological advances will further elucidate

the immunobiological function of the ever-enigmatic Thy-1, an objective which has been long overdue.

7. References

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