TUMOR NECROSIS FACTOR RECEPTOR TYPE-2 (p75) FUNCTIONS AS A CO-STIMULATOR OF ANTIGEN-DRIVEN T CELL RESPONSES

by

EDWARD YONG KIM

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Abstract

Co-stimulation is a fundamental mechanism for self-nonself discrimination in adaptive immunity and encompasses a remarkably dynamic system of receptor-ligand interactions that are spatially and temporally regulated to achieve fine-tuning of T cell responses. In addition to members of the CD28 superfamily, T cell co-stimulation can also be mediated through members of the TNF receptor (TNFR) superfamily. Although TNFα/TNF receptor-2 (TNFR2) interactions were shown to be capable of augmenting T cell proliferation, TNFR2-deficient mice were initially reported to have normal T and B cell compartments, with no apparent defect in T cell function. This thesis re-visited the question applying gene-targeted and TCR transgenic systems to examine the putative co-stimulatory role for TNFα/TNFR2 in controlling T cell responses *in vitro* and *in vivo*. My results demonstrate that TNFR2 is an important co-stimulatory molecule for both $CD4^+$ and $CD8^+$ T cells in promoting IL-2 induction, lowering the activation threshold, and promoting early survival for the dividing population during primary expansion. TNFR2 functions non-redundant of CD28, as TNFR2 is necessary to sustain AKT and NFκB activation, as well as to promote Bcl-X_L expression. $CD8^+$ TCR transgenic T cells deficient in TNFR2 exhibited dramatic reduction in clonal expansion *in vivo* upon recombinant *Listeria monocytogenes* (LM) challenge, which correlated with a survival defect of responding cells and diminished Bcl-2 and survivin expression. The frequency of LM-specific CD4 and CD8 effector T cells was diminished in $TNFR2^{-/-}$ mice upon primary challenge with recombinant LM, and led to the generation of a smaller pool of memory T cells. Moreover, TNFR2-deficient mice were more susceptible to a high dose of primary challenge with LM correlating with a marked reduction in the LM-specific

effector T cell pool *in vivo*. Thus, TNFR2 promotes adaptive immunity as a co-stimulator of T cell responses in both $CD4^+$ and $CD8^+$ subsets, and represents an interesting therapeutic target for modulating T cell immunity in disease contexts.

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List of Abbreviations

7-AAD	7-amino-actinomycin D
APC	antigen-presenting cell
ASM	airway smooth muscle
BAL	bronchial alveolar lavage
CFSE	carboxyfluorescein diacetate succinimidyl ester
ConA	concanavalin a
CQ-PCR	competitive and quantitative polymerase chain reaction
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DD	death domain
DLN	draining lymph node
EAE	experimental autoimmune encephalomyelitis
EMSA	electrophoretic mobility shift assay
GSK-3	glycogen synthase kinase-3
GM-CSF	granulocyte macrophage colony stimulating factor
GVHD	graft-versus-host disease
HIV	human immunodeficiency virus
HSV-1	herpes simplex virus-1
HVEM	herpes virus entry mediator
IκBα	inhibitor of NFκB
JNK/SAPK	Jun N-terminal kinase/stress-activated protein kinase
KLH	keyhole limpet hemocyanin
LAT	linker of activated T cells
LCMV	lymphocytic choriomeningitis virus
LIGHT	lymphotoxin-like, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes
LLO	listeriolysin-O
LM	*Listeria monocytogenes*
LPS	lipopolysaccharide

LT	lymphotoxin
mAb	monoclonal antibody
MAPK	mitogen activated protein kinase
memTNF	26-kDa membrane-bound TNFα
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
NF-κB	nuclear factor κB
NIK	NFκB-inducing kinase
NK	natural killer
NOD	non-obese diabetic
PCC	pigeon cytochrome C
PI-3K	phosphatidylinositol 3-kinase
PKB	protein kinase B
RAG	recombinase activating gene
RIP	receptor-interacting protein
rLM-SIY	recombinant *Listeria monocytogenes* expressing secreted form of SIY peptide
SIY	SIYRYYGL peptide
solTNF	17-kDa soluble TNFα
TCR	T cell receptor
TLR	Toll-like receptor
TRAF	TNFR receptor-associated factor
TNF	tumor necrosis factor
TNFR	TNF receptor
TNFR1, p55	tumor necrosis factor receptor-1
TNFR2, p75	tumor necrosis factor receptor-2
Treg	$CD4^+$ $CD25^+$ regulatory T cell
VSV	vesicular stomatitis virus
WT	wild-type
ZAP-70	zeta-associated protein of 70-kDa

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Preface

Co-stimulation is an essential mechanism for self-tolerance in adaptive immunity and involves coordinated receptor-ligand interactions delivered to T cells by APCs. In addition to this important signal, the strength and duration of TCR-mediated signals, the nature of the pathogen in (eg. acute vs. persistent strains of bacteria or virus), and the local cytokine milieu all represent important cues for determining T cell fate. Gene-targeting approaches in mice have led to a substantial advance in our understanding of the cellular and molecular mechanisms controlling the T cell response. This thesis consists of three main parts: (1) an introduction providing a historical perspective on the two-signal model of self-nonself discrimination and the growing number of co-stimulatory molecules belonging to either the CD28- or TNFR-superfamilies; (2) a series of published manuscripts describing the important role of TNFR2 as a T cell co-stimulatory molecule as revealed using gene-targeted mice; and (3) a conclusion section with discussion/perspectives on TNFR co-stimulation in the context of our current understanding of the dynamics of T cell differentiation and exploration of their therapeutic potential in autoimmunity.

Chapter 1 Introduction

1.1 Preface

The focus of this thesis was the characterization of TNF receptor-2 (TNFR2) in co-stimulating the T cell response as defined by augmenting IL-2 production, lowering the threshold of T cell activation, and providing important survival signals for generating a robust effector and memory population. The role of TNFα in the immune system has been extensively characterized in cell lineages across the hematopeoitic range, reflecting the ubiquitous expression of its receptors. In addition to CD28, Studies in the past few years have highlighted the importance of a second major class of co-stimulatory molecules belonging to the TNFR superfamily. TNFR2-deficient mice were initially reported to have normal T and B cell compartments; no overt phenotype in T cell function was apparently observed in this original report. This thesis re-visited the question applying gene-targeting approaches to dissect T cell responses in the absence of TNFR2, and revealed TNFR2 as an important co-stimulatory molecule for both CD4 and CD8 T cells *in vitro* and *in vivo*. This chapter will highlight historical perspectives of T cell co-stimulation, beginning with a review of CD28 superfamily and then leading into the more recently identified TNFR superfamily, emphasizing a large variety of receptors that co-stimulate T cell responses and bestow remarkable flexibility in regulating discrete phases of the differentiation program.

1.2 T cell co-stimulation

1.2.1 Self-tolerance: two-signal model

A myriad of cell surface receptor-ligand interactions serve as control points in determining cell fate for homeostasis and function in the vertebrate immune system. For T cells the ability to discriminate self from non-self is fundamental to maintaining a highly diverse T-cell repertoire, while eliminating or suppressing lymphocytes capable of recognizing and responding to self-antigens. It is indeed a remarkable evolutionary achievement bestowing T cells with a remarkably effective mechanism that ensures destruction of pathogenic entities while leaving normal host tissue intact. To put this into perspective, consider the substantial number of lymphocyte antigen receptors, which confer the ability to recognize and respond to both foreign and self-antigens, encompassing an estimated 25 x 10^6 T-cell receptors (TCRs) (1) and between 10^4 and 10^{10} B-cell receptors (2). Much has been learned since Brestcher and Cohn first postulated a 2-signal model for self-nonself discrimination more than 30 years ago (3). Although antigen specificity of the T cell response is defined by the T cell receptor (signal 1), the fidelity of the immune response is achieved via non-cognate co-stimulatory receptor-ligand interactions (signal 2). Signal 1 occurs constantly in peripheral homeostasis with low-affinity interactions between cognate antigen and TCR as a natural consequence of the thymic selection process. However, ligands that induce signal 2 are selectively expressed when host defense surveillance encounters a pathogenic challenge. Thus, the physiological requirement of co-stimulatory signals for optimal activation of T cells in addition to the Ag-specific signal delivered through the TCR is a major means by which self-tolerance is achieved. The nature of signal 2 has been the subject of intense

investigations over the past decade resulting in a major conceptual advance of T cell co-stimulation beyond two-signal models that previously posited greatest significance to members of the well-studied Ig-like CD28 superfamily.

1.2.2 CD28/B7 pathway in T cell responses

From a historical perspective, co-stimulation as a major mechanism of self-tolerance for the T cell compartment was discovered along a course of experiments that led to the identification of the CD28/B7 pathway. Work in the early 80's showed that T cell clones failed to undergo expansion in the absence of co-stimulatory signals, and instead enter an anergic state in which they are refractory to further activation. Importantly, T cell anergy was found to be a direct consequence of a failure to induce IL-2 production, and co-stimulation was therefore intimately linked with a signal that regulated IL-2 expression. This search resulted in the identification of the CD28/B7 pathway as a prominent co-stimulatory pathway for T cells (3, 4).

CD28 (reviewed in ref. 1) is the best characterized of a growing number of co-stimulatory molecules, and has been shown to synergize with the TCR to lower the threshold of T cell activation (11, 12), enhance initial clonal expansion (13), regulate IL-2 production (7, 14), and augment the expression of anti-apoptotic members of the Bcl-2 family (15). CD28 is constitutively expressed on the surface of ~ 80% of human T cells and virtually 100% of murine T cells, and undergoes up-regulation in cell surface expression following T cell activation. *In vitro* studies have shown CD28 dependence of antigen-specific proliferation by naive TCR transgenic T cells. Lucas *et al.* (4) showed using DO.10 TCR transgenic $CD4^+$ T cells that CD28 deficiency is associated with a

significant decrease in proliferative response to limiting concentrations of OVA peptide during the first 48 hrs of stimulation, and that $CD28^{-/-}$ DO.10 TCR transgenic $CD4^+$ T cells could not maintain their proliferative response after 60 hrs even at high Ag concentrations (4, 5). These findings suggest that CD28 plays a conditional role for initiating Ag-specific T cell proliferation but is essential for optimal IL-2 production and sustained T cell proliferation (4, 5). Sperling *et al.* (5) used MHC class I-restricted 2C TCR transgenic system to show that blockade of CD28 signaling by CD28-deficiency or CTLA-4 Ig treatment resulted in aborted proliferation after 48 hrs of stimulation, thus demonstrating an essential role for CD28 in sustaining proliferative responses and promoting T cell survival (4, 5). Moreover, the same study showed that co-ligation with anti-CD28 mAb during stimulation with BALB/c splenocytes promotes late proliferation and survival (4, 5).

In vivo studies corroborated these *in vitro* findings establishing the role of CD28 on the T cell proliferative response. Using the DO11.10 TCR transgenic (OVA-specific) $CD4^+$ T cell adoptive transfer system into syngeneic BALB/c mice to track clonal expansion *in vivo* following OVA/IFA immunization, Gudmundsdottir *et al.* (6) found that a responding Ag-specific $CD4^+$ T cell undergoes division to generate >20 daughter cells *in vivo* as detected in regional lymph nodes 72 hrs after immunization (6). The role of co-stimulation by CD28 in clonal expansion of Ag-specific CD4 T cells was measured in this system using CTLA-4 Ig: in contrast to control Ig treatment that was associated with accumulation of over 200,000 OVA-specific CD4 T cells in regional lymph nodes 72 hrs after immunization from a starting population of 20,000 OVA-specific T cells, CTLA-4 Ig treatment led to a dramatic decrease of less than 40,000 OVA-specific T cells

(6). TCR and CD28 signals cooperatively determine the degree of primary clonal expansion by increasing both the proportion of Ag-specific T cells that divide and the number of rounds of division the responding T cells undergo (6).

In contrast to naive T cells that depend on CD28 co-stimulation, studies on proliferation and cytokine production by memory T cells indicate CD28-independence during the recall response (7, 8). Indeed, Suresh *et al.* (2001) found that disrupting CD28/B7 interactions did not significantly affect the generation and maintenance of CD8 T cell memory and LCMV-specific CD28-deficient memory CD8 T cells showed normal homeostatic proliferation *in vivo*, conferring protective immunity to re-challenge (9). This could be due to high constitutive levels of activated src-kinase Lck in memory T cells, which localizes to and phosphorylates the TCR complex and thus decreases the threshold for T cell activation (10), thereby reflecting functional and biochemical differences between naive and memory T cells. London *et al.* (12) showed that memory cells rapidly produced effector cytokines (IFN-gamma, IL-4, IL-5) within 12-24 h of Ag exposure and proliferated at lower Ag concentrations than naive cells. Using APCs deficient in B7 and CD40, they also showed that memory cells were less dependent on co-stimulation by B7:CD28 and CD40L:CD40 interactions (11). These findings further support the notion that memory CD4+ T cells possess a lower threshold of activation compared to their naive cell counterparts, allowing for rapid response during re-challenge.

1.2.3 CD28 in Th1/Th2 differentiation

In addition to a role in clonal expansion and survival, CD28/B7 interactions have also been found to regulate Th1/Th2 differentiation of naive $CD4^+$ T cells. CD28

engagement has been shown to enhance the production of various cytokines including IL-1, IL-2, IL-4, IL-5, TNFα, and IFNγ (12), and studies support a fundamental role for CD28 in the early development and differentiation of both Th1 and Th2 T cell subsets (13). In the absence of CD28-mediated co-stimulation, Th2 cytokines (IL-4, IL-5, and IL-10) are not produced whereas IFN-γ production is not severely affected (14-16), and hence bias naive T cells toward a Th1 phenotype. This was demonstrated using hCTLA4Ig to block CD28/B7 interactions, which resulted in strong abrogation of IL-4 production (17). Seder *et al.* (1994) suggest that this bias away from Th2 phenotype was due to the lack of IL-2 production, since the addition of exogenous IL-2 overcame the defect in IL-4 production (17). The importance of CD28 signaling in the differentiation of Th2 cells is supported by several studies *in vivo* utilizing $CD28^{-/-}$ mice, which display reduced Th2-dependent antibody response to VSV while the Th1-dependent DTH response to LCMV remains intact (18). Moreover, T cells isolated from CTLA4Ig transgenic mice produce significantly reduced amounts of IL-4 and elevated levels of IFNγ in response to primary immunization (19, 20). Hence, CD28-mediated signaling can modulate both quantitative and qualitative outcomes during the T cell response, affecting threshold of activation and survival as well as regulating Th1/Th2 differentiation.

1.2.4 CD28/B7 co-stimulation in autoimmune disease models

Many of the investigations into clinical applications of CD28/B7 blockade have focused on experimental autoimmune encephalomyelitis (EAE) as an animal model for multiple sclerosis (MS) (21). EAE is an acute or chronic-relapsing, acquired, inflammatory and demyelinating autoimmune disease mediated by $CD4^{+}$ Th1 cells. It can

be induced by immunization with various myelin proteins or their immunodominant peptide epitopes (21). Initial studies performed at the onset of the disease showed that blockade of CD28/B7 interactions resulted in the reduction of EAE, correlating with a significant reduction in proliferation of CNS-reactive T cells in the draining lymph nodes 10–15 days after immunization, suggesting that amelioration of disease was due to limited T cell expansion *in vivo* (22, 23).

A more clinically relevant system is the investigation of CD28/B7 blockade after disease onset. These studies use a murine relapsing-remitting EAE (R-EAE) that is induced by immunization with the immunodominant proteolipid protein epitope (PLP139–151) (24). Following immunization, mice exhibit a paralytic acute phase followed by spontaneous remission and clinical relapses, pathology that bears similarity to MS. Interestingly, blockade of the CD28/B7 pathway during the acute or remission phases of EAE prevents further relapses (25, 26), which might not have been predicted based on the previous observations that the stimulation of recently activated T cells and memory T cells was CD28-independent (7, 11). One possible explanation could be that the activation of autoreactive T cells may be more dependent on CD28/B7 co-stimulation due to weaker affinity for their ligand. This is corroborated by evidence showing that *ex vivo* proliferation of myelin-autoreactive T cells purified from mice that were developing acute EAE remain dependent on CD28/B7 co-stimulation (27). Importantly, several groups showed that the clinical relapses were a consequence of the activation of naive autoreactive T cells triggered by endogenously presented myelin epitopes derived from autoimmune tissue destruction, a phenomenon known as epitope spreading (28, 29).

Hence, CD28/B7 interactions plays an important role in controlling relapse due to a dependence upon activation of a naive set of autoreactive T cells in this system.

The NOD mouse, a model of spontaneous autoimmune diabetes, has been used to study the role of CD28/B7 interactions in Th1/Th2 differentiation as a crucial determinant of disease outcome. Augmenting Th2 cytokines either by exogenous injection or over-expression in islets protects mice from the disease (30). Consistent with *in vitro* data showing dependence of Th2 phenotype on CD28 signals, blockade of the CD28/B7 pathway via disruption of CD28 gene expression led to an earlier onset, increased incidence, and increased severity of diabetes in NOD mice as compared to their littermate controls (31). Re-enforcing the protective role of Th2 cytokines in diabetes and the dependence of Th2 differentiation on CD28, it was shown that treatment of young NOD mice with a stimulating anti-CD28 mAb led to increased production of IL-4 by islet-infiltrating NOD T cells and prevented the development of autoimmune diabetes (32). More recently, another explanation has arisen to account for exacerbation of spontaneous diabetes in both B7-1/B7-2-deficient and CD28-deficient NOD mice (31): a profound decrease in the regulatory $CD4^+$ $CD25^+$ T cell (Treg) compartment in the absence of CD28 signaling (33). Transfer of this regulatory T cell subset from control NOD animals into CD28-deficient animals can delay/prevent diabetes, demonstrating that Treg's are important for controlling autoimmune diabetes (33).

Notably in the NOD system, the islet autoreactive T cells that develop during the course of disease produce Th1 cytokines but not immunoregulatory Th2 cytokines, reflecting a change in cytokine balance that leads to more severe insulitis and suggests a basis for the exacerbation of diabetes (31). Within this context, once an established

effector T cell population has developed, proliferation of Th1 cells but not of Th2 cells is CD28-dependent, manifested as increased T cell death late in culture that cannot be fully rescued by IL-2 or other survival cytokines (7, 11). Lenschow *et al.* (1995) found that NOD mice treated at the onset of insulitis (2-4 wk of age) with CTLA4Ig or a monoclonal antibody specific for B7-2 prevented the development of diabetes (34). However, late therapy had no effect on disease progression (34). Together these results suggest that blockade of CD28-mediated co-stimulatory signals is effective early in disease development, after insulitis but before the onset of diabetes, presumably by ablating Th1-type autoreactive pathology. Thus, the CD28/B7 pathway can play paradoxical roles in autoimmune diabetes. CD28 blockade can skew away from Th2 differentiation and hence bias naive T cells towards Th1, as well as reduce the numbers of Treg's leading to reduced protection (33); CD28 blockade may also limit the activation and expansion of Th1 cells established during the course of disease leading to increased protection against autoimmune diabetes (35).

1.2.5 CD28 signal transduction

CD28 was established as a key co-stimulatory molecule for IL-2 induction based on its ability to substantially augment expression in T cells stimulated via TCR (36). Indeed, CD28 has been shown to synergize with the TCR to lower the threshold of T cell activation (37, 38), enhance initial clonal expansion (4), regulate IL-2 production (36, 39), and augment the expression of anti-apoptotic members of the Bcl-2 family (40). Transcriptional regulation of IL-2 is achieved by cooperative binding of different transcription factors (including NFAT, AP-1, NFκB) (41), which are downstream targets of both the TCR and CD28 signaling

pathways (36, 42, 43). Their integration at the IL-2 promoter has been mapped to the CD28 response element (CD28RE), which functions in conjunction with a nonconsensus AP-1 site (44). Notably, a number of other cytokine genes are transcriptionally up-regulated through CD28RE, including IL-3, IFNγ, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-8 (36, 45, 46). The CD28RE in the IL-2 promoter contains a sequence similar to a consensus binding site for NFκB family transcription factors (47). CD28 ligation in conjunction with TCR cross-linking antibody or low concentrations of phorbol 12-myristate 13-acetate (PMA), leads to IκB degradation and subsequent increases in nuclear concentrations of NFκB dimers and transcriptional activation of genes regulated by NFκB (48-50). Thus, coordinated signals provided by external stimuli elicit intracellular signaling pathways that converge qualitatively and quantitatively towards changes in gene expression profile.

Initial biochemical studies of CD28 function demonstrated its role in the transcription and stability of IL-2 mRNA (36, 39, 51, 52). Subsequent studies of CD28 signaling showed synergy with those mediated via the TCR complex towards the activation of downstream effectors (53). In particular, optimal activation of the transcription factors NFAT, AP-1, and NFκB is dependent on 'amplification signals' provided by CD28-mediated recruitment and subsequent activation of Vav (54) and phosphoinositide 3-kinase (PI3K) (53). More recently, protein kinase B (AKT) was shown to provide the CD28-mediated co-stimulatory signal for up-regulation of IL-2, suggesting that CD28 also utilizes discrete signaling pathways (55). However, the effects mediated by CD28 were found to be insufficient to sustain long term T cell survival, and

therefore the effects of CD28 are functionally positioned early during T cell activation (within 24 hrs of antigen encounter) (56).

The co-stimulatory role of CD28 on T cell proliferation is therefore due to several mechanisms: increased transcription and mRNA stability of IL-2, increased expression of the anti-apoptotic protein Bcl-X_L that promotes cell survival *in vitro* (5, 40) and *in vivo* (57, 58), and decreased threshold of T cell activation (59). In naive T cells, CD28 co-stimulation also appears to promote the formation of an immunological synapse, a distinct region formed at the contact zone of the T cell and APC resulting from the specific re-organization of specific signaling and adhesion cell-surface proteins accompanied by re-orientation of the microtubule organizing centre and golgi apparatus to a region just beneath the contact site (60, 61). Wulfing and Davis (1998) used beads coated with an antibody against the cortical actin cytoskeleton-linked adhesion molecule ICAM-1 to measure the movement of anti-ICAM-1-coated beads towards the contact site (60, 61). They were able to show that the engagement of B7/CD28 triggers an active accumulation of molecules at the T cell-APC interface. This movement of beads towards the interface was inhibited by blocking antibodies against ICAM-1 and B7 (60, 61). The accumulation of receptor-pairs and other cytoskeletal-linked molecules thereby increases their local densities at the contact site for signal amplification (60, 61). This suggests that co-stimulation could be mediated to a significant extent by the re-distribution of lipid rafts to the synapse. Viola *et al.* (1999) showed that immobilizing cholera toxin B subunit or a monoclonal antibody to the glycophosphatidylinositol (GPI)-linked protein CD59 in culture wells with anti-CD3 provided efficient co-stimulation of the proliferative response by resting T cells (60, 61). Viola *et al.* (1999) suggest that cross-linking rafts

mimics the co-stimulatory effect of CD28 and that the recruitment of rafts to the immunological synapse may be a general mechanism to amplify signals, resulting from increased local accumulation of kinases (60, 61).

1.2.6 Signal 2 not solely defined by CD28

Shahinian *et al.* (1993), who first reported the generation of a CD28-deficient mouse, observed that T cell responses to lectins, activity of T helper cells and immunoglobulin class switching were significantly reduced in $CD28^{-/-}$ mice. In contrast, cytotoxic T cells were still induced in $CD28^{-/-}$ mice and exhibited delayed-type hypersensitivity after infection with lymphocytic choriomeningitis virus (18). These findings suggest that CD28 is not required for all T cell responses *in vivo*, and implies that alternative co-stimulatory pathways may exist. Further studies using CD28-deficient mice showed that co-stimulation through the CD28 pathway was not an absolute requirement for T cell activation (4, 37, 62-64). These studies strongly suggest that the intensity and duration of the antigenic signal mediated through the TCR is also a critical factor (42, 62, 65).

Additional members of the B7/CD28 family have been identified in recent years. ICOS, a CD28 homolog, is a co-stimulatory receptor that is upregulated on both $CD4^+$ and $CD8^+$ T cells following activation through the TCR and enhanced via CD28 signaling (66). ICOS binds a unique B7 family member, B7h, which is expressed constitutively by B cells and macrophages, and its expression can be induced by inflammatory stimuli on non-lymphoid cells, including endothelial cells, fibroblasts, and epithelial cells. Although CD28 and ICOS share remarkable similarity in signaling functions, ICOS co-stimulation alone is insufficient to induce substantial IL-2 production

or Bcl-X_L upregulation (67). ICOS, in contrast to the early role of CD28 in IL-2 induction, appears to be important for the generation of effector T-cell cytokine responses (68). Thus, ICOS appears to play a critical role disctint from CD28 in T cell activation.

1.2.7 Danger signals induce a myriad of co-stimulatory ligands

Dendritic cells (DCs), in their role as professional APCs, are pivotal in the generation of strong innate and adaptive immune responses, serving as the upstream regulator for decisions between immunity and tolerance. Current data support the danger signal model in which stimuli received by DCs via Toll-like receptors (TLRs) signal DC maturation as measured by enhanced expression of the co-stimulatory molecules B7.1, B7.2 and CD40 (69). The corollary of this model is that in the absence of danger signals elicited by pathogen, T cell-DC interactions would lead to tolerance induction (70). As Janeway first proposed (71), the induction of B7.1 and B7.2 on the DC surface is a particularly important step in the initiation of adaptive immunity, thereby coupling pathogen surveillance with the induction of the co-stimulators that allows activation of pathogen-specific T cells. Naive $CD4^+$ T cells can differentiate into either Th1 or Th2 cells depending on a number of determinants expressed by activated APCs, including types of co-stimulatory molecules expressed and cytokines secreted (72). Thus, the differentiation program that expands a naive T cell population into effector and memory cells depends on a complex and dynamic array of temporally and spatially regulated factors. Not surprisingly, investigations into novel co-stimulatory molecules over the past few years has led to a major conceptual advance of the two-signal model, integrating a

myriad of receptors to include not only CD28-superfamily members, but also cell surface receptors belonging to the TNFR superfamily.

1.3 TNF receptor superfamily and co-stimulation

1.3.1 TNFα in the immune system

TNFα was identified thirty years ago as a product of lymphocytes and macrophages that caused the lysis of tumor cells (73, 74). Though systemic toxicity stymied early hopes of using LTα or TNFα as anti-tumor agents, the discovery of new members of the TNFR superfamily opened new lines of investigation into host defense, inflammation, apoptosis, autoimmunity, and organogenesis. Large-scale sequencing of expressed sequence tags (ESTs) identified many related proteins constituting the TNFR superfamily proteins (reviewed in (75-77). Pharmaceutical intervention designed to inhibit TNFα have been successful in the clinic for inflammatory conditions such as rheumatoid arthritis and inflammatory bowel disease (78, 79). Indeed, TNFα and other members of the TNF/TNFR superfamily are now being targeted for therapies aimed at prevalent medical conditions such as atherosclerosis, osteoporosis, autoimmune disorders, allograft rejection, and cancer (reviewed in (80).

TNF is initially synthesized as a non-glycosylated, homo-trimeric transmembrane 26-kDa protein (memTNF), which is then cleaved by the matrix metalloprotease TNFα-converting enzyme (TACE), to release the 17-kDa soluble TNF (sTNF) (reviewed in (81)). The relative roles of memTNF and sTNF in immunity have recently been addressed using a gene knock-in strategy of a deletion/mutant TNFα allele to ensure

complete loss of TACE-mediated cleavage but normal cell surface expression and function of memTNF (82), (83). Mice which expressed memTNF in the absence of solTNF were protected against acute *Mycobacterium tuberculosis* infection but succumbed to late progressive infection (82). Although memTNF knock-in mice cleared low-dose *Listeria* infection and were protected against an otherwise lethal secondary challenge, higher dose of primary *Listeria* challenge led to delayed clearance of bacteria with slower T-cell accumulation and increased inflammation and mortality, indicating that solTNF is required for optimal control of a primary infection (84). Georgopoulos *et al.* showed using transgenic mice that express a T cell-targeted membrane-associated mutant human TNFα that memTNF plays a role in tissue destruction and autoimmune inflammation (85). memTNF has been directly associated with specific immune functions, including the contact-dependent lymphocyte and monocyte-mediated cell killing and the CD40 ligand-independent, T cell-mediated polyclonal B cell activation (85).

1.3.2 Co-stimulatory members of the TNFR superfamily

In the past few years, a number of TNF/TNFR superfamily members have been shown to play co-stimulatory roles in T cell responses: OX40/OX40 ligand (OX40L), 4-1BB/4-1BBL, CD27/CD70, CD30/CD30L and HVEM (herpes-virus entry mediator)/LIGHT (86, 87). The following serves as summary highlighting key features of this superfamily. TNFRs are type I transmembrane proteins comprised of extracellular cysteine-rich domains, which typically consist of pseudo-repeats of six cysteines that form three disulphide bridges (88). Croft (2003) suggests that the different TNFR

molecules may have arisen through gene-duplication events, as indicated by the close proximity of the genes that encode OX40, 4-1BB, CD30 and HVEM (86). Studies using gene-targeted murine models, as well as monoclonal antibodies with modulatory activity, have established their role in co-stimulating discrete aspects of the T cell response, including priming of naive T cells and the generation and maintenance of a robust memory cell population (86).

1.3.3 Expression and function of TNFR superfamily in T cells

Examination of TNFR superfamily member expression reveals important insight into their relative functions within the T cell-APC interface. On naive T cells, TNFR family members are either expressed at constitutive levels or are induced, reflecting temporal usage and discrete roles in the differentiation program of the T cell response. Based on *in vitro* data, CD27 (89) and HVEM (90) are found at low-to-medium levels on the T cell surface, whereas OX40 and 4-1BB are induced with peak expression 2-4 days into the T cell response (91, 92). During the course of activation, TCR signals are sufficient for expression of the inducible TNFRs. However, CD28/B7 interactions augment the kinetics and levels of expression of OX40 and 4-1BB (92-94), suggesting an interesting link between the two major co-stimulatory families.

The other side to the T cell-APC interaction is the expression of co-stimulatory TNF superfamily ligands, which in keeping with the danger signal model of self-nonself discrimination, are induced after APC activation (95, 96). OX40L, 4-1BBL, CD70 and CD30L are not constitutively expressed by resting or immature APCs but are induced 24 hrs to several days after activation, largely coinciding with the peak level of expression of

their receptors on T cells (95, 96) . CD70 is mainly expressed by B cells (97), whereas OX40L, 4-1BBL and CD30L are mainly expressed by a broad range of professional APCs. From a temporal perspective, a cascade or rounds of co-stimulation may be mediated depending on timing of peak expression: the constitutive expression of HVEM by naive T cells and LIGHT by immature dendritic cells indicates a role in the early activation of T cells and APCs; CD27 is also constitutively expressed but peaks after T cell activation, suggesting that this interaction might indicate a second round of co-stimulation during clonal expansion. OX40, 4-1BB and CD30, and their ligands are induced in the late phase of primary expansion and so these interactions function later to sustain the response (86).

1.3.4 TNFR in co-stimulating primary T-cell responses

The roles of the co-stimulatory members of the TNFR superfamily have been the subject of intense investigations in the past few years (86, 87). Each role has been delineated temporally during primary expansion; the following will highlight CD27, OX40, and 4-1BB, as the data set is particularly strong for identifying as co-stimulatory in T cell responses.

CD27: Engaging CD27 signaling with antibody or ligand can enhance $CD4^+$ or $CD8^+$ T cell proliferation and cytokine production (98, 99). In contrast to CD28, which promotes cell division, CD27 appears to promote T cell expansion by allowing T cells to survive through successive divisions, without influencing the rate of cell division (100). *In vitro* blocking studies of CD27 have shown inhibition of T cell proliferation and cytokine secretion, suggesting a role in the early stages of T cell activation (101, 102) .

$CD27^{-/-}$ T cells undergo normal division initially but then proliferate poorly 3 or more days after activation *in vitro*, indicating an important role in initial expansion (103). Moreover, using an influenza virus infection system in combination with MHC H2-D^b tetramers loaded with the immunodominant influenza $NP_{366-374}$ peptide (which allows visualization and quantification of virus-specific $CD8^+$ T cells), Hendriks *et al.* (2000) showed that CD27-deficiency is associated with lower numbers of virus-specific $CD4^+$ and $CD8^+$ T cells at the peak of primary responses (days 8–10) and fewer memory T cells generated over 3 or more weeks (103). Temporally, because expression of CD27 is upregulated early after T-cell activation, it may function to deliver signals that maintain early proliferation, before the peak of the effector response.

OX40: Initial studies using OX40L or anti-OX40 antibody showed that OX40 signaling can enhance cytokine production and proliferation of $CD4^+$ T cells (reviewed in (104), independently of CD28 (105). Although most evidence suggests a role for OX40 primarily on CD4 T cells, agonistic anti-OX40 antibodies enhanced the expansion of both $CD4^+$ and CD8 T^+ cells *in vivo* (106, 107). OX40 appears to act later in the T cell response. Although TCR plus OX40 co-signal can induce IL-2 production, OX40 does not replace CD28 or influence the initial rate of cell division. Rather, OX40 allows for survival of greater numbers of T cells later in the response (94, 108). $OX40^{-/-}$ and $OX40L^{-/-}$ mice have reduced primary $CD4^+$ T-cell responses to several viruses, yielding lower frequencies of antigen-specific effector T cells and a smaller memory T cell pool (107, 109). In contrast to CD27-deficient T cells, $OX40^{-/-}$ CD4 T cells are unimpaired in early proliferation, but show reduced proliferation and marked apoptotic cell death 4–5 days after activation (94). Although $CD8^+$ T cell responses to viruses were unimpaired in

$OX40^{-/-}$ or $OX40L^{-/-}$ mice (109, 110), there were small defects in alloantigen or contact sensitivity–induced CD8 T cell responses in the absence of OX40L (111). However, the effects of OX40 on CD8 T cells in these studies may be due to indirect effects of OX40 on CD4 T cell help (87). Thus, OX40 plays a clear role in CD4 T cell responses *in vivo* but appears to be dispensable for CD8 T cell responses *in vivo*.

4-1BB: Initial analysis of 4-1BB in murine T cells indicated a role in augmenting T cell proliferation and both Th1 and Th2 cytokine production (reviewed in (112), thereby regulating the number of cytokine-producing cells (113, 114). Under conditions of strong TCR signaling, co-stimulation with 4-1BBL induced similar levels of IL-2 as CD28-mediated co-stimulation (115). Similar to the role of OX40 in T cell survival during primary expansion, 4-1BB engagement prevented activation-induced cell death in T cells (116). Gene targeted deletion of 4-1BBL led to markedly fewer antigen-specific $CD8^+$ T cells in primary responses and fewer memory T cells generated (117-119). Blocking 4-1BBL did not alter the initial proliferative response of $CD8^+$ T cells but suppressed the accumulation of effector CTLs at the peak of the primary response, due to a survival defect in the dividing population (120). Mice deficient in 4-1BB or 4-1BBL showed defects in $CD8^+$ T cell responses to viruses, with no observed defects in antibody or CD4 T cell responses to vesicular stomatitis virus (VSV), LCMV, or influenza virus (117, 118, 121).

Taken together, the body of evidence supports a model of co-stimulation characterized by temporally regulated functions in the T cell response. Given the early expression of CD27, its main effect may be to regulate cell division and expansion of T-cell numbers, rather than being essential for T-cell survival after this clonal expansion

phase (86). CD27 may therefore function in temporal coincidence with CD28 to provide early pro-survival signals in support of primary expansion. The studies of OX40 and 4-1BB indicate that these molecules have similar functions providing late-acting survival signals that ensure transition from effector into memory T cells. OX40 and 4-1BB appear to function later than CD28, as they are upregulated by CD28 signals. Even more telling are functional analyses of CD28-deficiency versus OX40- or 4-1BBL-deficiency, which have shown that early primary T-cell responses are markedly impaired in the absence of CD28 signals, but only late responses are impaired in the absence of OX40 or 4-1BB signals (94, 122).

1.3.5 TNFR signal transduction

The receptors of this superfamily couple directly to signaling pathways for cell proliferation, survival, and differentiation (reviewed in ref. (80). The expansion of this superfamily across the mammalian genome and many indispensable roles in mammalian biology emphasize its evolutionary success (80). The cytoplasmic domains of TNFRs function as docking sites for signaling molecules, principally through two classes of adaptor molecules: TRAFs (TNF receptor–associated factors) and death domain-containing (DD) molecules (reviewed in (123)). In mammals, at least six TRAF molecules and a number of DD molecules have evolved at locations spread through the genome (124). Co-stimulatory members of the TNFR superfamily are non-DD-containing receptors with TRAF-binding domain in their cytoplasmic portion, which function to propagate the signal downstream through intracellular effectors linked to the TRAF network of adaptors.

The precise pathways for activation of caspases, NFκB, and other cellular responses, although still not well understood, appear to involve a variety of signaling molecules such as p38, JNK, ERK, RIP, and NIK (124). Signaling studies of co-stimulatory members of the TNFR superfamily (HVEM, CD27, OX40, 4-1BB, CD30) have shown a good deal of commonality, each bearing the potential to bind TRAF2 and TRAF3 (124). As an aside, TRAFs have the potential to form heterotrimeric signaling complexes in addition to homotrimeric complexes (124), complicating our understanding of the purpose of their promiscuous binding to TNFR. However, several studies indicate that TRAF2 is crucial to at least some of the signals from the co-stimulatory TNFR-family members (86).

More recently, a pathway involving phosphatidylinositol-3-kinase (PI3K) and the serine-threonine Protein Kinase B (AKT) has been linked to co-stimulatory molecules in T cells. Kane *et al.* (2001) showed that retrovirus-mediated expression of activated AKT in primary T cells from CD28-deficient mice was capable of selectively restoring production of IL2 and IFNγ (55). Kane *et al.* also showed that AKT overexpression leads to specific NFκB induction in the Jurkat T cell line, This pathway also appears to be used by co-stimulatory members of the TNFR superfamily. Ligation of OX40 induces PI3K and the activation of AKT; $OX40^{-/-}$ T cells are defective in sustained expression of AKT, correlating with the survival defect that quantitatively limits the generation of a robust effector and memory pool (86).

With regard to T-cell survival, both OX40 and 4-1BB can upregulate the expression of the anti-apoptotic proteins BCL-X_L and BFL1, directly correlating with their suppression of apoptotic cell death (16, 83, 84). This again supports the idea that

these molecules are functionally analogous. So far, there is no information on whether CD27, HVEM or CD30 regulate proteins of the BCL2 family, although it is probable that they might also target anti-apoptotic molecules.

1.3.6 TNFR2 as a putative T cell co-stimulatory molecule

Several studies have implicated TNFα as a ligand playing a co-stimulatory role in T cell proliferation (125-127) in the context of infection by activated APCs. T cell proliferation was found to be markedly enhanced when TNFα was added exogenously to cultures stimulated by TCR cross-linking, and that this increase was comparable to the effect of adding the T cell growth factor IL-2 exogenously (65). The proliferative response of T cells to stimulation through the TCR complex was ablated when a neutralizing antibody to TNFα was added to T cell cultures, suggesting that TNFα acts in an autocrine manner as a T cell growth factor (65). Consistent with this notion, TNFα is expressed early during T cell activation (129). Taken together, this suggests that TNFα provided by activated APCs and endogenously upon T cell activation can act through a co-stimulatory pathway and may be a critical regulatory point for the progression of the T cell response.

TNFα binds to two distinct receptors on the cell surface, TNFR1 and TNFR-2 (reviewed in (130)). Studies using agonist antibodies have demonstrated that the two receptors signal distinct TNF activities (125). While TNFR1 was shown to be responsible for signaling cytotoxicity and the induction of several genes, TNFR2 was shown to be capable of signaling proliferation of primary thymocytes and a cytotoxic T cell line (126). Agonist antibodies towards TNFR2 can enhance the proliferative response of T cells to a

level observed when adding TNF-α exogenously to cell cultures, whereas specific activation of TNFR1 had no effect. More recently, Micheau and Tschopp showed that TNFR1 can signal both death and survival by using a mechanism involving two sequential signaling complexes: an initial plasma membrane bound complex (complex I) consisting of TNFR1, the adaptor TRADD, the kinase RIP1, and TRAF2 that rapidly signals activation of NF-kappa B. and a second step forming a cytoplasmic complex (complex II), wherein TRADD and RIP1 associate with FADD and caspase-8 to signal cell death (131). However, TNFR2 is the predominant TNF receptor on activated T cells (132), (133) , suggesting that the pro-survival signal potentially derived from TNFR1 is likely minimal during T cell activation. However, these studies did not address whether the TNFR2 signal is *important*, or if it is actually a redundant pathway.

Mice deficient in TNFR1 (134, 135) and TNFR2 (136) were generated to clarify their respective roles in the immune system. These studies confirmed the importance of TNFR1 for the cytotoxicity associated with TNF-α. $TNFR1^{-/-}$ T cells did not exhibit a defect in proliferative response to mitogens such as Concanavalin A (ConA) nor agonist antibody specific for the TCR complex (137). This was consistent with the observations that agonists specific for TNFR1 did not affect the proliferative response to TCR agonists. Functional analysis of $TNFR2^{-/-}$ T cells did not reveal any changes in responsiveness compared to wild-type T cells, including response to the mitogen ConA (136). However, this first report describing $TNFR2^{-/-}$ mice did not examine T cell response to specific stimulation through the TCR complex, which is the normal physiological route of T cell activation. Therefore, a potentially critical co-stimulatory

pathway has been neglected, and the elucidation of the mechanisms that lead to a functional T cell response is incomplete.

1.3.7 Summary of thesis

The goal of this research was to investigate the functional role of TNFα/TNFR2 interactions for T cell responses to gain a clearer understanding of the factors that control T cell activation threshold, differentiation into cytokine-producing effector cells, and the generation of memory T cells. The objective was to examine the putative co-stimulatory function of TNFR2 by assaying T cells from TNFR2$^{-/-}$ mice using well-established *in vitro* stimulation systems and TCR transgenic model systems coupled with an *in vivo* cognate Ag-expression system. TNFα and its receptors are an important cytokine/receptor system in the innate and adaptive arms of the immune system for controlling differentiation, survival and death. Most of the work on this pleiotropic cytokine focused on its role as an effector cytokine promoting innate pro-inflammatory responses against pathogen. The first published report describing the TNFR2 knockout mice did not identify an important role for TNFα/TNFR2 interactions in T cell responses, despite earlier work showing: (1) TNFR2 is expressed on activated T cells, (2) TNFR2 plays an important role in TCR-agonist induced T cell proliferation as agonistic Abs against TNFR2 promotes proliferation and antagonistic Abs against TNFR2 impairs the proliferative response, and (3) TNFα plays an important role in TCR-agonist induced T cell proliferation as exogenous addition of the cytokine augments proliferation and neutralizing antibodies against TNFα abolishes the proliferative response. The hypothesis tested in this research was that TNFR2 functions as a co-stimulator of T cell responses in

promoting IL-2 production, lowering the threshold of T cell activation, and promoting T cell clonal expansion.

Chapter 1 describes *in vitro* experiments showing that T cell activation requires a threshold amount of T cell receptor (TCR)-mediated signals, an amount that is reduced by signals mediated through TNFR2-mediated co-stimulation in both naive $CD4^+$ and $CD8^+$ T cell subsets. TNFR2 deficiency in $CD8^+$ T cells increased the requirements for TCR agonist approximately 5-fold. This resulted in a marked reduction in the accumulation of effector $TNFR2^{-/-}$ T cells in cultures stimulated with TCR agonist. This hypo-proliferative response was associated with delayed kinetics of induction of the acute activation markers CD25 and CD69, as well as a marked decrease in the production of IL-2 and IFN-γ. The net result was that very few cells were recruited into the dividing population. Interestingly, CD28 co-stimulation was only partially effective in rescuing the proliferative defect of $TNFR2^{-/-}$ $CD8^+$ T cells. Thus TNFR2 provides an important co-stimulatory signal in addition to that provided by CD28 towards optimal T cell proliferation and IL-2 production. This chapter describes experiments showing that TNFR2 lowers the threshold of T cell activation, promotes IL-2 production, and induces clonal expansion of effector $CD8^+$ T cells. It also indirectly implicates the autocrine consumption of TNFα during T cell activation as an important checkpoint for inducing clonal expansion and IL-2 production.

Chapter 2 continues the *in vitro* examination of TCR and TNFR2-mediated signals that promote IL-2 production and identifies a functional link between TNFR2 and CD28 at a critical checkpoint of T cell activation: sustaining AKT and NFκB activation after short duration of TCR/CD28-mediated stimulation. TNFR2-deficient T cells possessed a

profound defect in IL-2 production in response to TCR/CD28-mediated stimulation, an important finding given the major role previously attributed to CD28 for promoting IL-2 production. Examination of key signaling intermediates revealed that TCR-proximal events such as global tyrosine phosphorylation and ZAP-70 phosphorylation, as well as downstream MAPK cascades are unperturbed in TNFR2-deficient T cells. In contrast, TNFR2 is non-redundantly coupled to sustained AKT activity and NFκB activation in response to TCR/CD28-mediated stimulation. Moreover, TNFR2-deficient T cells exhibit a defect in survival during the early phase of T cell activation that is correlated with a striking defect in Bcl-X_L expression. These reveal the synergistic requirement of TCR, CD28, and TNFR2 towards optimal IL-2 induction and T cell survival. It is proposed that TNFR2 acts as one of the earliest co-stimulatory members of the TNFR family, and is functionally linked to CD28 for initiating and determining T cell fate during activation.

Chapter 3 describes the utilization of MHC class I- and II-restricted TCR transgenic model systems in combination with an *in vivo* cognate Ag-expression system to assay for the role of TNFR2-mediated co-stimulation in Ag-driven T cell responses. Experiments described in this chapter showed that $CD4^+$ and $CD8^+$ T cells depended on TNFR2 for survival during clonal expansion, allowed larger accumulation of effector cells and conferred protection from apoptosis for a robust memory pool *in vivo*. Using the MHC class I-restricted 2C TCR and MHC class II-restricted AND TCR transgenic systems, it was shown that TNFR2 regulates threshold for clonal expansion of $CD4^+$ and $CD8^+$ T cell subsets in response to cognate antigen. Using a novel recombinant *Listeria monocytogenes* (rLM-SIY) expressing a secreted form of the 2C agonist peptide (SIY) to investigate the role of TNFR2 for T cell immunity *in vivo*, TNFR2 was shown to control

the survival and accumulation of effector cells during the primary response. TNFR2$^{-/-}$ CD8 T cells exhibit loss of protection from apoptosis that is correlated with diminished survivin and Bcl-2 expression. Null mutant mice were more susceptible to rLM-SIY challenge at high doses of primary infection, correlating with impaired LM-specific T cell response in the absence of TNFR2-mediated co-stimulation. Moreover, the resulting memory pools specific for SIY- and listeriolysin (LLO)-epitopes derived from rLM-SIY were diminished in TNFR2$^{-/-}$ mice. Thus, examination of antigen-driven T cell responses revealed a hitherto unknown co-stimulatory function for TNFR2 in regulating T cell survival during the differentiation program elicited by intracellular pathogen *in vivo*.

Overall, this research demonstrated the critical role of TNFR2 in regulating the threshold of T cell activation, and that clonal expansion depended on TNFR2 particularly at lower Ag doses. Importantly, TNFR2 functions in both CD4^{+} and CD8^{+} T cell subsets within the first 10 hrs of TCR/CD28-mediated stimulation to sustain AKT and NFκB activation and promote Bcl-X_L expression, which correlates with the non-redundant role of TNFR2 in promoting IL-2 production and T cell survival. It also indicates an unappreciated role for autocrine consumption of TNFα by activated T cells for clonal expansion and survival. TNFR2 controls the clonal expansion of Ag-specific effector T cells and generation of a robust memory pool during the adaptive immune response against intracellular pathogen by inducing the expression of anti-apoptotic molecules such as Bcl-2 and survivin. The early role for TNFR2-mediated co-stimulation of T cell responses indicates that this receptor acts upstream of other members of the TNFR family such as OX-40 and 4-1BB. This research demonstrates the exciting potential of targeting TNFR2 in ameliorating T cell-mediated diseases.

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Chapter 2 Tumor necrosis factor receptor-2 (p75) lowers the threshold of T cell activation[1]

2.1 Preface

T cell activation requires a threshold amount of T cell receptor (TCR)-mediated signals, an amount that is reduced by signals mediated through co-stimulatory molecules expressed on the T cell surface. This chapter examines the role of TNFR2 as a putative co-stimulatory receptor for T cell activation in response to TCR cross-linking stimulation *in vitro*. TNFR2 deficiency in $CD8^+$ T cells increased the requirements for TCR agonist-induced proliferation approximately 5-fold. $TNFR2^{-/-}$ T cells displayed a marked reduction in the proliferative response to TCR agonist, delayed kinetics of induction of the acute activation markers CD25 and CD69, and a marked decrease in the production of IL-2 and IFN-γ. Addition of exogenous IL-2 to T cell cultures rescued the proliferative response of $TNFR2^{-/-}$ T cells. Interestingly, CD28 co-ligation was only partially effective in rescuing the proliferative defect of $TNFR2^{-/-}$ $CD8^+$ T cells. Thus TNFR2 provides an important co-stimulatory signal in addition to that provided by CD28 towards optimal T cell proliferation and IL-2 production. This chapter describes experiments showing that TNFR2 lowers the threshold of T cell activation, promotes IL-2 production, and induces clonal expansion of effector $CD8^+$ T cells.

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2.2 Introduction

T cells occupy a central role in the initiation and regulation of the adaptive immune response (reviewed in ref. 1). T cell activation is tightly regulated, involving coordinated integration of multiple signals to ensure antigen-specific clonal expansion and differentiation (1). Although the T cell receptor (TCR) plays a major role in defining the fine specificity of an immune response, additional mechanisms have evolved to ensure self-tolerance (2). The general requirement of co-stimulatory signals for the optimal activation of T cells, in addition to the antigen-specific signal delivered through the TCR, is a major means by which self-tolerance is achieved. The antigen-specific signal is integrated with co-stimulatory signals, and the convergence of positive signals pushes the T cell towards activation (3). Such mechanisms serve to protect against untoward autoimmune reactions and to focus the immune response on the infected target tissue (2). Clearly, the elucidation of the multiple mechanisms that regulate T cell behavior is crucial towards elucidation of their potential role in disease processes, such as immunodeficiency and autoimmunity.

In the context of infection, a major co-stimulatory pathway involves the CD28 molecule on the T cell surface (reviewed in ref. 1). Antigen presenting cells (APCs) express CD28-ligand (B7) upon activation by foreign pathogen (4). Infectious agents are processed into antigenic peptides, and APCs thus display both antigen and co-stimulatory ligands to antigen-specific T cells (4). Therefore, the induction of B7 on the surface of the APC alerts antigen-specific T cells of the presence of infectious agents. These signals are integrated within the responding T cell, and in effect, co-stimulation serves to lower the threshold amount of signals required through the TCR complex for optimal T cell activation (5). Conversely, in the absence of infection, APCs do not upregulate co-stimulatory molecules,

and T cells normally remain functionally inactive or are tolerized upon contact with such an APC (1). A corollary of this model is that without co-stimulatory signals, the signals mediated through the TCR complex are normally insufficient for mounting a functional antigen-specific T cell response.

However, studies using CD28-deficient mice have shown that co-stimulation through the CD28 pathway is not an absolute requirement for T cell activation (6-10). These studies suggest that the intensity and duration of the antigenic signal mediated through the TCR is a critical factor (5, 8, 11). Furthermore, although CD28 represents a major co-stimulatory pathway, both *in vitro* and *in vivo* studies strongly suggest that there are additional co-stimulatory pathway(s) that can lead to a functional T cell response. Indeed, infectious agents can trigger numerous co-stimulatory molecules and cytokines in addition to B7, including the pro-inflammatory cytokine TNF-α.

Several studies have implicated TNF-α in playing a co-stimulatory role in T cell proliferation (12-16). Indeed, APCs not only express CD28 ligand in response to infectious agents, but also express TNF-α when activated. Interestingly, there is a substantial increase in T cell proliferation in response to TCR agonist when TNF-α is added exogenously, comparable to that found when IL-2 is added exogenously (11). Importantly, the proliferative response of T cells to stimulation through the TCR complex is essentially abolished when a neutralizing antibody to TNF-α is added (11). In addition, TNF-α is expressed early during T cell activation (17), suggesting that it may serve as a regulatory control point. Taken together, this suggests that TNF-α, provided both exogenously upon APC activation by infectious agents and/or endogenously upon T cell activation, can act

through a co-stimulatory pathway and may be a critical regulatory point for the progression of the T cell response.

TNF-α binds to two distinct receptors on the cell surface, TNFR-1 (p55) and TNFR-2 (p75) (reviewed in ref. 18). Studies using agonist antibodies have demonstrated that the two receptors signal distinct TNF activities (12). While TNFR1 signaling was shown to be responsible for inducing cytotoxicity and the induction of several genes, TNFR2 signaling was shown to be capable of inducing proliferation of primary thymocytes and a cytotoxic T cell line (13). Agonist antibodies towards TNFR2 can enhance the proliferative response of T cells to a level observed when adding TNF-α exogenously to cell cultures, whereas specific activation of TNFR1 had no effect (15). More recently, Micheau and Tschopp showed that TNFR1 can signal both death and survival by using a mechanism involving two sequential signaling complexes: an initial plasma membrane bound complex (complex I) consisting of TNFR1, the adaptor TRADD, the kinase RIP1, and TRAF2 that rapidly signals activation of NF-kappa B. and a second step forming a cytoplasmic complex (complex II), wherein TRADD and RIP1 associate with FADD and caspase-8 to signal cell death. However, TNFR2 is the predominant TNF receptor on activated T cells, suggesting that the pro-survival signal potentially derived from TNFR1 is likely minimal during T cell activation. However, these studies do not address whether the TNFR2 signal is *important*, or if it is actually a redundant pathway.

Recently, mice deficient in TNFR1 (19, 20) and TNFR2 (21) TNFRs were generated to clarify the respective roles of these receptors in the immune system. These studies confirmed the importance of p55 for the cytotoxicity associated with TNF-α. Interestingly, $TNFR1^{-/-}$ T

cells did not exhibit a defect in proliferative response to mitogens such as concanavalin A (ConA) or TCR cross-linking (22). This is consistent with the observations that agonists specific for p55 did not affect the proliferative response to TCR agonists (15). Functional analysis of TNFR2$^{-/-}$ T cells did not reveal any changes in responsiveness, including response to the mitogen Con A (21). However, the same group that generated TNFR2$^{-/-}$ mice did not examine T cell response to specific stimulation through the TCR complex, which is the normal physiological route of T cell activation. Furthermore, no study to date had examined the importance of TNFR2 for T cell proliferation using TCR specific stimulation. Therefore, a potentially critical co-stimulatory pathway has been neglected, and the elucidation of the mechanisms that lead to a functional T cell response is incomplete.

This question was addressed in this chapter by examining the *ex vivo* response of TNFR2$^{-/-}$ T cells to specific stimulation through the TCR complex. It was determined that TNFR2$^{-/-}$ T cells display a marked reduction in this proliferative response, and that TNFR2 deficiency in CD8^{+} T cells increased the requirements for TCR agonist to generate an equivalent response to wild-type. The nature of this hypo-proliferative response was further characterized to elucidate the role of TNFR2 amongst the complex signaling pathways that are activated during the T cell response. Functional analyses revealed that TNFR2$^{-/-}$ CD8^{+} T cells produce significantly less IL-2 and IFN-γ in response to TCR agonist, and display delayed kinetics in the acquisition of activation phenotype. We propose a model in light of these data that TNFR2 contributes in a non-redundant manner to the activation of gene expression that are associated with T cell activation.

2.3 Materials and Methods

Mice

Breeders for C57BL/6 (B6, H-2^b) and B6-TNFR2$^{-/-}$ mice were obtained from The Jackson Laboratories (Bar Harbor, ME). B6 mice deficient in the TNFR2 TNF-α receptor have been previously described (21). TNFR2$^{-/-}$ mice were genotyped using a PCR strategy. Mice 4 to 7 weeks of age were used for all experiments.

Antibodies and flow cytometry

The following Abs were used: FITC-conjugated mAbs to mouse CD8 (53.67), PE-conjugated CD4 (GK1.5), and biotin-conjugated CD25 (PC61), CD44 (Pgp-1), and CD69 (H1.2F3) (PharMingen, supplied by Cedarlane, Hornby, Ontario). Cell staining and flow cytometric analysis were performed according to standard procedures. Briefly, cells were incubated with the relevant Abs for at least 15 min at 4 °C, and subsequently washed twice with FACS medium (PBS + 2% FCS). CELLQUEST (Becton Dickinson, Mountain View, CA) was used for data acquisition and analysis.

Cells

Lymph nodes were harvested and single cell suspensions prepared from each of the mouse lines. For studies of CD4$^-$ CD8$^+$ (CD8) and CD4$^+$ CD8$^-$ (CD4) T cell subsets, each of the respective populations were purified from whole lymph node cell suspensions using miniMACS microbeads (Miltenyi Biotec, Auburn, CA) and either mouse biotin-conjugated CD8β (53.58) mAb or CD4 mAb (GK1.5). The respective T cell subsets were positively selected using a MACS MS$^+$ Separation column and MiniMACS magnet, as per

manufacturer's protocol (Miltenyi Biotec), achieving > 95% purity as determined by flow cytometry. Cells were cultured at 37°C and 5% CO_2 in Iscove's DMEM (Life Technologies, Burlington, Ontario) supplemented with 10% (v/v) FBS (Life Technologies), 5 x 10^{-5} μM 2-ME, and antibiotics (I-media).

Proliferation assays and cell surface marker expression

Proliferation assays were performed by incubating 1 x 10^5 cells with varying concentrations (0 to 10 μg/ml) of plate-bound anti-CD3ε (2C11). Cells were cultured in triplicates in a volume of 0.2 ml in flat-bottom 96-well plates, and 1 μCi of [^{3}H]thymidine was added for the last 10 hrs of a 72 hr culture period. In some cultures, exogenous IL-2 was added at a concentration of 20 U/ml. Cell surface marker expression was assayed using 1 x 10^5 cells incubated in flat-bottom 96-well plates coated with 10 μg/ml of anti-CD3 for various periods of time. The activation markers CD25 and CD69 were analyzed by FACS (described above). In other cultures, 10 μg/ml of soluble anti-CD28 mAb (37.51) was included.

Carboxyfluorescein diacetate succinimidyl ester fluorescence assay

Purified $CD8^+$ T cells (1 x 10^6) were labeled with 1 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) and incubated with 10 μg/ml of plate-bound anti-CD3 in a flat-bottom 24-well plate for various periods of time. Cells were harvested and stained with the indicated Abs or with 7-amino actinomycin D (7-AAD), and subsequently analyzed by FACS (described above).

7-AAD assay

$CD8^+$ T cells (1×10^6) were incubated with 10 μg/ml of plate-bound anti-CD3 in a flat-bottom 24-well plate for various periods of time. Cells were harvested and stained with 7-AAD (10 μg/ml in FACS medium), fixed with 4% paraformaldehyde, and subsequently analyzed by FACS (described above).

Cytokine ELISA

$CD8^+$ T cells (2×10^6) were cultured in 1 ml of I-media in a flat-bottom 24-well plate coated with 10 μg/ml of anti-CD3 for 20 hrs. The amount of IL-2 and IFN-γ secreted into the supernatant was determined by ELISA. The capture and detection Abs used for IL-2 were JES6-1A12 and JES6-5H4, respectively (obtained from PharMingen). The capture and detection Abs used for IFN-γ were R4-6A2 and XMG1.2, respectively (PharMingen). Briefly, plates were coated with the capture Ab (4 μg/ml in carbonate buffer) and blocked with 1% BSA/ 0.1% azide in PBS. Wells were washed with PBS-Tween 20 and samples were then added in three dilutions (1:2, 1:5, 1:10), with each plate containing wells for standard. The wells were washed and the detection Ab (1 μg/ml in 1% BSA/ 0.1% azide in PBS) was added. The wells were then washed and streptavidin-alkaline phosphatase (PharMingen) was added (1/2000 in 1% BSA/ 0.1% azide in PBS). After washing the wells, substrate (Sigma 104) was added and plates were subsequently analyzed with an ELISA plate reader at 405 nm.

Intracellular cytokine assay

LN cells (1 x 10^6) were incubated with 10 μg/ml of plate-bound anti-CD3 for 36 hrs, with the last 6 hrs in the presence of Golgi Stop (PharMingen) and subsequently stained for intracellular cytokine expression, as described previously (23). Briefly, cells were harvested and stained with anti-CD8PE and anti-CD4Tricolor. Cells were then fixed with 2% paraformaldehyde and permeabilized with 0.3% (w/v) saponin in FACS medium. Anti-cytokine Abs used: for IL-2, FITC-anti-mouse IL-2 (JES6-5H4); for IFN-γ, PE-anti-mouse IFN-γ (XMG1.2). Cells were washed with permeabilization buffer and analyzed by FACS.

Competitive and quantitative RT-PCR for cytokine mRNA levels

Competitive and quantitative polymerase chain reaction (CQ-PCR) was used to determine the intracellular levels of IL-2 and TNF mRNA. T cells (2 x 10^6) were cultured in 1 ml of I-media in flat-bottom 24-well plates coated with 5 μg/ml of anti-CD3 for 9 hrs. Cells were then harvested and total RNA was prepared according to the manufacturer's recommendations using the RNeasy Mini Kit (Qiagen). cDNAs were generated from the total RNA preparation as previously described (24). CQ-PCR was then performed as previously described (24). Briefly, the amount of cDNA was normalized between TNFR2$^{-/-}$ and wild-type T cells using the house-keeping gene HPRT. The linearized pPQRS plasmid was used as the competitor (gift from Richard Locksley, University of California San Francisco). The sequences for the 5' and 3' oligonucleotide primers used for IL-2 were, respectively:

5' CCACTTCAAGCTCTACAGCGGAAG 3' and 5' GAGTCAAATCCAGAACATGCCGCA 3'. The sequences for the 5' and 3' oligonucleotide primers used for TNF-α were, respectively: 5'GTTCTATGGCCCAGACCCTCACAC 3' and 5' TCCCAGGTATATGGGTTCATACCAAG 3'.

The sequences for the 5' and 3' oligonucleotide primers used for HPRT were, respectively: 5' GTTGGATACAGGCCAGACTTTGTTG 3' and 5' GAGGGTAGGCTGGCCTATAGGCT 3'. The PCR products were subjected to electrophoresis on a 2% agarose gel, and visualized with ethidium bromide. Densitometry was performed using the AlphaImager software.

2.4 Results

Proliferative response to TCR cross-linking is reduced in lymph node T cells from TNFR2$^{-/-}$ mice

To determine whether TNFR2 plays a role in the proliferative response of T cells to TCR cross-linking, lymph node cells from B6 and B6-TNFR2$^{-/-}$ mice were stimulated with immobilized anti-CD3ε mAb (2C11) to induce proliferation. Immobilized Ab mimics the MHC:peptide arrays on the surface of an APC. The proliferative response of TNFR2$^{-/-}$ lymph node T cells was significantly reduced (Fig. 2.1*A*). We next investigated whether exogenous IL-2 could rescue the hypo-proliferative response displayed by TNFR2$^{-/-}$ T cells. As shown in Fig. 2.1*B*, the addition of exogenous IL-2 was able to rescue the proliferative response of TNFR2$^{-/-}$ T cells to TCR cross-linking. This suggests that TNFR2$^{-/-}$ T cells are able to respond to IL-2, and that the reduction in the proliferative response to TCR cross-linking may be due, at least in part, to a lack of IL-2 production. The hypo-proliferative response of TNFR2$^{-/-}$ T cells is not due to defects in TCR expression since these cells express similar levels as wild-type (data not shown). The TNFR2$^{-/-}$ and wild type T cells

used in these studies were similar in cell surface expression of activation/memory markers (i.e. CD25, CD69, CD44) (data not shown). Furthermore, a similar proliferative defect in response to TCR cross-linking was observed in TNFR2$^{-/-}$ T cells even after depletion of CD44-positive cells (data not shown). Thus, naive T cells lacking TNFR2 expression are hypo-proliferative in response to TCR cross-linking. This proliferative defect was not observed for TNFR1$^{-/-}$ T cells (data not shown), demonstrating that the biological effect of TNF on T cell proliferation is restricted to TNFR2.

TNFR2 lowers the activation threshold for proliferation by CD4^{+} and CD8^{+} T cells

To examine whether TNFR2 modulates the threshold of T cell activation for proliferation, the proliferative response of purified TNFR2$^{-/-}$ CD8^{+} T cells to varying doses of anti-CD3 was determined. As shown in Fig. 2.2*A*, CD8^{+} T cells deficient in TNFR2 required approximately five-fold greater anti-CD3 stimulation to mount a response equivalent to wild-type CD8^{+} T cells. This result suggests that TNFR2 decreases the threshold of activation for proliferation by lowering the requirement for signals derived from the TCR. Similarly, the proliferative response of CD4^{+} T cells deficient in TNFR2 was also affected, indicating that TNFR2 plays an important role for both T cell subsets (Fig. 2.2*B*). To determine whether exogenous IL-2 could rescue the hypo-proliferative response of TNFR2$^{-/-}$ T cells, the proliferation assays were performed in cultures supplied with exogenous IL-2. As shown in Figs. 2.2*A* and 2.2*B*, exogenous IL-2 markedly enhanced the proliferative potential for both cell types. At lower doses of anti-CD3, exogenous IL-2 was able to rescue the proliferative response of TNFR2$^{-/-}$ CD4^{+} and CD8^{+} T cells. Since IL-2 is a critical growth factor for T cell proliferation, this observation is consistent with the hypothesis that

the induction of IL-2 production (or secretion) in response to TCR cross-linking is impaired in T cells deficient in TNFR2.

We noted in Fig. 2.2*A* that at higher doses of anti-CD3 and in the presence of exogenous IL-2, TNFR2$^{-/-}$ CD8^{+} T cells displayed a greater proliferative response than wild-type CD8^{+} T cells. Wild-type CD8^{+} T cells undergo activation-induced cell death (AICD) as a means of limiting the extent of expansion in an auto-regulatory manner (25). We have previously shown that TNFR2$^{-/-}$ CD8^{+} T cells are highly resistant to Fas-mediated AICD (26). To determine whether this increase in proliferative response could be due to the resistance of TNFR2$^{-/-}$ CD8^{+} T cells to AICD, CD8^{+} T cells were stimulated with anti-CD3 in the presence of exogenous IL-2 and subsequently stained with 7-AAD as a means of determining the percentages of viable cells. As shown in Fig. 2.3*A*, cultures of TNFR2$^{-/-}$ CD8^{+} T cells contained more live cells (74 % 7-AAD negative) than wild-type (19% 7-AAD negative). The TNFR2$^{-/-}$ population also contains fewer early apoptotic cells (1%) compared to the wild-type population (19%). This observation is consistent with the hypothesis that the increase in the proliferative response of TNFR2$^{-/-}$ CD8^{+} T cells compared to wild-type when activated with high concentrations of anti-CD3 and exogenous IL-2 is due to their resistance to AICD. By contrast, the proliferative response of TNFR2$^{-/-}$ CD4^{+} T cells is fairly similar to that of wild-type CD4^{+} T cells at all concentrations of anti-CD3 tested in the presence of exogenous IL-2. We have previously found that in contrast to CD8^{+} T cells, TNFR2$^{-/-}$ CD4^{+} T cells were similar in sensitivity to Fas-mediated AICD when compared to wild-type CD4^{+} T cells (26). Staining of the TNFR2$^{-/-}$ CD4^{+} T cell culture with 7-AAD indicated that there were only minor differences in the proportion of live and early apoptotic cells in this culture compared to wild type (Fig. 2.3*B*). This observation provides additional

support to the hypothesis that activated TNFR2$^{-/-}$ CD4^{+} and CD8^{+} T cells differ in their susceptibility to AICD and that TNFR2 has distinct functions in CD4^{+} and CD8^{+} T cells.

The hypo-proliferative response of TNFR2$^{-/-}$ T cells was further investigated by examining their susceptibility to apoptosis when stimulated with TCR agonist alone. The number of viable cells recovered after 72 hrs of anti-CD3 stimulation was much lower for TNFR2$^{-/-}$ CD8^{+} T cell cultures (Fig. 2.4*A*), consistent with the proliferation data (Fig. 2.2*A*). Staining of the cultures with 7-AAD after 48 h of anti-CD3 stimulation revealed that a greater percentage of cells were viable (42% 7-AAD negative) in the wild-type cultures compared to those of TNFR2$^{-/-}$ T cells (19% 7-AAD negative) (Fig. 2.4*B*). This indicates that TNFR2$^{-/-}$ T cells stimulated with anti-CD3 without exogenous IL-2 are more susceptible to apoptosis than wild-type cells.

TNFR2$^{-/-}$ CD8^{+} T cells display a marked reduction in the dividing population in response to TCR cross-linking

CFSE is a dye that binds irreversibly to cellular proteins, and permits analyses of the parameters and kinetics of cell division events (27). We used this technique to determine the number of cellular divisions by TNFR2$^{-/-}$ and wild type CD8^{+} T cells that have been activated by TCR cross-linking. As shown in Fig. 2.5, there was a marked reduction in the proportion of TNFR2$^{-/-}$ CD8^{+} T cells that have divided after 60 hrs of anti-CD3 stimulation. Wild-type CD8^{+} T cells underwent considerable division, as nearly all cells had decreased CFSE fluorescence relative to unstimulated cells that remained CFSE^{hi}. This indicates that the hypo-proliferative response of TNFR2$^{-/-}$ CD8^{+} T cells in the absence of exogenous IL-2 is due to a marked reduction in the number of cells that have undergone division.

CFSE-labeled cells were also stimulated in the presence of exogenous IL-2 to study whether it could rescue the hypo-proliferative response observed for TNFR2$^{-/-}$ CD8^{+} T cells. As expected, cultures that were stimulated in the presence of exogenous IL-2 had undergone a greater number of divisions than in the absence of exogenous IL-2, as reflected in the relative decrease in CFSE fluorescence. Consistent with the results from the [^{3}H]thymidine incorporation assay (Fig. 2.2), TNFR2$^{-/-}$ CD8^{+} T cells stimulated in the presence of IL-2 were able to divide to at least the same extent as wild-type. In addition, essentially all of the TNFR2$^{-/-}$ CD8^{+} T cells that were stimulated in the presence of exogenous IL-2 had undergone division. Together, these results demonstrate that TNFR2$^{-/-}$ CD8^{+} T cells are able to use IL-2 when supplied exogenously, and that the induction of this critical growth factor in response to TCR cross-linking may be limited in TNFR2-deficient T cells.

CD28 co-stimulation partially rescues the hypo-proliferative response of TNFR2$^{-/-}$ CD8^{+} T cells

To examine whether CD28 co-stimulation could rescue the hypo-proliferative response by TNFR2-/- CD8+ T cells, the cells were stimulated with anti-CD28 in addition to anti-CD3. As expected, CD28 co-stimulation increased the proliferative potential of anti-CD3 stimulated wild-type CD8+ T cells (Fig. 2.6). This is in agreement with the well-documented decrease in the threshold of activation that CD28 co-stimulation mediates (5). CD28 co-stimulation caused a partial increase in proliferative potential by TNFR2-/- CD8+ T cells, but was only restored to the level of wild-type T cells stimulated with anti-CD3 alone. This indicates that TNFR2 plays an important co-stimulatory role for T cell activation that is distinct from CD28 co-stimulation.

TNFR2$^{-/-}$ CD8^{+} T cells display a reduction in activation phenotype in response to TCR cross-linking

Acute activation markers such as CD25 (IL2Rα) and CD69 are expressed at the cell surface during T cell activation and serve as a measure of the frequency of activated cells. Given that T cells deficient in TNFR2 proliferated poorly in response to TCR cross-linking, it was of interest to determine whether these cells also display a defect in acquiring an activated phenotype. The cell surface expression of CD25 in response to TCR cross-linking was important to study since it is the critical component of the high-affinity IL-2R. As shown in Fig. 2.7, the cell surface expression of CD25 was similar between the two cell types at the earliest time point tested (3 h). However, at 8 h of anti-CD3 stimulation the percentage of cells that were CD25^{+} was reduced for TNFR2$^{-/-}$ CD8^{+} T cells compared to wild-type (50% vs. 84%, respectively). Since CD25 expression is essential for optimal IL-2 signaling, the marked reduction in the proportion of cells that are CD25^{+} for TNFR2$^{-/-}$ CD8^{+} T cells may be an important factor that contributes to the marked reduction in the proportion of cells that divide in response to TCR cross-linking.

CD69 is an early activation marker of T cells. The pattern of induction of CD69 for TNFR2$^{-/-}$ CD8^{+} T cells stimulated with anti-CD3 closely parallels that of CD25 (Fig. 2.7). With greater duration of TCR-mediated stimulation, there is greater recruitment of TNFR2$^{-/-}$ CD8^{+} T cells into the CD69^{hi} population, demonstrating the increased requirement in the amount of TCR-mediated signals to generate a particular response for the TNFR2$^{-/-}$ T cell population compared to wild-type.

The forward light scatter (FSC) pattern was also examined in this kinetic study to assess blastogenesis in response to TCR cross-linking. As both the kinetics of induction of CD25

and CD69 indicate that TNFR2$^{-/-}$ CD8^{+} T cells are activated with delayed kinetics (due to increased requirements for TCR-mediated stimulation in the absence of TNFR2), it would be expected that FSC should also display the same pattern. Indeed, as shown in Fig. 2.7, the percentage of cells that have increased in size in response to TCR cross-linking is markedly reduced at the 20 h time point, and that there is increasing recruitment of TNFR2$^{-/-}$ CD8^{+} T cells into the FSC^{hi} population with greater duration of TCR-mediated stimulation.

IL-2 production in response to TCR cross-linking is reduced in TNFR2$^{-/-}$ T cells

Based on the observation that exogenous IL-2 was able to rescue the marked reduction of the proliferative response by TNFR2$^{-/-}$ T cells, the amount of IL-2 produced in response to TCR cross-linking was examined to assess whether this was one of the limiting factors. The amount of IL-2 that was secreted in response to TCR cross-linking was measured by ELISA performed on the culture supernatant. As shown in Fig. 2.8*A*, there was a marked reduction in the amount of IL-2 in the culture supernatant of TNFR2$^{-/-}$ CD8^{+} T cells compared to wild type at 20 h of anti-CD3 stimulation. This suggests that the amount of IL-2 secreted is limiting for the proliferative response of TNFR2$^{-/-}$ CD8^{+} T cells. Furthermore, when the culture supernatant of TNFR2$^{-/-}$ CD8^{+} T cells was assayed for IL-2 at 40 h of anti-CD3 stimulation, the amount of IL-2 was equivalent to the amount produced by wild-type cells at 20 h. This indicates that the production of IL-2 occurs in a delayed manner for TNFR2$^{-/-}$ CD8^{+} T cells, as a greater duration of TCR-mediated stimulated allows for increased accumulation of TCR-mediated signals. This is consistent with the threshold model of T cell activation, in this case for IL-2 production, such that TNFR2 lowers the requirement for TCR-mediated stimulation.

To study whether the observed reduction in IL-2 production by TNFR2$^{-/-}$ CD8^{+} T cells was paralleled by a reduction in the transcript, IL-2 mRNA was measured by competitive and quantitative reverse transcription-polymerase chain reaction (CQ-PCR). As shown in Fig. 2.8*B*, TNFR2$^{-/-}$ CD8^{+} T cells displayed a 2- to 3-fold reduction in the amount of IL-2 transcript in response to TCR cross-linking compared to wild type. This result suggests that TNFR2 provides a co-stimulatory signal that lowers the threshold of activation for IL-2 gene induction.

TNF-α induction in response to TCR cross-linking is modestly reduced in TNFR2$^{-/-}$ T cells

TNF-α is another important cytokine that is expressed during T cell activation (23). Previous studies have shown that TNF-α can enhance the proliferative response of T cells to TCR cross-linking (11). To determine whether TNFR2 is required for TNF-α expression upon TCR stimulation, the level of TNF-α mRNA was measured using CQ-PCR. It was found that TNFR2$^{-/-}$ CD8^{+} T cells displayed a modest decrease in the level of TNF-α transcript compared to wild-type (Fig. 2.9). Interestingly, this decrease was not as marked as for IL-2. This is likely due to differential requirements for the assembly and constitution of the transcription factor complexes needed for the induction of these two cytokine genes (28). Indeed, previous reports have shown that TNF-α is expressed earlier than IL-2 during T cell activation (23) suggesting that the latter cytokine possesses a higher TCR signaling threshold for its induction during T cell activation. Furthermore, TNF-α was found to be one of the first cytokines produced by T cells upon activation (23), suggesting that it may be an important early checkpoint for the progression of the T cell response.

IFN-γ expression in response to TCR cross-linking is reduced in TNFR2$^{-/-}$ T cells

A third cytokine that is transcriptionally regulated and expressed in particularly large amounts by activated CD8^{+} T cells is IFN-γ. The total amount of secreted cytokine was measured using ELISA. As shown in Fig. 2.10*A*, TNFR2$^{-/-}$ CD8^{+} T cells produced markedly less IFN-γ than wild type. To examine whether this difference was due to a reduction in the number of cells expressing IFN-γ, or to a reduction in the amount of IFN-γ produced per cell, an intracellular cytokine immunostaining assay was used. As shown in Fig. 2.10*B*, there was both a marked reduction in the number of cells expressing IFN-γ (25% for wild-type vs. 9% for TNFR2$^{-/-}$ CD8^{+} T cells), as well as a marked decrease in the amount produced per cell, as measured by mean fluorescence intensity (MFI). Thus, deficiency of TNFR2 in T cells results in the diminished recruitment of cells in the IFN-γ expressing population in response to TCR-mediated stimulation, as well as a reduction in the intracellular levels of cytokine produced per cell.

2.5 Discussion

T cell activation is a complex process and involves not only the engagement of the TCR with its antigenic ligand but also a myriad of adhesion molecules and co-stimulatory ligands that are found on APCs activated by pathogenic stimuli. Engagement of B7-1 and B7-2 on APCs by CD28 molecules on T cells provides a critical signal for cell cycle progression, IL-2 production and clonal expansion (5). However, it is becoming clear that CD28 is part of a larger family of related counter-receptors that regulate the T cell response. These additional molecules play an important role, together with CD28, to regulate the acquisition of effector function and/or induction of tolerance in T cells (14). In this study we examined the importance of the TNFR2 TNF-α receptor (TNFR-2) for T cell activation and more broadly assessed the role that TNF-α plays as an environmental cue towards eliciting an efficient T cell response.

Previous reports have shown that TNF-α can act through TNFR2 to enhance the proliferative response of T cells to TCR agonists (11, 15, 16). However, the mechanism by which TNFR2 elicits this enhancement has not been determined. Moreover, it is not clear whether TNFR2 performs an essential or redundant function in T cells. We addressed these questions by investigating T cells from $TNFR2^{-/-}$ mice. It was determined that TNFR2 performs an important co-stimulatory function that effectively lowers the threshold of activation, and thus lowers the requirement for TCR agonist to produce a given proliferative response. This conclusion is partly based on the observation that a much higher concentration of anti-CD3 was required by $TNFR2^{-/-}$ T cells to achieve the same level of proliferation as wild-type T cells.

We investigated the nature of the hypo-proliferative response of TNFR2$^{-/-}$ T cells. Since T cell proliferation is particularly dependent on signals mediated through the high-affinity IL-2R, we assessed the kinetics of expression of CD25, a component of the high affinity IL-2R and the amount of IL-2 produced in response to TCR cross-linking. It was found that TNFR2$^{-/-}$ CD8^{+} T cells showed delayed kinetics in the recruitment of cells to the CD25^{hi} population. This observation can be interpreted according to a threshold model for T cell activation. In the absence of TNFR2, greater amounts of TCR-mediated signals are required to elicit a particular response. With longer incubation times with TCR agonist, there would be a net accumulation and increase in the amount of TCR-mediated signals. Thus, over time there is an increase in the proportion of cells that are CD25^{+}. For wild-type CD8^{+} T cells, this activation threshold is achieved with much shorter incubation times with anti-CD3 antibodies compared to TNFR2$^{-/-}$ CD8^{+} T cells. This demonstrates the co-stimulatory importance of TNFR2 in that much less stimulation is required to reach the activation threshold for the induction of CD25 in the wild-type CD8^{+} population.

Interestingly, the response by TNFR2$^{-/-}$ CD8^{+} T cells to TCR cross-linking seems to display a bimodal distribution (Fig. 2.7), giving rise to two apparent populations: those that are CD25^{+} and the others that are CD25^{neg}. The observation that a proportion of TNFR2$^{-/-}$ CD8^{+} T cells were CD25^{+} at the 8 h time point may be due to a stochastic phenomenon in which the amount of TCR-mediated signals received permits a particular frequency of CD25^{+} cells, similar to the stochastic pattern of effector responses described by Keslo *et al.* (29). An alternative explanation is that there exists a subpopulation of TNFR2$^{-/-}$ CD8^{+} T cells that readily upregulate CD25 and another subpopulation that requires a greater amount

of TCR-mediated signal. It is of interest to determine whether subpopulations of $CD8^+$ T cells have an intrinsically lower threshold of activation.

The threshold model for a particular T cell response can be applied to the induction of cytokines. As Itoh and Germain (28) pointed out, signaling thresholds exist for elicitation of a particular cytokine response, and signals that rise above this point lead to an increase in the overall amount of cytokine produced by a cell population (28). Three cytokines that are expressed during T cell activation are TNF-α, IL-2, and IFN-γ. Consistent with the hierarchal organization of TCR signaling thresholds proposed by Itoh and Germain (29), we observed that the induction of TNF-α was similar between $TNFR2^{-/-}$ and wild-type $CD8^+$ T cells, whereas there was a marked decrease in the induction of IL-2 and IFN-γ for $TNFR2^{-/-}$ T cells. This suggests that the TCR signaling threshold for TNF-α gene expression is lower than for the other two cytokines studied. This is also consistent with previous studies on the kinetics of cytokine induction and which demonstrate that TNF-α is expressed earlier than IL-2 and IFN-γ (23).

The observation that exogenous IL-2 rescued the hypo-proliferative response of $TNFR2^{-/-}$ $CD8^+$ T cells suggested that its expression might be limiting. Indeed, as determined at the level of secreted cytokine as well as IL-2 transcript, there is a marked reduction in the level of IL-2 gene induction in the absence of TNFR2. This is consistent with the threshold model of activation, in that greater amounts of TCR-mediated signals is required to reach the threshold for $TNFR2^{-/-}$ $CD8^+$ T cells. Furthermore, since IL-2 is a critical growth factor and important for the proliferative response, it could account, at least in part, for the reduction in proliferation observed for $TNFR2^{-/-}$ T cells.

The expression of IFN-γ followed the same pattern as IL-2, consistent with the threshold model of activation. There was a significant reduction in the recruitment of TNFR2$^{-/-}$ CD8^{+} T cells into the IFN-γ expressing population, as well as a reduction in the intracellular levels per cell. This is interpreted to mean that for wild-type CD8^{+} T cells, the amount of signals generated by the time point tested was sufficient to reach the threshold of activation. This is reflected in both the significant recruitment of wild type cells into the IFN-γ expressing population, as well as a marked increase in the intracellular levels per cell relative to TNFR2$^{-/-}$ CD8^{+} T cells.

What is the mechanism by which TNFR2 provides co-stimulation for T cell activation? A model that can account for the co-stimulatory role of TNFR2 in T cell proliferation is shown in Fig. 2.11. This model suggests that TNFR2 provides an important signal that recruits and utilizes proteins disctint from those used by the TCR, but converges at the level of NFκB and c-Jun activation. Consistent with this hypothesis, it has been shown that the TNFR2 signaling pathway leads to the activation of NFκB and c-Jun (14), both of which are critical for the induction of genes, particularly IL-2, during T cell activation. This hypothesis is supported by increasing number of reports that provide evidence that little gene transcription is detected until all the transcription factors that are necessary for forming a complete complex at the promoter are available at an adequate concentration (30). Previous reports have shown that co-stimulation of T cells mediated through CD28 arises from upregulating the activity of transcription factors that are essential for IL-2 gene induction (31). It seems likely that TNFR2 provides unique co-stimulatory signals to complement CD28 co-stimulatory signals in order to achieve maximum IL-2 production.

Many groups have investigated the signaling events that are generated specifically through the TCR complex. However, current models for T cell activation and proliferation do not implicate the TNFR2 pathway. Based on the current schematic for the TNFR2 signaling pathway, convergence with the pathways activated through the TCR occurs far downstream at the level of transcription factor activation, specifically NFκB and c-Jun (see Fig. 2.11). If TNFR2 provides a signal that is distinct and independent of that mediated through the TCR complex, then TCR-proximal signaling events should not be affected by TNFR2 deficiency. Our preliminary experiments support this conclusion. We found that the global tyrosine phosphorylation of substrates, as well as Erk1/Erk2 activation, shortly after stimulation of T cells with anti-CD3 indicate that these events were not affected by TNFR2-deficiency (data not shown). Further studies are required to elucidate the biochemical mechanism though which TNFR2 lowers the threshold of T cell activation.

2.6 Acknowledgements

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2.7 Figures

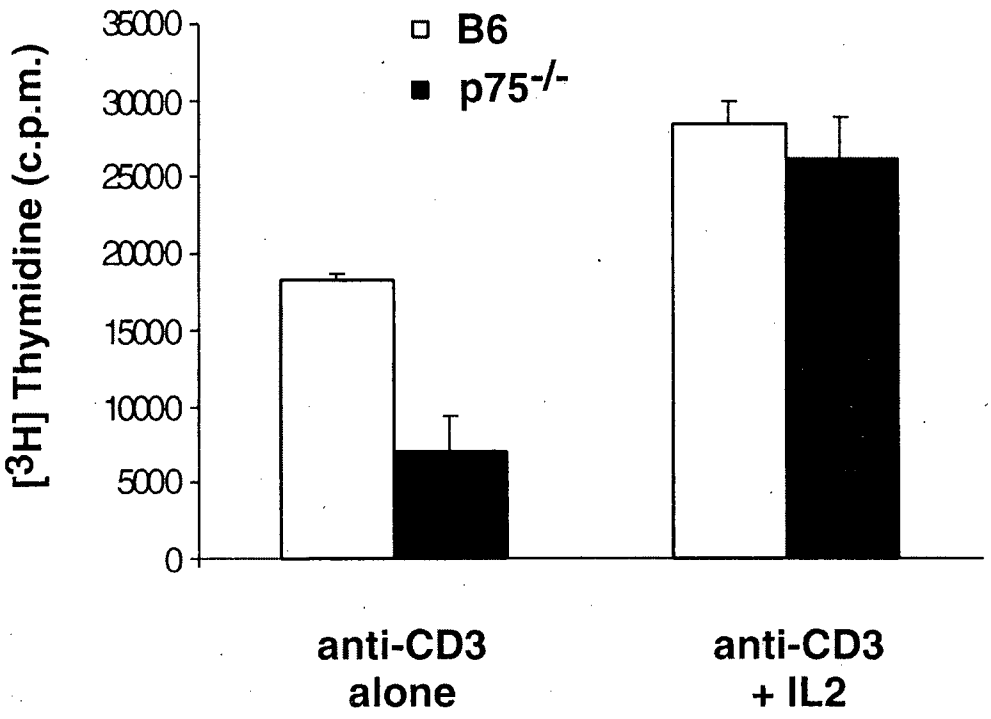

Figure 2.1 Proliferative response to TCR cross-linking is reduced in lymph node T cells from TNFR2$^{-/-}$ mice.

Lymph node cells (1 x 10^5) were stimulated with either plate-bound anti-CD3 alone, or in the presence of exogenous IL-2, for 48 h and pulsed with [^{3}H]thymidine for the last 8 h of culture. Data denotes mean ± standard deviation for triplicate determinations and is representative of three independent experiments.

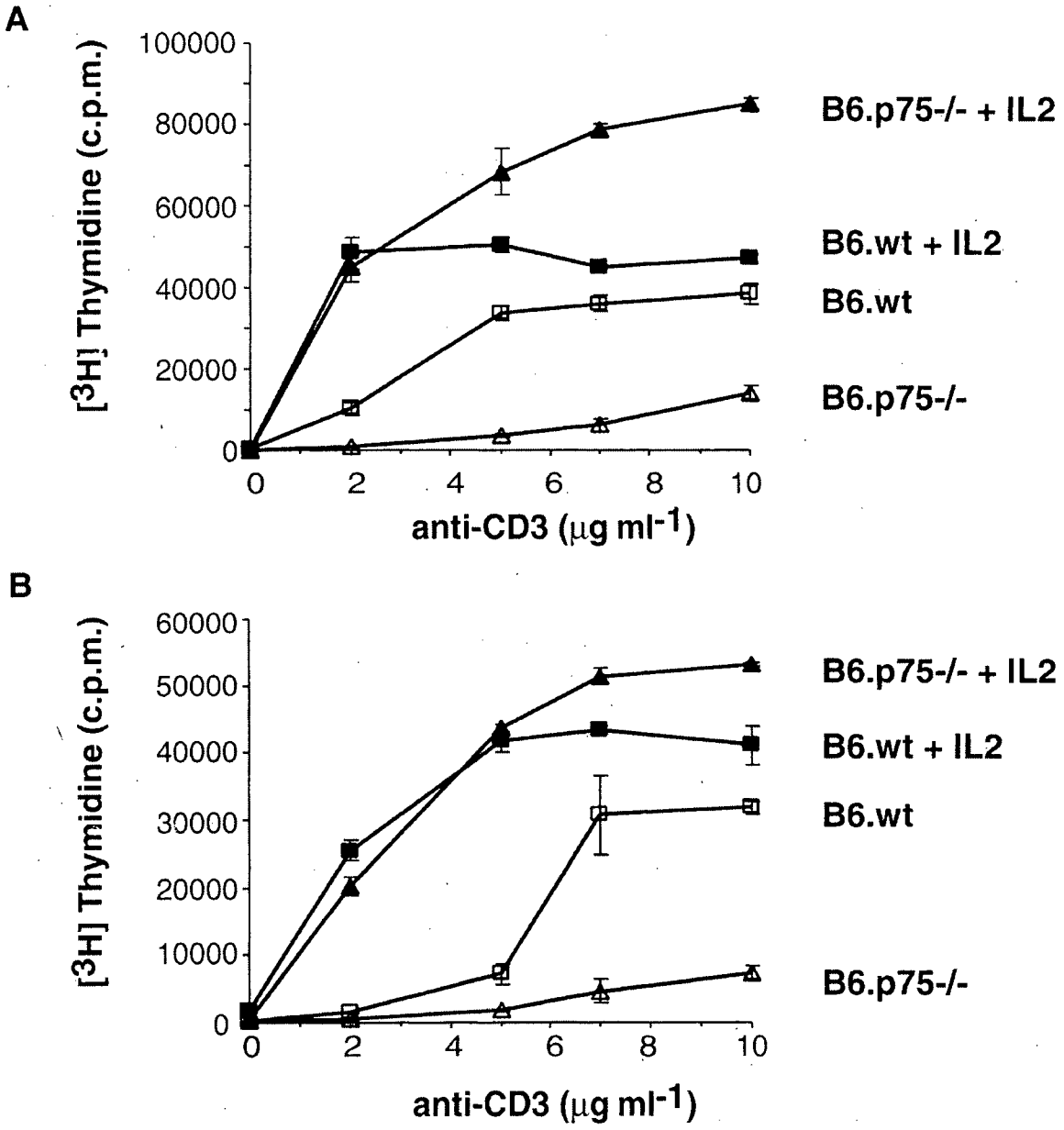

Figure 2.2 TNFR2 lowers the threshold of activation for T cell proliferation in response to TCR cross-linking.

(A) $CD8^+$ T cells (1 x 10^5) were stimulated with either plate-bound anti-CD3 alone, or in the presence of exogenous IL-2, for 72 h with [^{3}H]thymidine added for the last 10 h of culture (see Materials and Methods). (B) $CD4^+$ T cells (1 x 10^5) were stimulated with either plate-bound anti-CD3 alone, or in the presence of exogenous IL-2, for 78 h with [^{3}H]thymidine added for the last 10 h of culture. Mean ± standard deviation of triplicate determinations was used to express the data. The data were reproduced in three independent experiments.

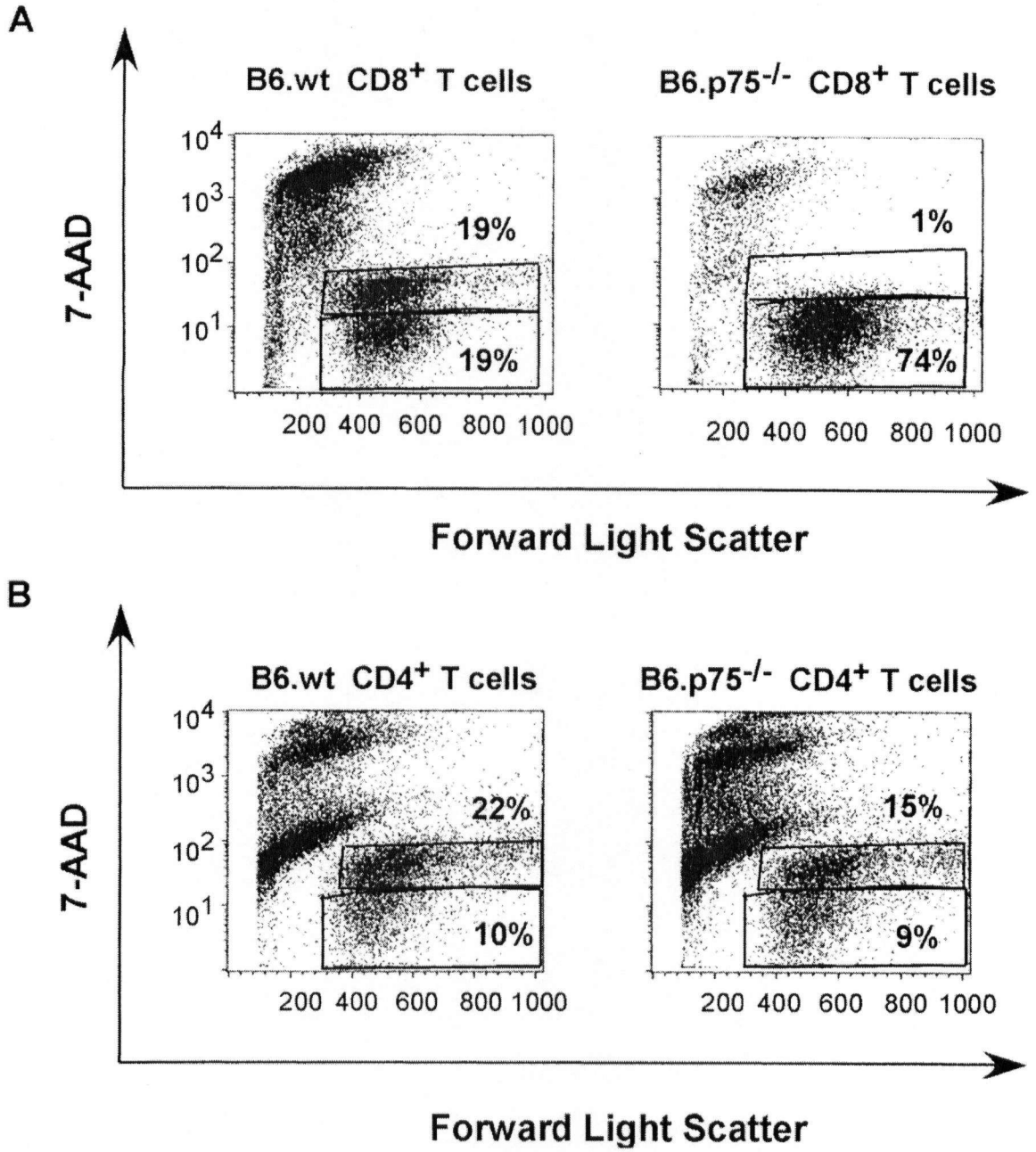

Figure 2.3 (A) $CD8^+$ T cells deficient in TNFR2 are resistant to activation-induced cell death. (B) TNFR2-deficient $CD4^+$ T cells are not resistant to activation-induced cell death.

(A) Cells were harvested at 72 h of stimulation with plate-bound anti-CD3+ exogenous IL-2, stained with 7-AAD and analyzed using FACS (see Materials and Methods). Percentage refers to 7-AAD negative cells (live cells). (B) Culture conditions are similar to (A) except CD4+ T cells were used and the cells were harvested at 78 h. The data were reproduced in three independent experiments.

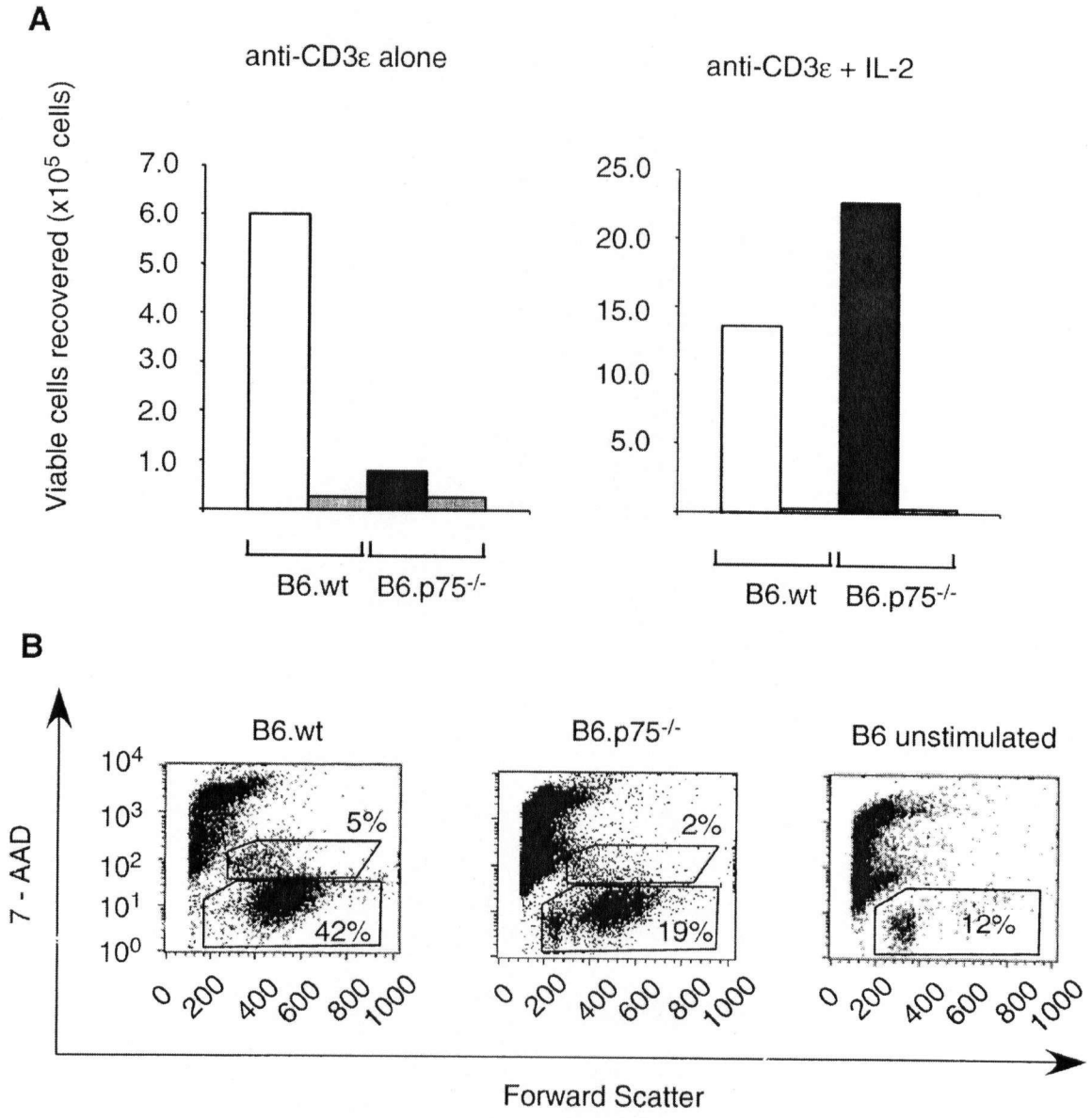

Figure 2.4 TNFR2$^{-/-}$ CD8^{+} T cells are more susceptible to apoptosis when stimulated with anti-CD3 without exogenous IL-2.

CD8^{+} T cells (1 x 10^{6}) were stimulated with either 10 μg/ml of plate bound anti-CD3 alone, or in the presence of exogenous IL-2, and harvested after 48 and 72 h. (A) The total number of viable cells in each culture was determined using a hemocytometer. White bars correspond to stimulated B6 wild-type CD8^{+} T cells; gray bars correspond to unstimulated controls; black bars correspond to stimulated TNFR2$^{-/-}$ CD8^{+} T cells. (B) Cultures were also stained with 7-AAD and analyzed using FACS (see Materials and Methods). The data shown is representative of three independent experiments performed.

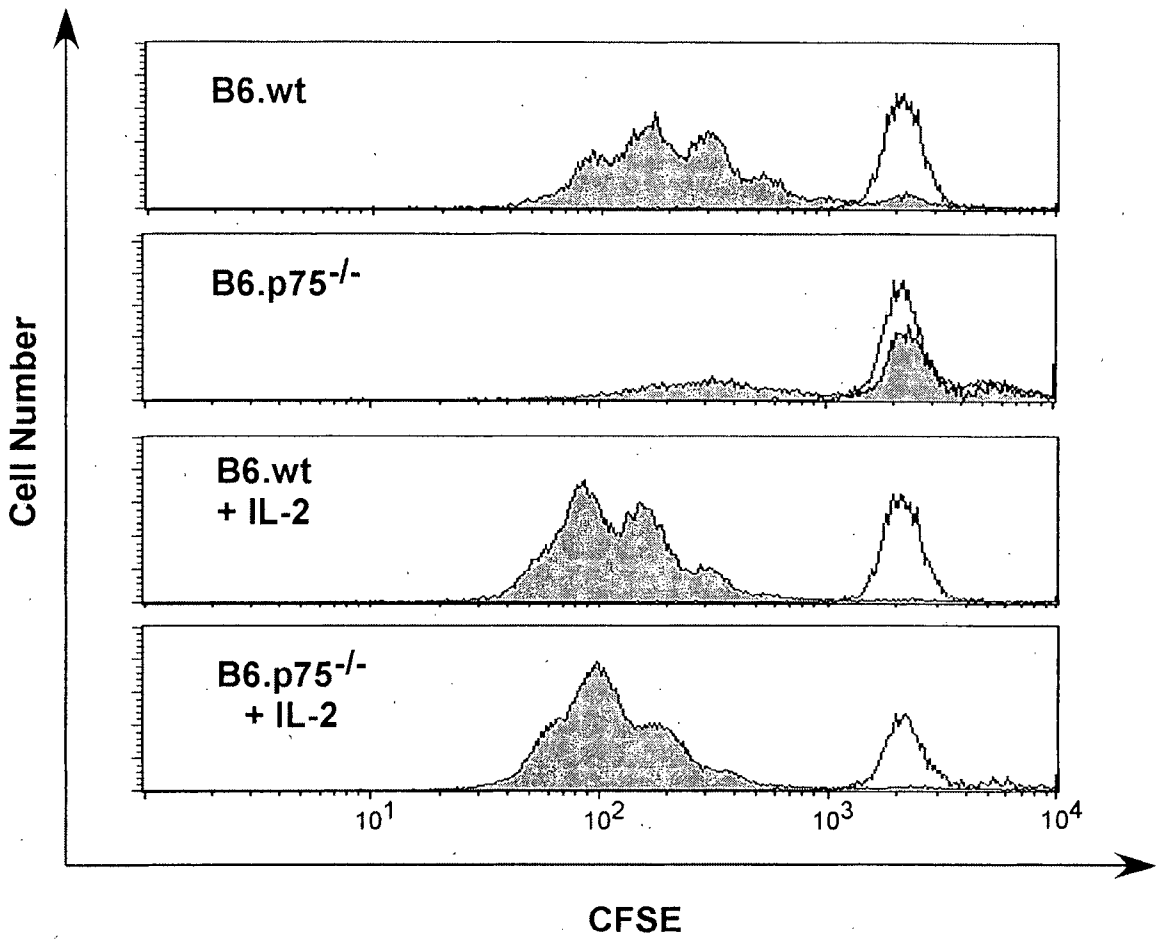

Figure 2.5 TNFR2$^{-/-}$ CD8^{+} T cells display marked reduction in the proportion that undergo division in response to TCR cross-linking.

CD8^{+} T cells (5 x 10^{5}) were labeled with 1 μM CFSE and stimulated with either 10 μg/ml of plate-bound anti-CD3 alone, or in the presence of exogenous IL-2, for 60 h (see Materials and Methods). The shaded gray curve corresponds to the stimulated cells; the black line corresponds to CFSE-labeled unstimulated cells (negative control). The data were reproduced in three independent experiments.

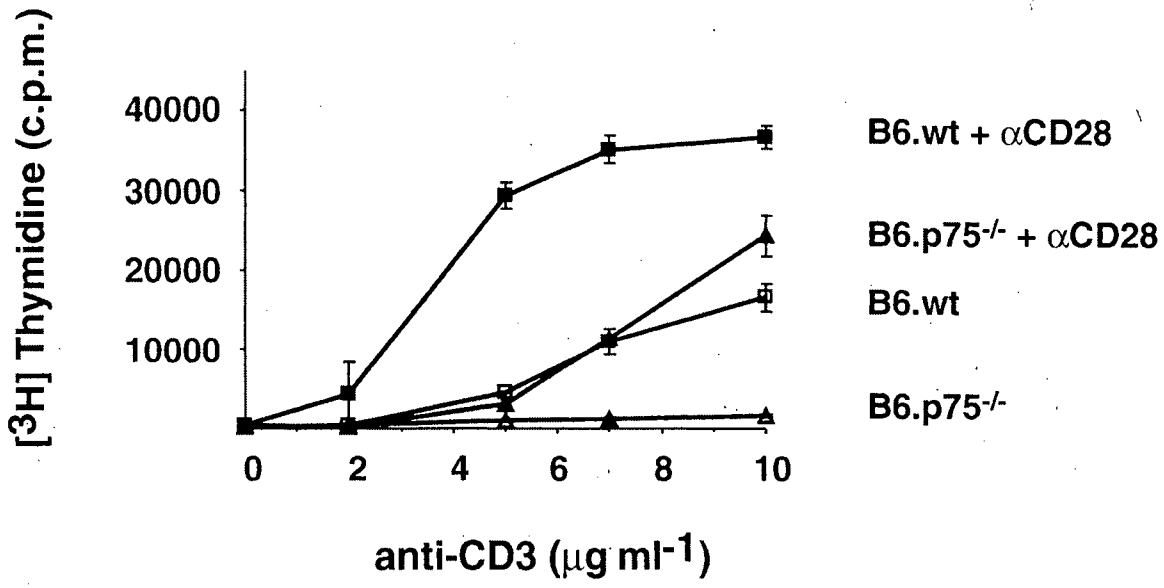

Figure 2.6 CD28 co-stimulation partially rescues the hypo-proliferative response of TNFR2-deficient $CD8^+$ T cells.

$CD8^+$ T cells (1×10^5) were stimulated with either 10 μg/ml of plate-bound anti-CD3 alone, or in the presence of 10 μg/ml anti-CD28, for 72 h with [^{3}H]thymidine added for the last 10 h of culture (see Materials and Methods). Mean ± standard deviation for triplicate determinations were used to express the data. The data shown are representative of three independent experiments performed.

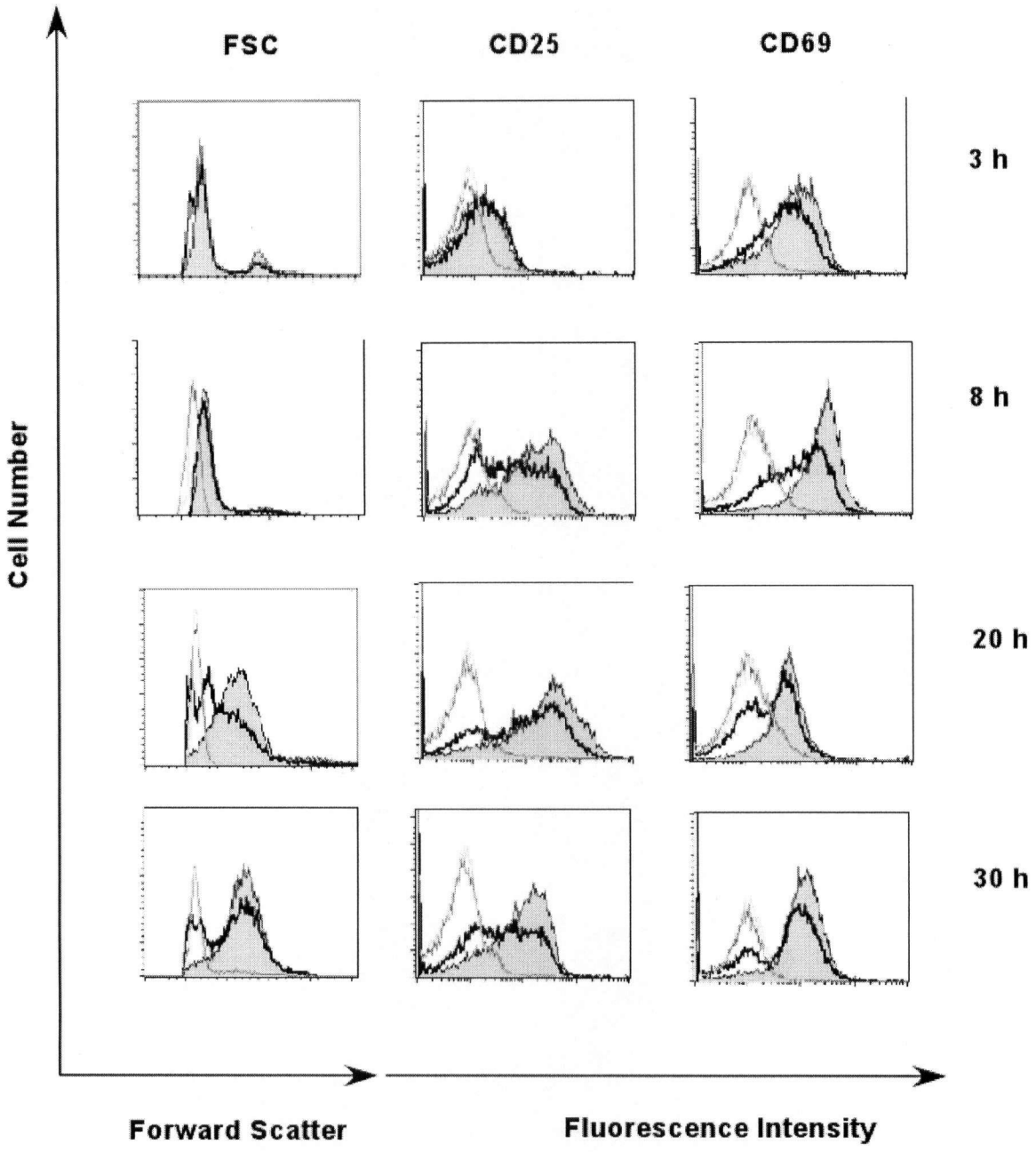

Figure 2.7 Induction of CD25 and CD69 expression as well as blastogenesis in response to TCR cross-linking occurs with delayed kinetics in TNFR2$^{-/-}$ CD8^{+} T cells.

CD8^{+} T cells (1 x 10^{6}) were stimulated with 10 μg/ml of plate-bound anti-CD3 for the indicated periods of time, stained with the relevant antibodies, and analyzed by FACS (see Materials and Methods). Shaded gray curve corresponds to stimulated wild-type CD8^{+} T cells; bold black line corresponds to stimulated TNFR2$^{-/-}$ CD8^{+} T cells; light gray line corresponds to unstimulated wild-type CD8^{+} T cells; dark gray line corresponds to unstimulated TNFR2$^{-/-}$ CD8^{+} T cells. FSC = Forward Light Scatter. Data shown are representative of one of three independent experiments performed.

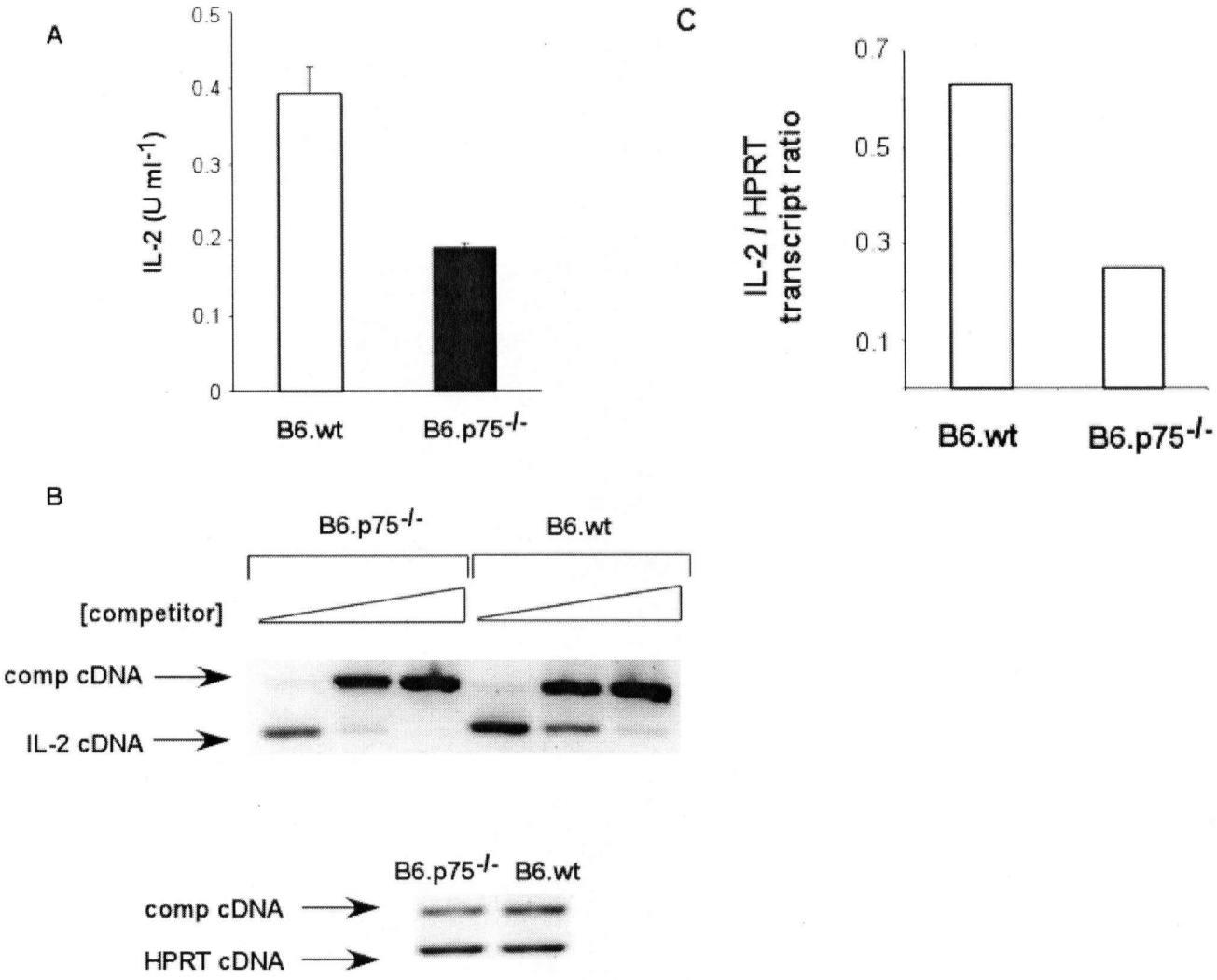

Figure 2.8 TNFR2$^{-/-}$ CD8^{+} T cells display a marked reduction in the level of IL-2 produced in response to TCR cross-linking.

(A) CD8^{+} T cells (1 x 10^{6}) were stimulated with 10 μg/ml of plate-bound anti-CD3 and cytokine ELISA was performed on the culture supernatants (see Materials and Methods). The data points denote mean ± standard deviation of triplicate determinations. (B) TNFR2$^{-/-}$ CD8^{+} T cells display a reduction in the levels of IL-2 mRNA in response to TCR cross-linking. TNFR2$^{-/-}$ CD8^{+} T cells (2 x 10^{6}) were stimulated with 10 μg/ml of plate-bound anti-CD3 for 9 h. Total RNA was harvested, reverse transcribed, and subsequently analyzed using CQ-PCR (see Materials and Methods). Gradient bar refers to the concentration of competitor in each lane (1, 10, 100 x). (C) The IL-2/HPRT transcript ratios of anti-CD3 stimulated wild type TNFR2$^{-/-}$ CD8^{+} T cells. Abbreviations used: comp = competitor, HPRT = hypoxanthine phosphoribosyl transferase. Data is representative of one of three independent experiments performed.

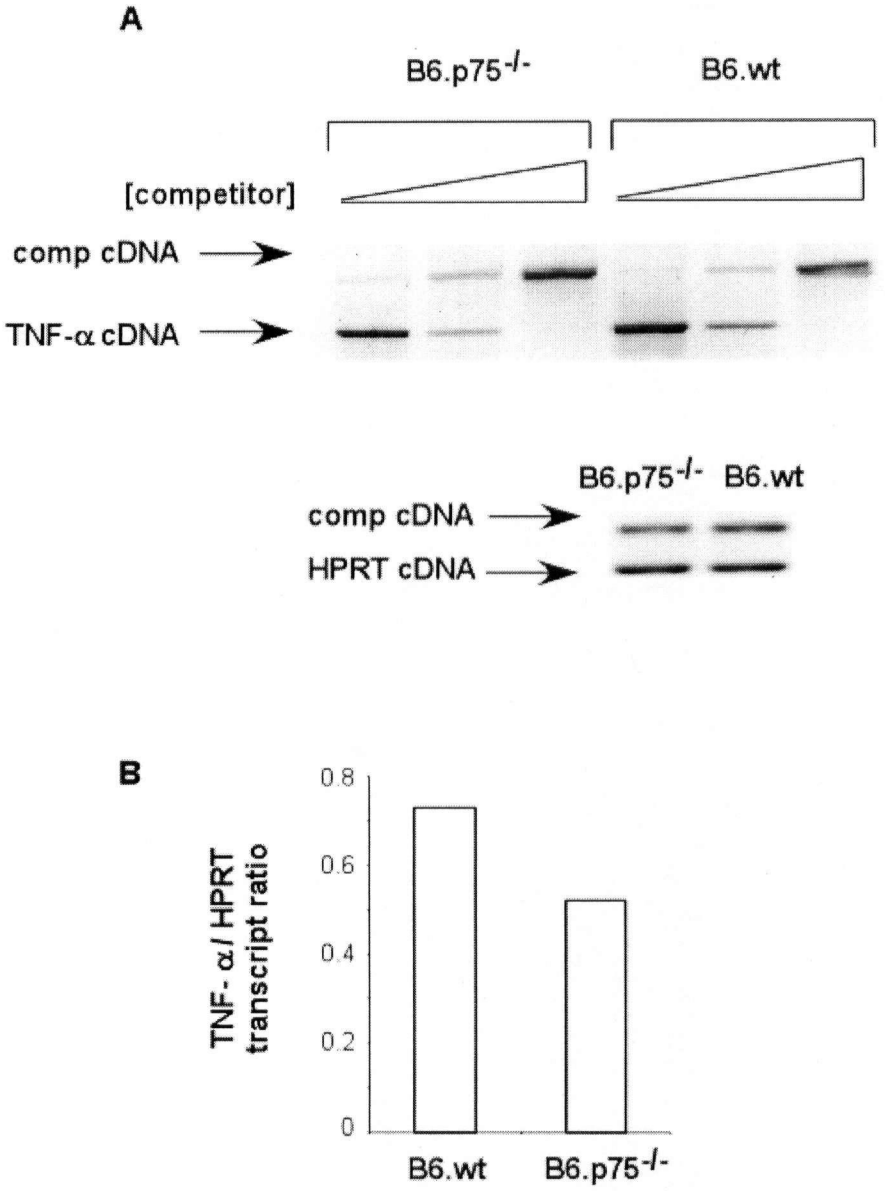

Figure 2.9 TNFα production in response to TCR cross-linking is largely unaffected for TNFR2$^{-/-}$ CD8^{+} T cells.

CD8^{+} T cells (2 x 10^{6}) were stimulated with 10 μg/ml of plate-bound anti-CD3 for 9 hrs. CQ-PCR was used to analyze the relative amounts of TNF-α transcripts between wild type and TNFR2$^{-/-}$ CD8^{+} T cells, as described for IL-2 in Fig. 8.

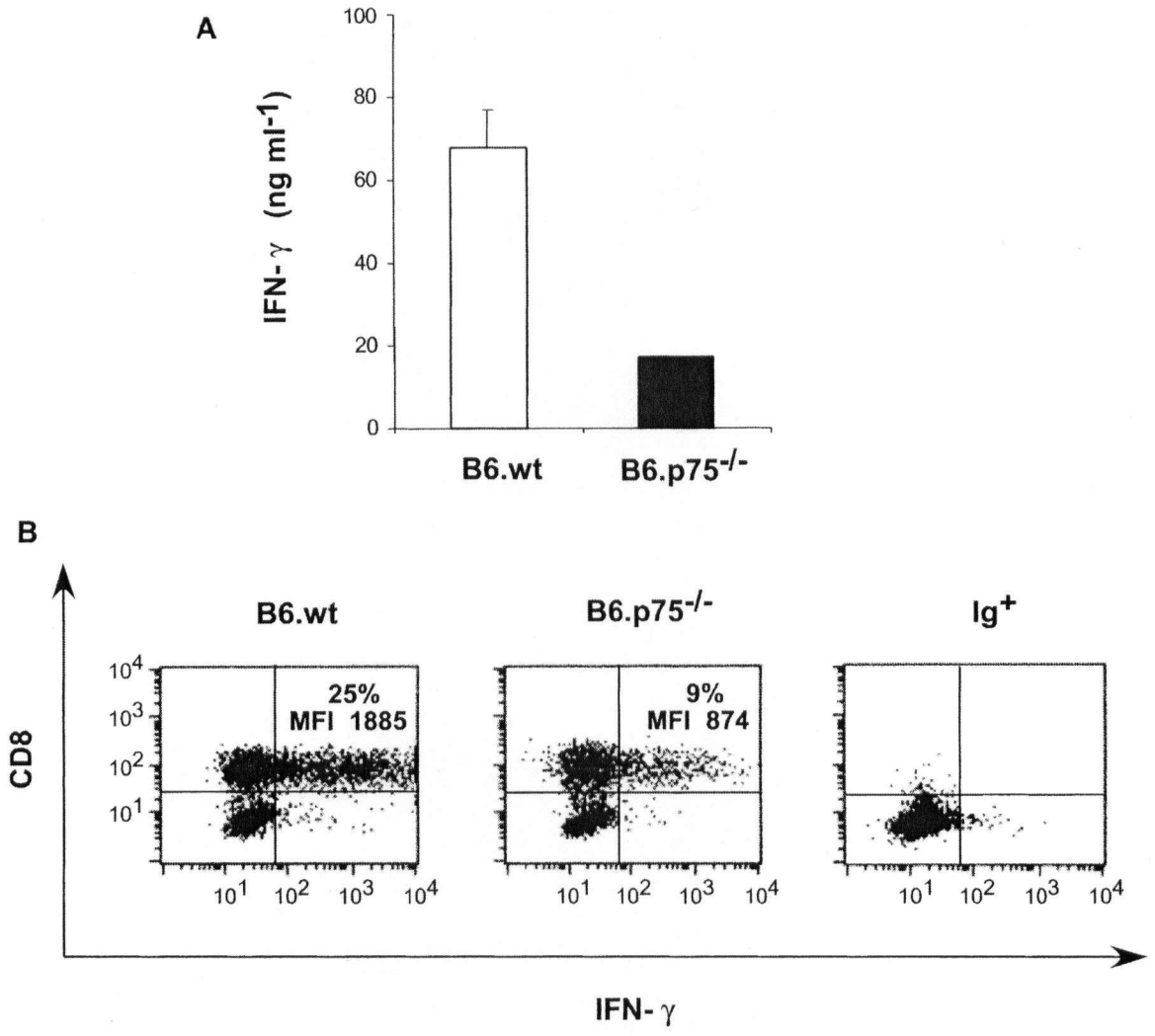

Figure 2.10 The decrease in IFN-γ expression by TNFR2$^{-/-}$ CD8^{+} T cells is due to a decrease in the percentage of cells that express IFN-γ, as well as a decrease in the intracellular levels expressed per cell.

LN cells (1 x 10^6) were stimulated with 10 μg/ml of plate-bound anti-CD3 for 36 hrs, with the last 6 hrs in the presence of the Golgi inhibitor, Golgi Stop (see Materials and Methods). The cells were stained for intracellular cytokine and analyzed by FACS. Ig^{+} cells stimulated with LPS were used as negative control. As an additional control, IFN-γ expression was analyzed in CD4^{+} T cells, since this T cell subset does not express significant amounts of IFN-γ at this stage of activation. MFI = Mean Fluorescence Intensity. The percentages refer to CD8^{+} T cells that express IFN-γ, as demarcated by gates within the FACS dot plots. Data is representative of one of three independent experiments performed.

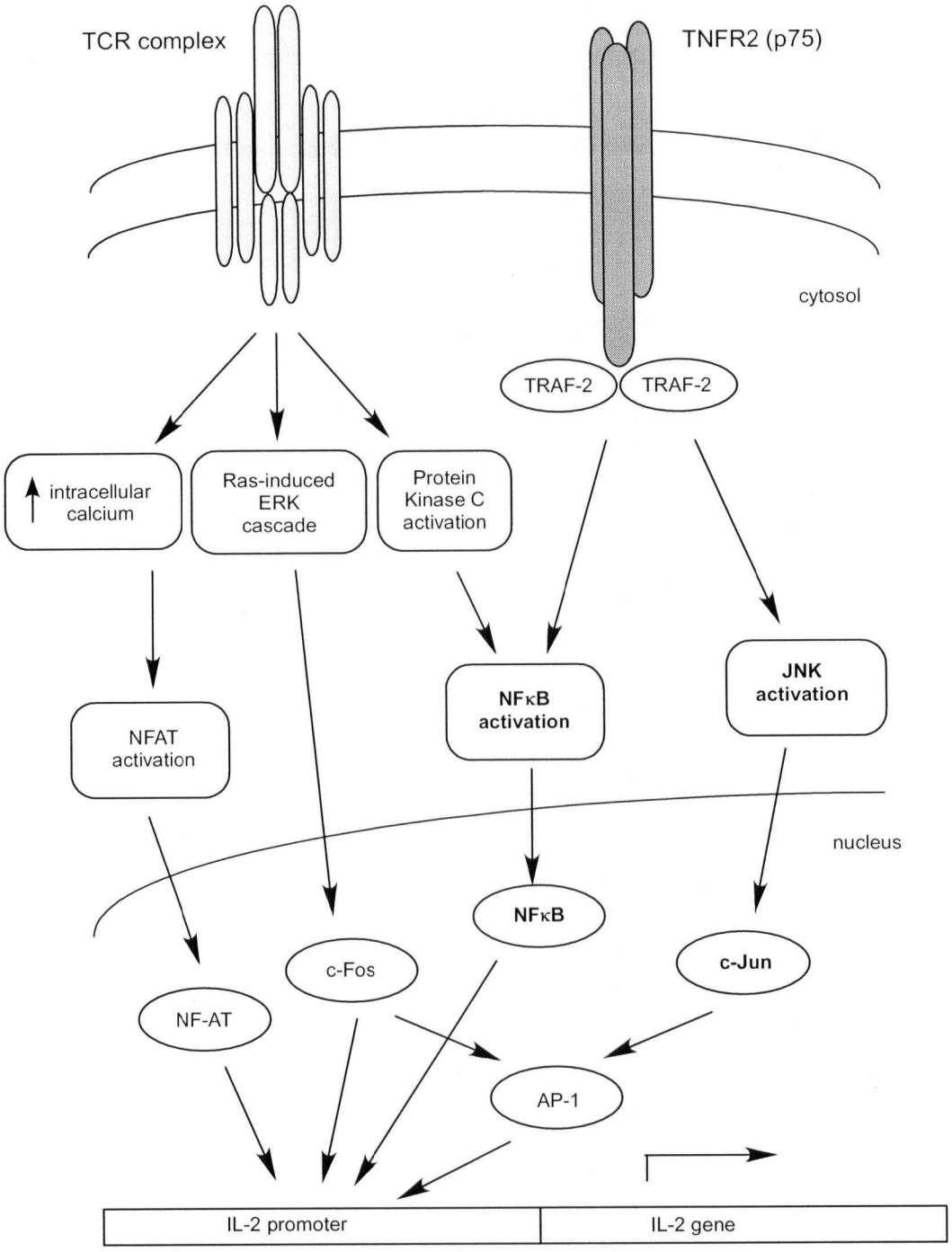

Figure 2.11 Model of TNFR2 co-stimulation for T cell activation.

This model postulates that TNFR2 contributes signals towards the activation and recruitment of transcription factors to the enhancer/promoter regions of genes that are upregulated during T cell activation.

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Chapter 3 Critical role of tumor necrosis factor receptor type-2 (p75) as a co-stimulator for IL-2 induction and T cell survival: a functional link to CD28 [1]

3.1 Preface

CD28 provides important signals that lower the threshold of T cell activation, augment the production of IL-2, and promote T cell survival. The recent identification of a second family of co-stimulatory molecules within the TNFR family has re-shaped the 'two-signal' model of T cell activation. This chapter examines the role of TNFR2 as a T cell co-stimulatory molecule in controlling cell fate during TCR/CD28-mediated stimulation *in vitro*. TNFR2-deficient T cells possessed a defect in IL-2 production in response to TCR/CD28-mediated stimulation. TNFR2 is non-redundantly coupled to sustained AKT activity and NFκB activation in response to TCR/CD28-mediated stimulation. Moreover, TNFR2-deficient T cells exhibit a defect in survival during the early phase of T cell activation that is correlated with a striking defect in Bcl-X_L expression. This chapter describes experiments showing a role for TNFR2 in sustaining the activation of key signaling intermediates during T cell activation, and the synergistic requirement of TCR, CD28, and TNFR2 towards optimal IL-2 induction and T cell survival. Moreover, it is proposed that TNFR2 acts as one of the earliest co-stimulatory members of the TNFR family, and is functionally linked to CD28 for initiating and determining T cell fate during activation.

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3.2 Introduction

T cells play a key role in immunity as actors and facilitators in the antigen-specific eradication of potential threats. They are tightly regulated by multiple mechanisms in a spatial- and temporal-dependent manner. The T cell receptor (TCR) confers specificity of response towards specific antigen, whereas numerous co-stimulatory molecules affect the response outcome (1, 2). In biochemical terms, while TCR-mediated signal transduction can activate a multitude of signaling cascades, a number of co-stimulatory molecules have been shown to promote more robust T cell responses, as well as fine-tuning of T cell differentiation toward specific effector mechanisms. Current models of T cell co-stimulation suggest several co-stimulatory molecules are required for the development of effective T cell-mediated immunity, reflecting the requirement of several signals in simultaneous action during the course of the T cell response, and the requirement of different signals at different times/stages of T cell differentiation (2).

The extensive literature identifies two general families of co-stimulatory molecules: CD28-related and the members of the TNF receptor (TNFR) family (2-4). Their respective characterization indicates overlapping and distinct activity on T cell proliferation, cytokine production, survival, and differentiation. The integration of signals stemming from the TCR and accessory cell surface proteins is thereby an important mode of molecular regulation for T cell activation. An important integration point for TCR-mediated and co-stimulatory signals that allows a robust T cell response is the tight regulation of the critical T cell growth factor IL-2 (5, 6). Early characterization of TCR- and CD28-mediated signals illustrated the two-signal concept for functional T cell activation. CD28 was established as a key co-stimulatory molecule for induction of IL-2 based on its ability to substantially augment its

expression in T cells stimulated via TCR (7). Transcriptional regulation of IL-2 is achieved by cooperative binding of transcription factors (including NFAT, AP-1, NFκB) (8), which are downstream targets of both the TCR and CD28 signaling pathways (5, 9). Their integration at the IL-2 promoter has been mapped to the CD28 response element (CD28RE), which contains a sequence similar to the NFκB consensus-binding site (10). Thus, coordinated signals provided by external stimuli activate intracellular signaling pathways that converge qualitatively and quantitatively towards changes in gene expression profile.

CD28 is the best characterized of the growing numbers of identified co-stimulatory molecules, and has been shown to synergize with the TCR to lower the threshold of T cell activation (11, 12), enhance initial clonal expansion (13), regulate IL-2 production (7, 14), and augment the expression of anti-apoptotic members of the Bcl-2 family (15). Initial biochemical studies of CD28 function demonstrated its role in promoting the transcription and stability of IL-2 mRNA (16). Subsequent studies showed synergy of CD28 signaling with signals mediated via the TCR complex towards the activation of downstream effectors (17). In particular, optimal activation of the transcription factors NFAT, AP-1, and NFκB is dependent on 'amplification signals' provided by CD28-mediated recruitment and subsequent activation of Vav (18) and phosphoinositide 3-kinase (PI3K) (17). More recently, protein kinase B (AKT) was shown to provide the CD28-mediated co-stimulatory signal for up-regulation of IL-2, suggesting that it also utilizes discrete signaling pathways (19). However, the effects mediated by CD28 were found to be insufficient to sustain long term T cell survival, and is thus functionally positioned early during T cell activation (within 24 hrs of antigen encounter) (20).

A number of novel co-stimulatory molecules have been identified from the TNFR family, including 4-1BB, CD134 (OX40), and CD27 (2). The discrete co-stimulatory roles played by these members of the TNFR family correspond with their expression pattern during the T cell response: peak expression occurs between day 2 and 4, and remains at significant levels thereafter (2). Analysis of CD27-deficient T cells revealed that CD27 promotes antigen-specific expansion of naive T cells as well as memory T cells , as reflected by delayed response kinetics and reduction of $CD8^+$ T cell numbers during secondary challenge (21). Stimulation of 4-1BB induces enhanced proliferative response when combined with anti-CD3 and anti-CD28 stimulation (22), sustains established $CD4^+$ and $CD8^+$ T cell responses as well as enhances cell division and effector function (23, 24). Engagement of OX40 promotes effector and memory T cell function, and can also enhance clonal expansion and cytokine production. Characterization of OX40-deficient T cells showed relatively unimpaired IL-2 production, cell division and clonal expansion, but revealed an important role towards sustaining the expression of anti-apoptotic molecules Bcl-X_L and Bcl-2 thereby promoting T cell survival (25). Thus, the roles of 4-1BB and OX40 for T cell differentiation and function appear to sustain the response (2), whereas CD28 appears to be important for the initial stages of activation and IL-2 induction.

Several lines of evidence suggest that the prototypic member of the TNFR family, TNFR-2 (p75), plays an important co-stimulatory role for T cells. First, it was found that adding exogenous TNF-α to T cell cultures stimulated via TCR cross-linking augmented the proliferative response to a degree comparable with T cell cultures supplemented with exogenous IL-2 (26). Furthermore, anti-TCR-induced proliferation in T cells is essentially abolished by a neutralizing Ab to TNF-α (26). Second, agonistic antibodies showed that of

the two receptors for TNF-α, TNFR2 signals the enhancement of the proliferative response (27). In correlation with these findings, it was found that TNFR2 is the dominant TNFR during T cell activation (28). Moreover, the known TNFR2 ligands are expressed by activated T cells and antigen presenting cells. TNF-α is one of the earliest cytokines expressed during activation of lymphocytes and antigen-presenting cells (28), and lymphotoxin-α is expressed on activated T and B cells (29). Biochemical characterization of the cytoplasmic domain of the TNFR2 TNF receptor revealed a TNF receptor-associated factor (TRAF)-binding domain that was shown to recruit TRAF2 (30), an adaptor molecule through which co-stimulatory members of the TNFR family are thought to mediate their signaling functions.

More recently, we have shown that TNFR2 decreases the threshold of T cell activation using TNFR2-deficient mice (31). Further cellular characterization of TNFR2-deficient $CD8^+$ T cells revealed a co-stimulatory role for TNFR2 for a number of aspects of T cell activation, including IFN-γ and IL-2 production, as well as the proliferative response that was only partially rescued by CD28 co-ligation (31). These data strongly suggested an important relationship between TCR, CD28 and TNFR2 towards optimal T cell responses. Here we show that T cells deficient in TNFR2 possess a marked defect in IL-2 production in response to TCR/CD28-mediated stimulation, demonstrating for the first time the co-stimulatory role of TNFR2 in relation to TCR and CD28-mediated signals towards IL-2 induction. Biochemical characterization showed that TNFR2 provides distinct qualitative signals via AKT and NFκB, whereas TCR-proximal signaling events and MAPK family cascades were largely intact in $TNFR2^{-/-}$ T cells. Moreover, we found that TNFR2-deficient $CD8^+$ T cells are more susceptible to apoptosis during the early phase of T cell activation in

response to TCR/CD28-mediated stimulation. This decreased survival of TNFR2-deficient $CD8^+$ T cells correlated with a defect in the expression of the anti-apoptotic molecule Bcl-X_L. These results support a model of co-stimulation that implicates TNFR2 in a critical role during TCR/CD28-mediated stimulation of IL-2 induction, clonal expansion, and survival. These functional aspects are correlated with a distinct role for TNFR2 in the intracellular signaling milieu during the early phase of the T cell response.

3.3 Materials and Methods

Mice

Breeders for C57BL/6 (B6, H-2^b) and B6-TNFR2-deficient mice were obtained from The Jackson Laboratories (Bar Harbor, ME). B6 mice deficient in the TNFR2 have been previously described (32). TNFR2-deficient mice were genotyped using a PCR strategy. Mice 4 to 7 weeks of age were used for all experiments.

Cells

Lymph nodes were harvested and single cell suspensions prepared from each of the mouse lines. The $CD4^-$ $CD8^+$ ($CD8^+$) T cell subset was purified from whole lymph node cell suspensions using miniMACS microbeads (Miltenyi Biotec, Auburn, CA). $CD8^+$ T cells were positively selected using a MACS MS^+ Separation column and MiniMACS magnet, as per manufacturer's protocol (Miltenyi Biotec), achieving > 95% purity. Cells were cultured at 37°C and 5% CO_2 in Iscove's DMEM (Life Technologies, Burlington, Ontario)

supplemented with 10% (v/v) FBS (Life Technologies), 5 x 10^{-5} μM 2-ME, and antibiotics (I-media).

Antibodies and flow cytometry

PE-conjugated hamster anti-mouse TNFR2 monoclonal antibody (TR75-89) was used to study TNFR2 expression (BD Pharmingen, Mississauga, ON). Cell staining and flow cytometry were performed according to standard procedures. Briefly, cells were incubated with the relevant antibodies for at least 15 min at 4 °C, and subsequently washed twice with FACS medium (PBS + 2% FCS). The CELLQUEST software program (Becton Dickinson, Mountain View, CA) was used for data acquisition and analysis. For TNF-α neutralization studies, two antibodies were used: rat anti-mouse TNF-α used (MP6-XT3) (BD Pharmingen, Mississauga, ON) and rat anti-mouse TNF-α (V1q) (Cedarlane, Hornby, ON).

7-AAD assay

$CD8^+$ T cells (1 x 10^6) were stimulated with 10 μg/ml of immobilized anti-CD3ε (2C11) and 10 μg/ml of anti-CD28 (35.71) in a flat-bottom 24-well plate for various periods of time. Cells were harvested and stained with 7-AAD (10 μg/ml in FACS buffer), fixed with 4% paraformaldehyde, and subsequently analyzed by FACS (described above).

Cytokine ELISA

$CD8^+$ T cells (2 x 10^6) were cultured in a flat-bottom 24-well plate coated with 10 μg/ml of anti-CD3 for 20 hrs. The amount of IL-2 and IFN-γ in the supernatant was determined by ELISA. The capture and detection Abs used for IL-2 were JES6-1A12 and JES6-5H4,

respectively (obtained from BD Pharmingen, Mississauga, ON). The capture and detection Abs used for IFN-γ were R4-6A2 and XMG1.2, respectively (BD Pharmingen, Mississauga, ON). Briefly, plates were coated with the capture Ab (4 μg/ml in carbonate buffer) and blocked with 1% BSA/ 0.1% azide in PBS. Wells were washed with PBS-Tween 20 and samples were then added in three dilutions, with each plate containing wells for standard. The wells were washed and the detection Ab (1 μg/ml in 1% BSA/ 0.1% azide in PBS) was added. The wells were then washed and streptavidin-alkaline phosphatase (BD Pharmingen, Mississauga, ON) was added (1/2000 in 1% BSA/ 0.1% azide in PBS). After washing the wells, substrate (Sigma 104) was added and plates were subsequently analyzed with an ELISA plate reader at 405 nm.

Cytokine Competitive and Quantitative RT-PCR

Competitive and quantitative polymerase chain reaction (CQ-PCR) was used to determine the steady-state levels of IL-2 and TNF mRNA. T cells (2×10^6) were cultured in 1 ml of I-media in flat-bottom 24-well plates coated with 5 μg/ml of anti-CD3 for 9 hrs. Cells were then harvested and total RNA was prepared according to the manufacturer's recommendations using the RNeasy Mini Kit (Qiagen). cDNAs were generated from the total RNA preparation as previously described (33). CQ-PCR was then performed as previously described (33). Briefly, the amount of cDNA was normalized between TNFR2-deficient and WT T cells using the housekeeping gene HPRT. The linearized pPQRS plasmid was used as the competitor (gift from Richard Locksley, UCSF). The sequences for the 5' and 3' oligonucleotide primers used for IL-2 were, respectively:

5'-CCA CTT CAA GCT CTA CAG CGG AAG-3' and 5'-GAG TCA AAT CCA GAA CAT GCC GCA-3'. The sequences for the 5' and 3' oligonucleotide primers used for TNF-α were, respectively: 5'-GTT CTA TGG CCC AGA CCC TCA CAC-3' and 5'-TCC CAG GTA TAT GGG TTC ATA CCA AG-3'. The sequences for the 5' and 3' oligonucleotide primers used for HPRT were, respectively: 5'-GTT GGA TAC AGG CCA GAC TTT GTT G-3' and 5'-GAG GGT AGG CTG GCC TAT AGG CT-3'. The PCR products were subjected to electrophoresis on a 2 % agarose gel and stained with ethidium bromide. Densitometry was performed using the AlphaImager software.

Western Blot

$CD8^+$ T cells (2×10^6) were cultured in a flat-bottom 24-well plate coated with 10 μg/ml of anti-CD3 (2C11) alone and in combination with 10 μg/ml of soluble anti-CD28 (37.51) for various periods of time. Whole cell lysates were prepared as previously described. Briefly, cells were harvested, washed with PBS and treated with lysis buffer (1% Triton X-100, TNE, 1mM sodium orthovanadate, 1mM sodium molybdate, Complete protease inhibitor cocktail (Roche Diagnostic, Laval, QC), and 10% glycerol) for 20 mins on ice. Total protein was quantified using D_C Protein Assay (BioRad, Hercules, CA). Equivalent amounts were resolved by SDS-PAGE, and then transferred to Immobolin-P membrane (Millipore Corporation, Bedford, MA).

The following antibodies (all from Cell Signaling Technologies, Pickering, ON) were used: rabbit phospho-p44/42 MAPK (Thr202/Tyr204) antibody, rabbit p44/42 MAPK antibody, rabbit phospho-p38 MAPK antibody, rabbit p38 MAPK antibody, rabbit phospho-SAPK/JNK (Thr183/Tyr185) antibody, rabbit SAPK/JNK antibody,

rabbit phospho-ZAP-70 (Tyr319) antibody, rabbit phospho-AKT (Ser473) antibody, rabbit AKT antibody, rabbit phospho-IκBα (Ser32) antibody, rabbit IκBα antibody. Tyrosine phosphorylation was assayed using 4G10, a mouse IgG2b monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

AKT activation was measured using the AKT kinase assay kit, which involves immunoprecipitating AKT from cell extracts, incubation with cold ATP and GSK-3αβ fusion protein as substrate, and detecting for phosphorylated GSK-3αβ by western blot as per manufacturer's protocol (Cell Signaling Technologies, Pickering ON).

Electrophoretic Mobility Shift Assay

$CD8^+$ T cells (4×10^6) were cultured in a flat-bottom 24-well plate coated with anti-CD3 (2C11) mAb (10 μg/ml) in the presence or absence of soluble anti-CD28 (37.51) mAb (10 μg/ml) for 8 hrs. Nuclear extracts were prepared as previously described (34). Briefly, cells were harvested and washed with PBS. Cell pellets were resuspended in Buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF) and incubated on ice for 15 minutes. Samples were centrifuged, resuspended in Buffer C (20 mM HEPES, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF), and incubated on ice for 20 minutes. Cellular debris was removed by centrifugation. Protein concentration of nuclear extracts was quantified using D_C Protein Assay (BioRad, Hercules, CA).

The oligonucleotide probe used for NFκB was end-labeled with $\gamma[^{32}P]$-GTP. The 5' and 3' sequences used for generating three-nucleotide overhangs of the oligonucleotide probe were, respectively: 5'-ATG TGA GGG GAC TTT CCC-3' and 3'-ACT CCC CTG AAA

GGG TCC-5'. The fill-in reaction was mediated using the large fragment (klenow) of DNA polymerase I (Invitrogen, Carlsbad, CA).

Nuclear extracts (4 μg) were incubated with radioactively labeled probe for 20 mins at room temperature, and then resolved by PAGE (5% acrylamide). Gel shifts were analyzed using Molecular Dynamics Phosphorimager SI system (Amersham Biosciences, Sunnyvale, CA) and ImageQuant (v 5.2) software (Amersham Biosciences, Sunnyvale, CA).

3.4 Results

TNFR2 expression during T cell activation

Patterns of cell surface expression of receptors can provide insight to their usage and function. The kinetics of expression of co-stimulatory members of the TNFR family appear to correlate with their temporal functions during T cell activation (2). Early work on the two TNF receptors reported that TNFR2 is the dominant TNF receptor on activated T cells (28), indicating that the pleiotropic effects of TNF-α can be attributed to the differential expression of the TNF receptors. However, the kinetics of TNFR2 expression is unknown, and their examination may reveal insight into the temporal aspects of its function during T cell activation. We therefore examined TNFR2 expression during TCR/CD28-mediated stimulation of naive T cells. Immediately *ex vivo* CD8 T cells expressed a low level of TNFR2, and expression increased for at least 48 hrs of TCR/CD28-mediated stimulation (Fig. 3.1*A*). As expected, TNFR2-deficient T cells did not display any expression on the T cell surface. The basal expression of TNFR2 in

naive T cells suggests that it plays a very early role in T cell activation, and continues to exert its effect as its expression is upregulated during the early phase of the response.

IL-2 induction during T cell activation is dependent on both CD28 and TNFR2

The induction of IL-2 gene during T cell activation requires both TCR-mediated and co-stimulatory signals. These signals provided at the initiation of the T cell response can control commitment to cytokine production and proliferation. CD28 is well characterized in its ability to co-stimulate robust IL-2 production. We previously reported the defect in the proliferative response to TCR/CD28-mediated stimulation by TNFR2-deficient $CD8^+$ T cells (31). Moreover, IL-2 induction was defective in TNFR2-deficient $CD8^+$ T cells stimulated via TCR-mediated stimulation. We sought to determine whether TNFR2 is critical for optimal IL-2 induction in T cells stimulated via both TCR and CD28. The amount of IL-2 that was secreted in response to TCR cross-linking alone and in combination with CD28 co-ligation was measured by ELISA performed on culture supernatants. As shown in Fig. 3.1*B*, TNFR2-deficient $CD8^+$ T cells stimulated with anti-CD3 displayed a marked reduction in the amount of IL-2 detected in the culture supernatant compared to WT $CD8^+$ T cells. CD28 co-ligation led to enhancement of IL-2 production in WT $CD8^+$ T cells, which is consistent with its known function in co-stimulating IL-2 induction. Strikingly, CD28 co-ligation did not rescue the defect in IL-2 production of TNFR2-deficient $CD8^+$ T cells, demonstrating the importance of TNFR2 as a co-stimulator of IL-2 during T cell activation. This suggests that TNFR2 and CD28 may act synergistically in co-stimulating optimal IL-2 induction.

A key point of IL-2 regulation is at the transcriptional level (9). To study whether the observed reduction in IL-2 production by TNFR2-deficient $CD8^+$ T cells stemmed from a

reduction in the steady-state levels of cytokine transcript, IL-2 mRNA was measured by competitive and quantitative reverse transcription-polymerase chain reaction (CQ-PCR). This technique permits quantitative analysis of relative transcript levels as a function of competitor dose. When stimulated with anti-CD3 alone, TNFR2-deficient $CD8^+$ T cells displayed a reduction in steady-state levels of IL-2 transcript compared to WT $CD8^+$ T cells (Fig. 3.2). As expected, CD28 co-ligation caused a substantial (10-fold) increase in steady-state levels of IL-2 transcript in WT $CD8^+$ T cells. In striking contrast, CD28 co-ligation only caused a modest increase for TNFR2-deficient $CD8^+$ T cells, which displayed a 10-fold reduction in IL-2 transcript compared to WT (Fig. 3.2). This result reveals for the first time the importance of TNFR2 in the optimal induction of IL-2 at the mRNA level in response to TCR/CD28-mediated stimulation. The defect in steady-state levels of IL-2 transcript suggests that TNFR2 and CD28 may converge on common intracellular signaling effectors to achieve fine control over the activation of transcription factors that assemble at the IL-2 promoter for its optimal induction.

TCR-proximal signaling events function independently of TNFR2

The biochemical events following TCR engagement are well characterized, involving signaling events that ultimately lead to alteration of gene expression (35). TCR-mediated signal transduction involves phosphorylation of key tyrosine residues in the TCR complex by Src-family protein tyrosine kinases that lead to recruitment and activation of the tyrosine kinase ZAP-70 at the T cell surface (36). These tyrosine kinases then phosphorylate adaptor proteins such as LAT and couple the TCR complex to the Ras- and Rho-family GTPase signaling cascades, that lead to activation of the ERK1/2, JNK, p38 MAPKs (18).

Given the important contribution made by TNFR2 for IL-2 induction during TCR/CD28-mediated stimulation, we sought to investigate the signaling events through which TNFR2 exerts its effect in relation to those mediated through the TCR and CD28. We first examined whether TNFR2 affects TCR-proximal signaling events in response to stimulation with anti-CD3 alone, or in combination with either anti-CD28 or exogenous TNF-α (10 ng/ml), by assaying for global tyrosine phosphorylation. Notable TCR-proximal substrates observed by examination of the tyrosine phosphorylation profile include ZAP-70 (70-kDa) and its immediate downstream target LAT (36-38-kDa). As shown in Fig. 3.3*A*, the tyrosine phosphorylation profiles of TNFR2-deficient $CD8^+$ T cells appeared largely similar to WT. In addition, anti-CD28 and exogenous TNF-α treatment of TNFR2-deficient $CD8^+$ T cells did not affect global tyrosine phosphorylation. These data suggest that TNFR2 is not required for tyrosine phosphorylation of substrates that are immediately coupled to the TCR complex, and that TCR-mediated stimulation is sufficient for these early signaling events. Consistent with this finding, ZAP-70 phosphorylation assayed directly by western blot showed that this TCR-proximal signaling event is not coupled to TNFR2 (Fig. 3.3*B*). Hence, TCR-proximal signaling events of $CD8^+$ T cells are functional and independent of TNFR2 at early time points of stimulation.

MAPK family cascades are not coupled to TNFR2 during T cell activation

TCR engagement is directly linked to the mitogen-activated protein kinase (MAPK) pathways, which include the ERK, JNK, and p38 MAPKs (37). Signals are delivered from the TCR complex to the ERK pathway via SLP76 and Grb2 (38), whereas JNK and p38 pathways appear to be coupled to both CD28 and TCR (39, 40). MAPK pathways are

notable for their role in activating transcription factors such as Elk1, c-Fos and c-Jun (the latter two of which are components of the heterodimeric transcription factor AP-1) (35, 41), which are known to be important for the regulation of IL-2 gene expression (40). We determined the relative roles of TCR, CD28 and TNFR2 in these MAPK pathways at various time points of stimulation using western blot analysis. We were particularly interested in examining the intracellular signaling milieu around the 10-hour time point, since we observed that steady-state levels of IL-2 transcript were markedly reduced in TNFR2-deficient $CD8^+$ T cells at this time point. As shown in Fig. 3.4, anti-CD3 treatment was sufficient to phosphorylate ERK1/2, JNK, and p38 in both TNFR2-deficient and WT $CD8^+$ T cells, and phosphorylation persisted up to 10 hrs of stimulation. Moreover, CD28 co-ligation did not lead to an increase in phosphorylation of these MAPKs. These data suggest that the MAPK pathways are sufficiently activated by TCR-mediated stimulation and do not require TNFR2.

TNFR2 is critical for sustaining protein kinase B (AKT) phosphorylation during the early phase of TCR/CD28-mediated T cell activation

TCR signaling is coupled to phosphoinositide-3 kinase (PI3K), which then leads to AKT recruitment and activation (42). A previous report demonstrated that AKT acts as a mediator for CD28 co-stimulation of IL-2 induction (19), indicating that both TCR and CD28 signaling promote AKT phosphorylation (42). The finding that CD28 co-ligation failed to rescue the defect in IL-2 induction by $TNFR2^{-/-}$ T cells suggested that TNFR2 may be functionally linked to CD28 in regulating IL-2. We therefore examined the possibility that AKT phosphorylation may be a downstream site of convergence between TNFR2 and

CD28. It was found that AKT underwent robust phosphorylation in response to anti-CD3 stimulation in both WT and TNFR2-deficient $CD8^+$ T cells at early time points (Fig. 3.5*A*). This phosphorylation was sustained over the 10 hrs of anti-CD3 stimulation examined for WT $CD8^+$ T cells, but was dramatically decreased for TNFR2-deficient $CD8^+$ T cells. Interestingly, CD28 co-ligation failed to rescue this defect in AKT phosphorylation. To directly measure the activation of AKT, we utilized a non-radioactive AKT assay that measures the phosphorylation of the substrate, a GSK-3α/β fusion protein (see *Materials and Methods*). As shown in Fig. 3.5*B*, wild-type and TNFR2-deficient $CD8^+$ T cells displayed similar immediate activation of AKT after 10 minutes of stimulation as indicated by similar levels of phosphorylated GSK-3α/β. Strikingly, AKT activity was dramatically less in TNFR2-deficient T cells compared to wild type after 10 hrs of stimulation, and CD28 co-ligation did not rescue this defect. These data demonstrate the importance of TNFR2 towards sustaining the activation of AKT during T cell activation, and indicate a functional link between the CD28 and TNFR2 co-stimulatory pathways.

Previous work has shown that IL-2 and related cytokines can activate AKT (43). Given that TNFR2-deficient $CD8^+$ T cells possess a defect in IL-2 induction, we sought to determine whether the defect in AKT phosphorylation was indirectly due to limited IL-2 production by TNFR2-deficient $CD8^+$ T cells. We addressed this question using a blocking antibody for IL-2R during stimulation with anti-CD3 and CD28 co-ligation. The dose of anti-IL2Rβ used was sufficient to completely abrogate T cell proliferation (data not shown). It was found that anti-IL-2Rβ treatment did not substantially affect AKT phosphorylation in WT $CD8^+$ T cells after 10 hrs of stimulation, demonstrating that the IL-2 is not necessary for AKT phosphorylation during the early phase (up to 10 hrs) of the response. This suggests

that this period represents an IL-2 independent phase of T cell activation. This strongly suggests that the defect in AKT activation observed for TNFR2-deficient $CD8^+$ T cells is due to lack of a direct signal towards AKT via TNFR2.

TNFR2 is critical for NFκB activation

NFκB is one of a crucial set of transcription factors that coordinately bind to the IL-2 promoter (44). It is downstream of both TCR and CD28 signaling via protein kinase C theta and AKT (45). In its latent form, it is found in a complex with IκBα in the cytoplasm (46). Activation signals target this complex by phosphorylating IκBα, leading to its degradation mediated by ubiquitin. The liberated NFκB then translocates through nuclear pores and subsequently acts upon on gene targets. Hence, NFκB activation can be measured directly by its translocation into the nucleus, and indirectly by the phosphorylation of IκBα. Previous work has shown that AKT activation leads to activation of NFκB (47). Given the defect in AKT phosphorylation observed in TNFR2-deficient $CD8^+$ T cells, as well as the important role that NFκB in IL-2 gene expression, we sought to determine whether TNFR2 couples to this transcription factor during T cell activation. First, we examined the phosphorylation of IκBα and steady-state levels of the unphosphorylated form in response to TCR/CD28-mediated stimulation. As shown in Fig. 3.6, IκBα underwent robust phosphorylation in the immediate response (i.e. 5 minutes) to TCR-, TCR/CD28-, and TCR/CD28/TNFα-mediated stimulation in WT and TNFR2-deficient $CD8^+$ T cells. IκBα phosphorylation was sustained in WT $CD8^+$ T cells after 10 hrs of stimulation, whereas TNFR2-deficient $CD8^+$ T cells display markedly reduced IκBα phosphorylation in comparison. CD28 co-ligation was unable to rescue IκBα phosphorylation in TNFR2-deficient $CD8^+$ T cells. The steady-state

level of IκBα protein was diminished in WT $CD8^+$ T cells at 10 hrs of stimulation, indicating its degradation subsequent to phosphorylation. In contrast, TNFR2-deficient $CD8^+$ T cells displayed similar level of IκBα protein relative to unstimulated control. These data suggest that TCR-mediated stimulation is sufficient for immediate phosphorylation of IκBα, and that TNFR2 provides an important contribution to sustain this response during T cell activation.

To directly measure the activation of NFκB, its presence in the nucleus of T cells upon TCR- and TCR/CD28-mediated stimulation was determined using electrophoretic mobility shift assay. Consistent with the IκBα phosphorylation data, the steady-state levels of NFκB in the nucleus of WT $CD8^+$ T cells was substantially greater than for TNFR2-deficient $CD8^+$ T cells in response to TCR/CD28-mediated stimulation. These data strongly suggest that TNFR2 plays an important role in activating NFκB translocation to the nucleus during T cell activation.

TNFR2 promotes T cell survival and is required for Bcl-X_L expression during T cell activation

T cell survival is an essential means by which the strength of the immune response is regulated. T cell activation can lead to a number of cell fate outcomes, depending on the context of signals provided by the local environment. Co-stimulation mediated by CD28 controls initial clonal expansion and provides early signals for expression of the Bcl-2 family member Bcl-X_L (15). The co-stimulatory TNFR family member OX40 was shown to be important for maintaining high levels of Bcl-X_L later during activation (days 4-8), indicating the temporally linked functions of CD28 and OX40 towards T cell survival (48).

Given that TNFR2-deficient T cells exhibit a defective proliferative response to TCR/CD28-mediated stimulation, we next addressed whether this defect is due to an inability to survive during T cell activation. We analyzed cell death using 7-AAD staining and correlated this with cell proliferation, as measured using CFSE by flow cytometry. CFSE fluorescence measured by flow cytometry revealed that anti-CD3 and anti-CD28 co-treatment led to the proliferation of TNFR2-deficient $CD8^+$ T cells to the dividing population after 65 hrs of culture (Fig. 3.7*A*), although the total clone size as measured by the number of viable cells recovered was reduced in TNFR2-deficient $CD8^+$ T cell cultures (Fig. 3.7*B*). The latter result suggested that TNFR2-deficient T cells are defective for survival during T cell activation. Examination of 7-AAD fluorescence revealed that the percentage of live cells was reduced in TNFR2-deficient T cell cultures compared to WT (42% and 71% respectively), and that proportionately more cells that were induced to divide stained as apoptotic. This suggests that TNFR2 is important for the survival of T cells during the proliferative response, and indicates that dividing cells possess an increased propensity to undergo apoptosis in the absence of survival signals provided by TNFR2.

The observation that CD28 co-ligation failed to rescue the survival defect of TNFR2-deficient T cells suggested that the expression of anti-apoptotic molecules attributable to CD28 signaling is also defective. We therefore examined TCR/CD28-mediated upregulation of Bcl-X_L using western blot analysis. Consistent with previous reports (15, 49), CD28 co-ligation led to an increase in Bcl-X_L expression over TCR-mediated stimulation alone. Strikingly, the expression of this anti-apoptotic molecule was completely abolished in TNFR2-deficient $CD8^+$ T cells, and CD28 co-ligation failed to rescue this defect. This

strongly suggests that TNFR2 acts to promote survival via expression of Bcl-X_L during the very early phase of T cell activation.

3.5 Discussion

The two-signal model for T cell activation has been substantially modified since it was first proposed more than 30 years ago (50). The 'second signal' that was initially attributed to CD28 has now grown in complexity based on observations that other cell surface molecules can modulate specific aspects of the T cell response. The identification of other co-stimulatory molecules is important in light of the ability of CD28-deficient T cells to undergo a robust activation response depending on the strength of signal mediated initiated by the TCR. We demonstrated here the critical role of TNFR2 in TCR/CD28-mediated stimulation of the induction of IL-2. The striking finding that IL-2 production in TNFR2-deficient T cells could not be rescued by CD28, strongly suggested that both receptors are important for co-stimulating IL-2 production. The results advance the current co-stimulatory model of IL-2 induction during T cell activation, for which CD28 has been understood to be the dominant co-stimulatory receptor. Our results revealed a hitherto unknown functional link between TNFR2 and TCR/CD28 in up-regulating the state-steady levels of IL-2 transcript, and that this relationship was correlated with defects in the activation of AKT and NFκB in TNFR2-deficient $CD8^+$ T cells. We found that TNFR2 is one of the earliest expressed receptor among the known co-stimulatory members of the TNFR family, with basal expression in naive CD8 T cells set to co-stimulate T cell activation. Moreover, our data showed that TNFR2 was important for T cell survival during TCR/CD28-mediated activation, and that TNFR2

deficiency correlated with a defect in the expression of the anti-apoptotic molecule Bcl-X_L. Thus, TNFR2 is positioned as one of the earliest of the known co-stimulatory members of the TNFR family and functionally linked to CD28 for IL-2 induction and T cell survival, and thereby serves as an important control point for T cell fate.

Biochemical mechanism of TNFR2 and CD28 in promoting IL-2 induction

Many studies have examined the biochemical nature of CD28 co-stimulation in relation to TCR signaling. The body of evidence strongly suggests that CD28 utilizes both qualitative and quantitative signals. Michel *et al.* (2001) showed that CD28 co-stimulation potentiates TCR signaling by amplifying PLC-γ1 activation and intracellular calcium response, demonstrating quantitative signaling and synergy between the TCR and CD28 signaling pathways. Kane *et al.* (2001) showed that CD28 signal transduction involving AKT is needed for activating CD28RE in the IL-2 promoter, demonstrating qualitative signals that are uniquely mediated by CD28 towards IL-2 induction. In examining the biochemical nature of the co-stimulatory role of TNFR2 in IL-2 induction, we observed that TCR-proximal events such as tyrosine phosphorylation of LAT and ZAP-70 phosphorylation were not coupled to the TNFR2 pathway. Moreover, MAPK cascades (ERK, JNK, and p38) were also found to be largely unaffected by TNFR2-deficiency in $CD8^+$ T cells, demonstrating that TCR-mediated signaling is adequate to activate the MAPK pathways. These findings strongly suggest that TNFR2 might provide a distinct signal for its co-stimulatory properties in T cells. Indeed, we showed specific defects in the activation of the AKT and NFκB pathways in TNFR2-deficient $CD8^+$ T cells, demonstrating that a co-stimulatory member of the TNFR family activates the

AKT/NFκB signaling axis, in a manner that cannot be replaced by CD28 signaling. Furthermore, blockade of IL-2 signaling (using anti-IL-2Rβ) during the first 10 hrs of culture did not abrogate the phosphorylation of AKT for wild-type T cells, indicating that defective AKT activation observed for TNFR2-deficient T cells was not due to indirect effects associated with defective IL-2 production. These data suggest that the TNFR2 pathway is not coupled to TCR-proximal signaling events or MAPK family cascades, but rather acts through a distinct signal to activate the AKT/NFκB pathway. The observation that TNFR2 is required for sustaining AKT phosphorylation at 10 hrs of TCR/CD28 stimulation suggests that TNFα is produced during the first 10 hrs of activation and then signals in an autocrine fashion through TNFR2. If this were the case, then we should be able to block AKT activation in WT cells by adding a neutralizing anti-TNFα Ab during the first 10 hr of activation. We used two neutralizing anti-TNFα mAbs from commercial sources (see *Materials and Methods*) but neither antibody was effective in inhibiting anti-TCR induced proliferation in WT cells. The lack of availability of an effective neutralizing anti-TNFα Ab prevented us from testing this hypothesis.

The finding that CD28 co-stimulation only led to a marginal increase in the steady-state levels of IL-2 transcript and secreted cytokine in TNFR2-deficient T cells (though leading to a substantial increase in wild-type cells) suggests a novel relationship between these two co-stimulatory molecules, and an interesting functional link between the CD28-related and TNFR families. The marginal increase demonstrated that CD28 was still functional in TNFR2-deficient T cells, and suggests that TNFR2 provides an important contribution relative to CD28 for IL-2 production during T cell activation. The relative signaling from these two co-stimulatory molecules appear to converge at the level of

AKT and NFκB, critical mediators of IL-2 production. The cytoplasmic tail of CD28 is known to contain a binding domain for PI3K, which is immediately upstream of AKT phosphorylation. Further work is required to examine signaling pathways by which TNF receptors and CD28 differentially regulate AKT and NFκB immediately proximal to these cell surface receptors.

Role of TNFR2 in co-stimulation relative to CD28 and other members of the TNFR family

On a temporal basis, CD28 controls the early outcomes of T cell activation by co-stimulating IL-2 induction and anti-apoptotic members of the Bcl-2 family, reflecting its constitutive expression on naive T cells. CD28 is therefore positioned among the earliest co-stimulatory molecules in a temporal model of T cell activation, affecting threshold of signaling required for progression of T cell activation upon TCR engagement such as initial clonal expansion, survival, and IL-2 induction. Engagement of CD28 then leads to efficient expression of TNFR family members (such as OX40, 4-1BB, and CD27) in a temporal sequence of co-stimulatory modulation of the T cell response. The growing number of co-stimulatory members identified in the TNFR family suggests that there are multiple specific and simultaneous signals that function in a temporal manner towards modulating T cell response outcomes (2). The data described in this chapter provide the first case of a co-stimulatory member of the TNFR family acting in the earliest phase of T cell activation, positioned temporally with CD28 in a non-redundant manner to promote the induction of IL-2 and promoting T cell survival. Moreover, this is the first TNFR member identified to have a functional link to CD28 towards IL-2 induction, in contrast

to the temporal link that exists between CD28 and other co-stimulatory members of the TNFR family.

The findings in our study are particularly important given the recent advances in our understanding of the signaling networks that are linked to various co-stimulatory molecules (2). Signaling by CD28 and TNFR family members appear to converge on common intermediates and their respective roles during T cell activation are temporally delineated by kinetics of expression at the cell surface. The constitutive expression of TNFR2 on T cells and subsequent up-regulation early during T cell activation, together with the observation that IL-2 induction is defective in TNFR2-deficient T cells, which cannot be rescued by CD28 co-ligation, strongly argues that TNFR2 is a critical co-stimulatory receptor for early T cell activation events during which T cell fate is determined.

Integral role of AKT as a common signaling mediator of co-stimulatory molecules

AKT activity is functionally associated with TCR/CD28-mediated induction of IL-2, expression of anti-apoptotic members of the Bcl-2 family, and T cell survival (20, 51). Indeed, the important role of AKT in T cell survival was shown by Song *et al.* (2004) using ectopic expression of active and dominant negative variants of AKT (20). A link between co-stimulatory members of the TNFR family and AKT was previously postulated (2). More recently, it was shown that OX40, a member of the TNFR family, functions to maintain AKT activity over time. OX40-deficient $CD4^+$ T cells display normal levels of phosphorylated AKT over the first 24 hrs of antigen-specific stimulation, but do not maintain large amounts of phosphorylated AKT from day 2 to day 4 (20). Sustained AKT

signaling mediated via OX40 is correlated with prolonged cell survival over time. Our observation that AKT phosphorylation is defective in TNFR2-deficient T cells during the first 24 hrs of stimulation suggests that the functional link between co-stimulatory molecules is temporally regulated through AKT as a common signaling mediator. Moreover, the early role of TNFR2 in the AKT/NFκB pathway during T cell activation suggests that it is an important regulatory point for initiation and progression of the T cell response. CD28, OX40 and now TNFR2 are therefore identified as co-stimulatory molecules that are linked with the AKT pathway to exert discrete effects (i.e. IL-2 induction, clonal expansion, and survival) during the T cell response. Each co-stimulatory molecule acts in a temporally distinct manner based on their peak expression on the cell surface suggesting critical check points during T cell activation.

Regulation of T cell survival by TNFR2 during T cell activation

T cell fate decisions are dependent on the signals that are provided during antigenic challenge, which regulate pathways involved in cell survival and death. CD28-mediated co-stimulation was thought to be the primary signal for commitment of responding T cells to clonal expansion, IL-2 production, and survival, depending on the strength of TCR-mediated signals (11). An inadequate level of signaling (i.e. sub-threshold) via CD28 and TCR-mediated signals leads to either anergy or death (1), whereas a sufficient level of signaling through these pathways leads to the induction of co-stimulatory members of the TNFR family, which subsequently functions to sustain the T cell response by promoting survival (2). We found in our study that TNFR2 functions at early time points during TCR/CD28-mediated activation towards T cell fate outcome early during activation by controlling

survival. The data suggest that the progression of the T cell response beyond day 1 is largely dependent on TNFR2. Indeed, TNFR2-deficient $CD8^+$ T cells show an increased propensity towards apoptosis during TCR/CD28-mediated stimulation, leading to reduced clone size. This suggests that the balance between life and death in controlling T cell fate outcomes is determined by early signals provided by TCR, CD28 and TNFR2.

The expression of Bcl-X_L was upregulated as early as 10 hrs after TCR/CD28-mediated stimulation, and was completely abrogated in TNFR2-deficient $CD8^+$ T cells. A previous study by Noel *et al.* (15, 49) showed that anti-CD3 treatment led to Bcl-X_L expression, and that CD28 co-ligation led to even greater levels of Bcl-X_L after 24 hrs of stimulation. The same study also showed that CD28-deficient T cells and CTLA4-Ig treatment displayed a substantial decrease in Bcl-X_L expression after 24 hrs of stimulation. Our study examined Bcl-X_L expression after 9 hrs of stimulation, showing that anti-CD3 treatment in wild-type $CD8^+$ T cells led to expression of Bcl-X_L and that CD28 co-ligation was additive towards greater expression. Although CD28 co-ligation led to an increase in expression, the level of Bcl-X_L was still significant after 9 hrs of anti-CD3 treatment alone, conditions that would be similar to examining CD28-deficient T cells at the same early time point. However, TNFR2-deficient T cells displayed a striking abrogation of Bcl-X_L expression when treated with either anti-CD3 alone or in combination with anti-CD28. This suggests that TNFR2 is critical for co-stimulating Bcl-X_L expression with the TCR complex during the very early phase of T cell activation.

Overall, the data show that TNFR2 acts as one the earliest of the identified co-stimulatory members of the TNFR family, functionally linked to CD28 during the early phase of the T cell response. TNFR2-deficiency in $CD8^+$ T cells led to specific biochemical

defects in the activation of AKT, NFκB, and Bcl-X_L as early as 10 hrs after TCR/CD28-mediated stimulation. The importance of TNFR2 during TCR/CD28-mediated stimulation reveals an important framework of cell surface molecules from distinct receptor families towards crucial activation events such as IL-2 induction, clonal expansion, and survival. It also points to the importance of the temporal and environmental context in dictating T cell fate outcomes during the early stages of activation.

3.6 Acknowledgements

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3.7 Figures

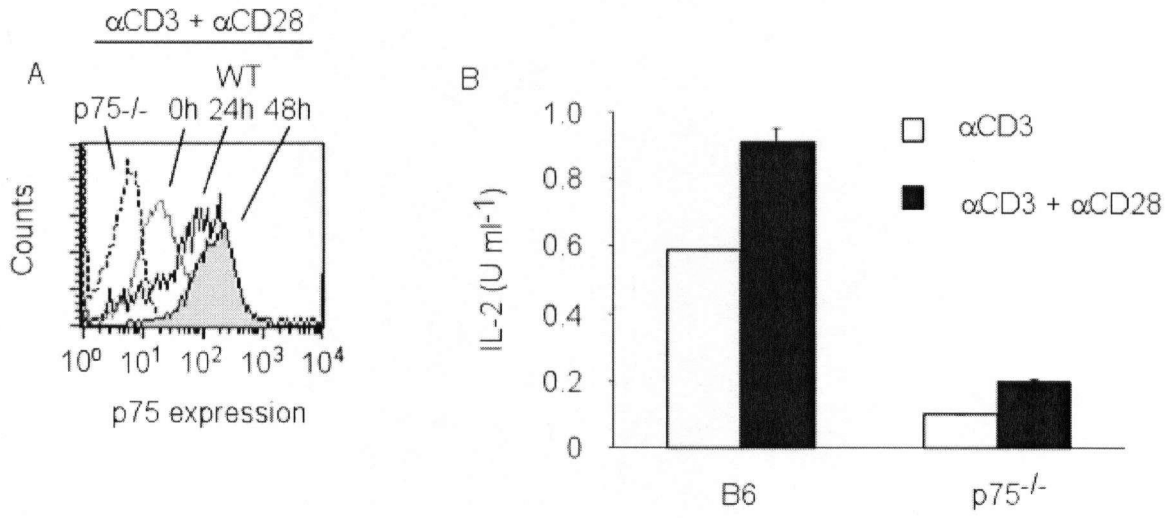

Figure 3.1 *A*) TNFR2 expression on naive and activated T cells. *B*) IL-2 production in response to TCR/CD28-mediated stimulation is defective in TNFR2-deficient $CD8^+$ T cells.

A) $CD8^+$ T cells ($1x10^6$) were stimulated with 10 μg/ml of plate-bound anti-CD3 in combination with soluble anti-CD28 (10 μg/ml) for various times, harvested and subjected to analysis by flow cytometry (see *Materials and Methods*). Dotted line = TNFR2-deficient T cells, grey line = immediately ex vivo WT T cells, black line = WT T cells stimulated for 24 hrs, shaded line = WT T cells stimulated for 48 hrs. *B*) $CD8^+$ T cells ($1x10^6$) were stimulated with 10 μg/ml of plate-bound anti-CD3 alone, or in combination with anti-CD28 (10 μg/ml). Cytokine ELISA was performed on the culture supernatants (see *Materials and Methods*). The data points denote mean ± standard deviation of triplicate determinations.

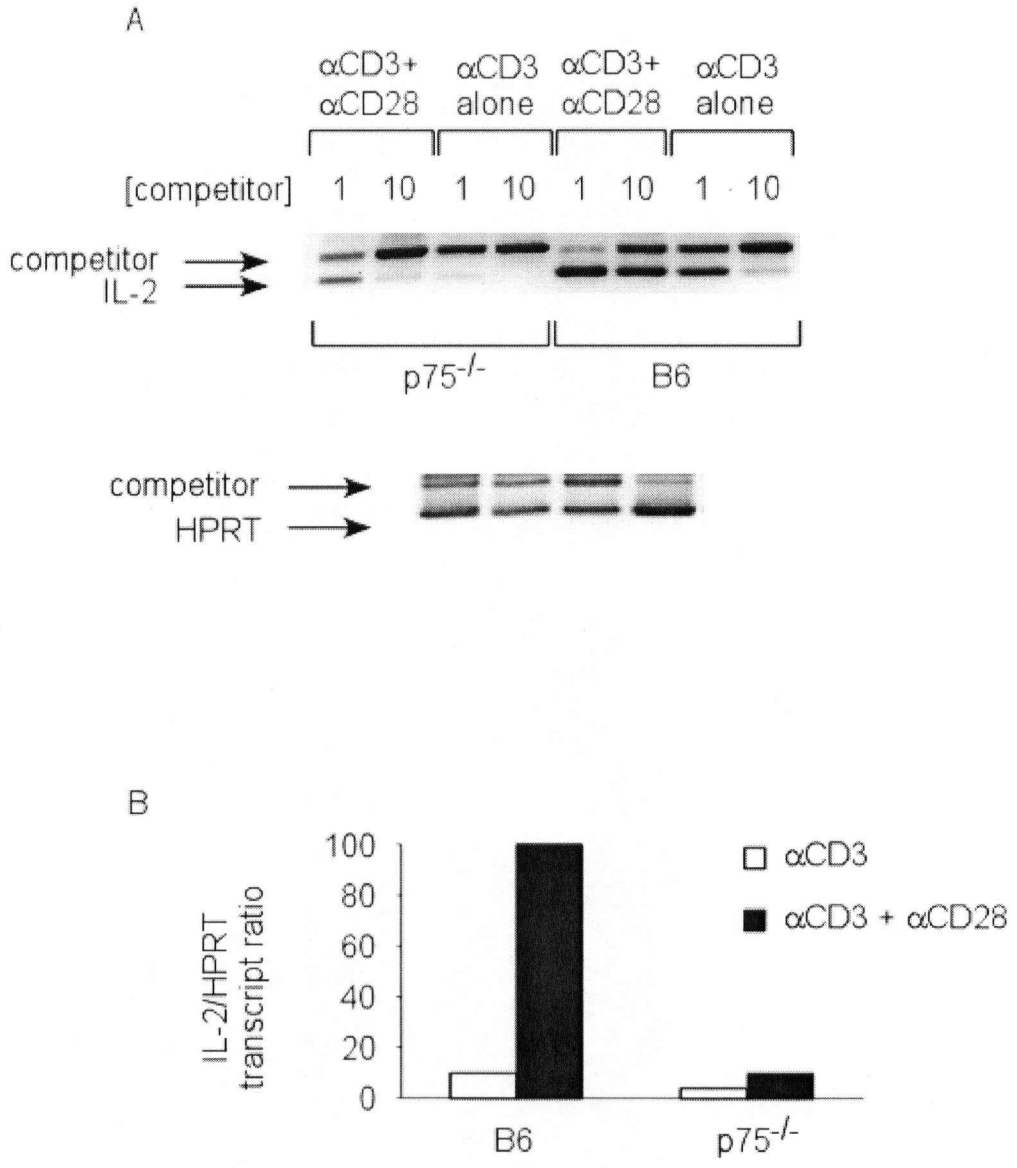

Figure 3.2 TNFR2-deficient $CD8^+$ T cells display a marked reduction in steady-state levels of IL-2 transcript in response to TCR/CD28-mediated stimulation.

A) $CD8^+$ T cells ($2x10^6$) were stimulated with 10 μg/ml of plate-bound anti-CD3 alone, or in combination with anti-CD28 (10 μg/ml). Total RNA was harvested, reverse transcribed, and subsequently analyzed using CQ-PCR (see *Materials and Methods*). Numerical values (i.e. 1 and 10) refer to the concentration of competitor relative to RNA sample in each lane. *B*) The IL-2/HPRT transcript ratios of WT and TNFR2-deficient $CD8^+$ T cells stimulated with either αCD3 alone, or in combination with αCD28. Abbreviations used: comp = competitor, HPRT = hypoxanthine phosphoribosyl transferase. Data is representative of one of three independent experiments performed.

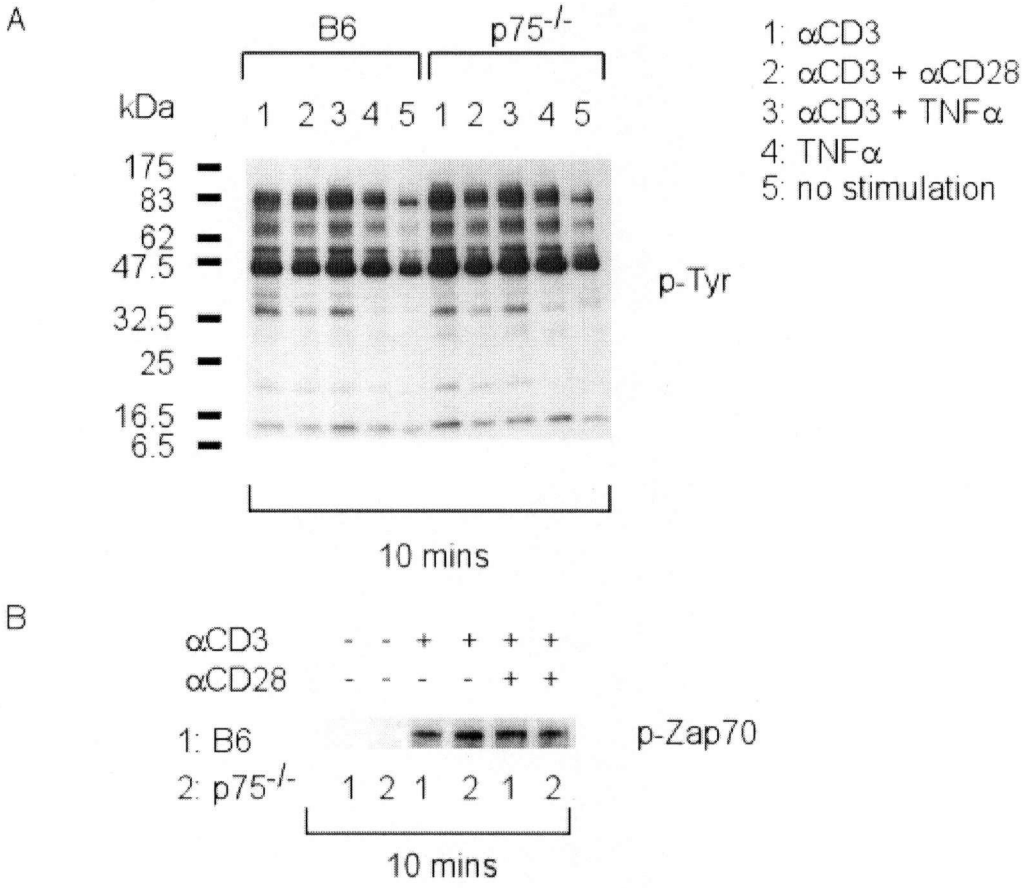

Figure 3.3 TCR-proximal signaling events are largely unaffected by TNFR2-deficiency.

A) Global tyrosine phosphorylation is unaffected by TNFR2-deficiency. $CD8^+$ T cells ($2x10^6$) were stimulated for 10 mins with 10 μg/ml of plate-bound anti-CD3 alone, or in combination with anti-CD28 (10 μg/ml) and TNFα (10 ng/ml). Whole cell lysates were prepared, resolved by SDS-PAGE, and subjected to western blot (see *Materials and Methods*). B) Zap-70 phosphorylation is unaffected by TNFR2-deficiency. $CD8^+$ T cells ($2x10^6$) were stimulated for 10 mins with 10 μg/ml of plate-bound anti-CD3 alone, or in combination with anti-CD28 (10 μg/ml). Whole cell lysates were prepared, resolved by SDS-PAGE, and subjected to western blot (see Materials and Methods). Data are representative of three independent experiments.

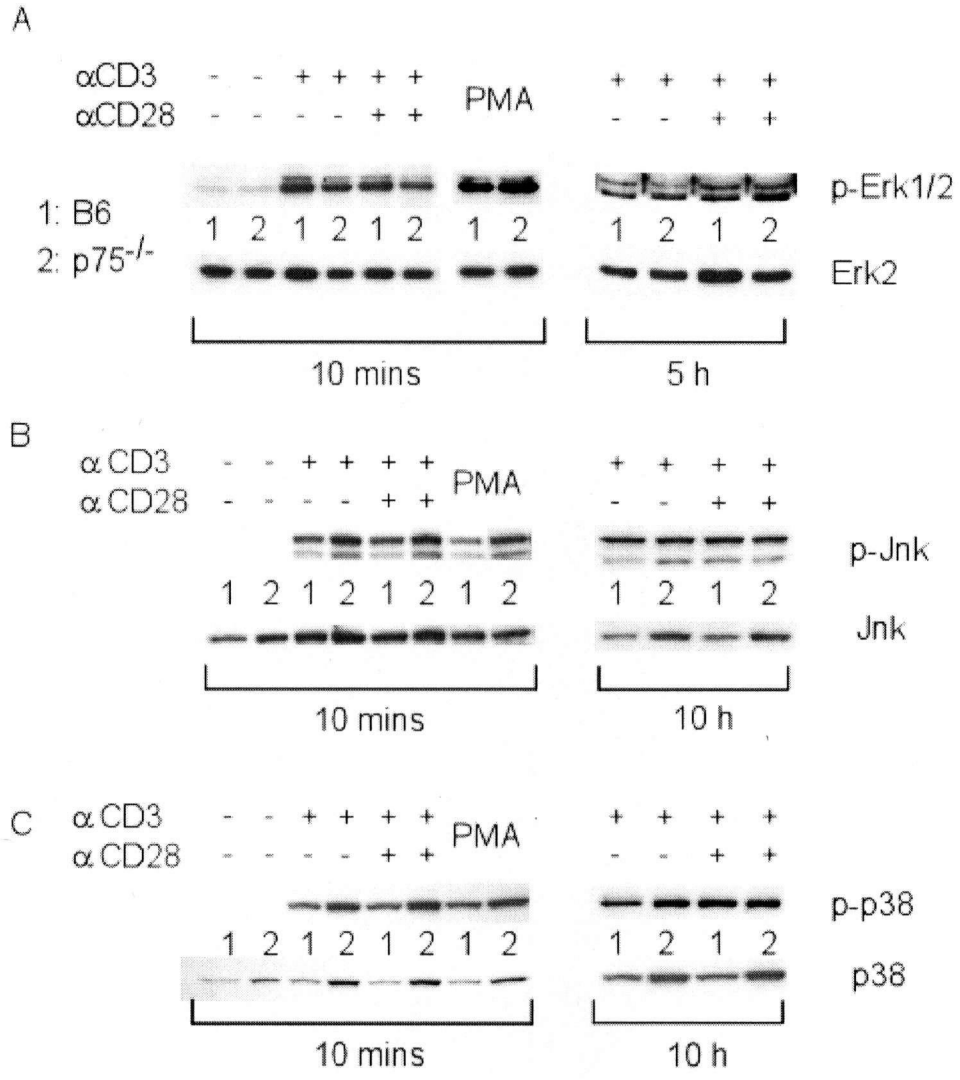

Figure 3.4 MAPK activation is unaffected by TNFR2-deficiency.

$CD8^+$ T cells ($2x10^6$) were stimulated with 10 μg/ml of plate-bound anti-CD3 alone, or in combination with anti-CD28 (10 μg/ml). PMA (10 ng/ml) stimulation was used as positive control. Whole cell lysates were prepared, resolved by SDS-PAGE, and subjected to western blot (see *Materials and Methods*). Data is representative of three independent experiments.

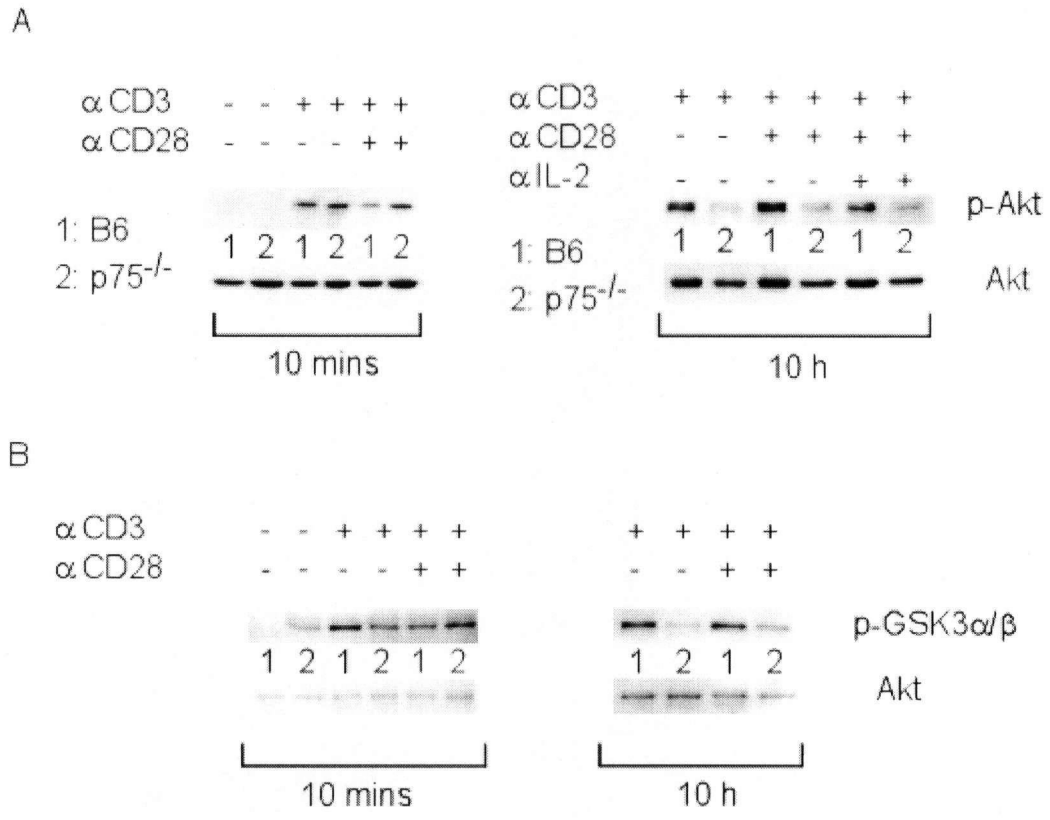

Figure 3.5 AKT activation is defective in TNFR2-deficient $CD8^+$ T cells.

A) AKT phosphorylation is defective in TNFR2-deficient T cells. $CD8^+$ T cells ($2x10^6$) were stimulated with 10 μg/ml of plate-bound anti-CD3 alone and in combination with anti-CD28 (10 μg/ml) or anti-IL2Rβ (20 μg/ml). Whole cell lysates were prepared, resolved by SDS-PAGE, and subjected to western blot (see *Materials and Methods*). *B*) AKT activity is defective in TNFR2-deficient T cells. $CD8^+$ T cells ($2x10^6$) were stimulated with 10 μg/ml of plate-bound anti-CD3 and anti-CD28 (10 μg/ml) for 10 hrs and total cell extracts were subsequently prepared. AKT was immunoprecipitated, incubated with cold ATP and GSK-3α/β fusion protein substrate, and phosphorylated GSK-3α/β was measured using western blot (see *Materials and Methods*). Data is representative of three independent experiments.

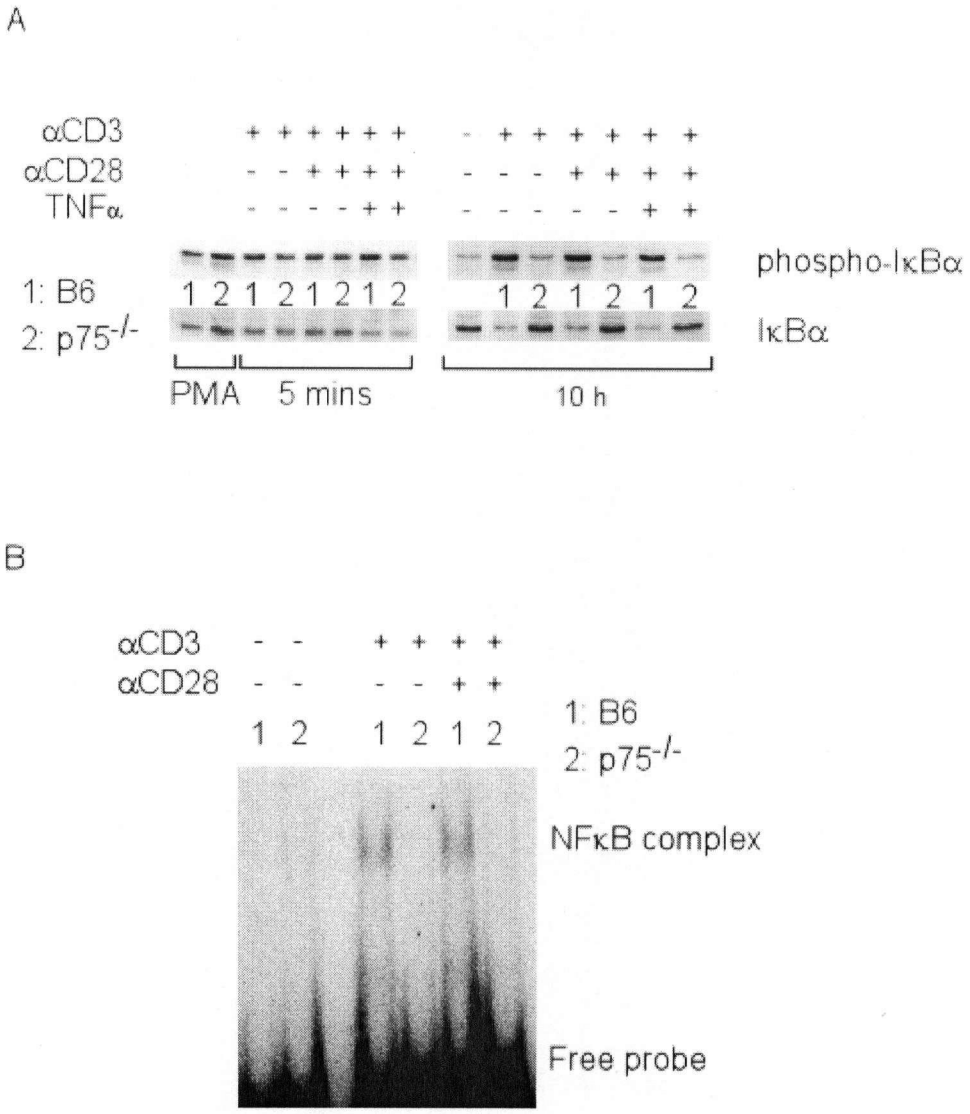

Figure 3.6 *A*) IκBα phosphorylation and subsequent degradation is defective in TNFR2-deficient $CD8^+$ T cells. *B*) NFκB activation is defective in TNFR2-deficient $CD8^+$ T cells.

A) $CD8^+$ T cells ($2x10^6$) were stimulated with 10 μg/ml of plate-bound anti-CD3 alone, or in combination with anti-CD28 (10 μg/ml) and TNFα (10 ng/ml). Whole cell lysates were prepared, resolved by SDS-PAGE, and subjected to western blot (see *Materials and Methods*). *B*) $CD8^+$ T cells (2×10^6) were treated with 10 μg/ml of plate-bound anti-CD3 alone or in the presence of anti-CD28 (10 μg/ml) for 8 hrs and nuclear extracts prepared (see *Materials and Methods*). Nuclear extracts were incubated with radio-labeled probe containing NFκB binding sites, and NFκB activation was determined using a gel shift assay as described in Materials and Methods. Data is representative of three independent experiments.

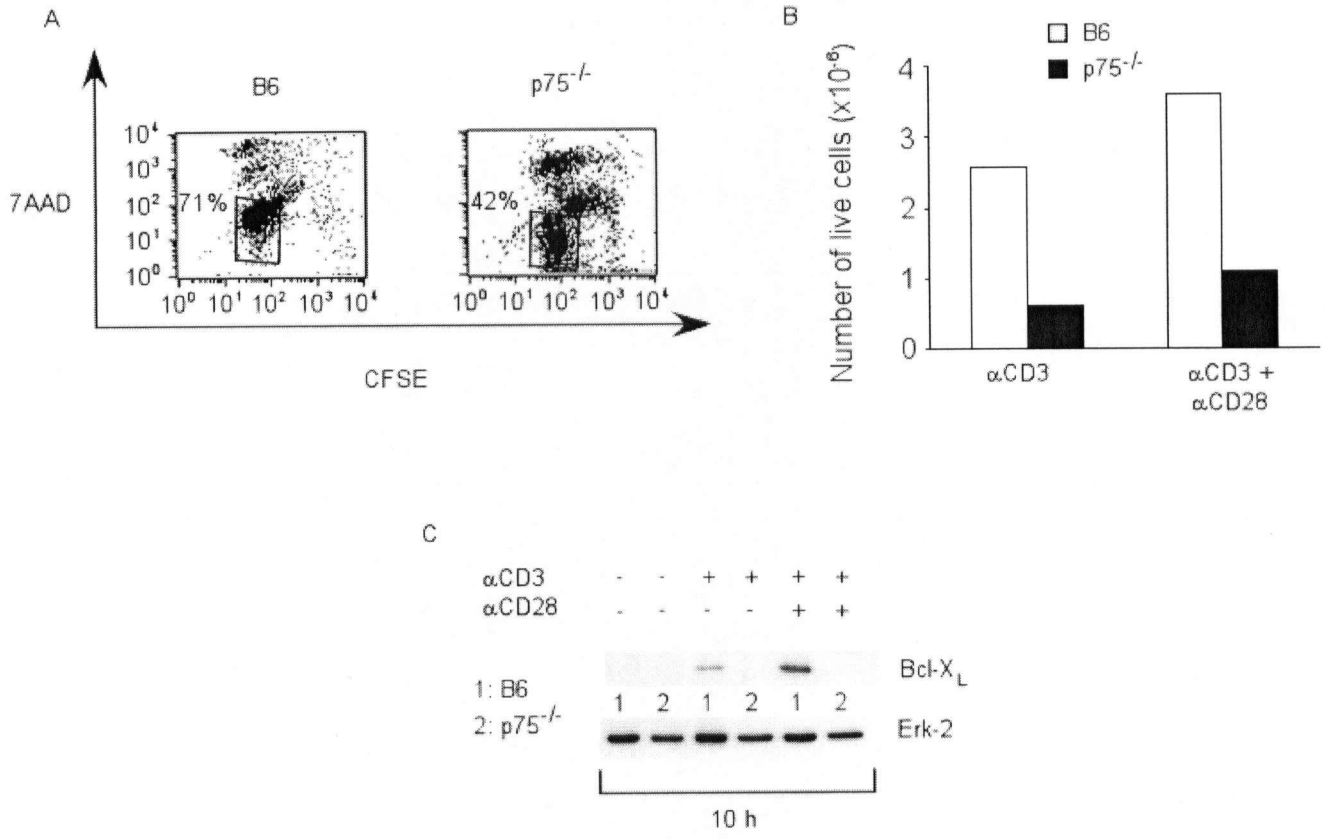

Figure 3.7 Early survival and expression of Bcl-X_L is defective in TNFR2-deficient $CD8^+$ T cells.

A) CFSE-labeled $CD8^+$ T cells ($1x10^6$) were treated with 10 μg/ml of plate-bound anti-CD3 and soluble anti-CD28 (10 μg/ml) for 65 hrs, harvested and stained with 7-AAD, and analyzed by flow cytometry (see Materials and Methods). *B*) Total live cell counts in culture after 48 hrs of stimulation with 10 μg/ml of plate-bound anti-CD3 and soluble anti-CD28 (10 μg/ml). C) $CD8^+$ T cells ($2x10^6$) were stimulated with 10 μg/ml of plate-bound anti-CD3 alone, or with soluble anti-CD28 (10 μg/ml) for 9 hrs. Whole cell lysates were prepared and Bcl-X_L expression was analyzed using western blot. Data is representative of three independent experiments.

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Chapter 4 Tumor necrosis factor receptor type-2 (p75) functions as a co-stimulator for antigen-driven T cell responses *in vivo* [1]

4.1 Preface

Naive T cells require co-stimulation for robust antigen-driven differentiation and survival. Members of the TNFR family have been shown to provide co-stimulatory signals conferring survival at distinct phases of the T cell response. This chapter examines the dependency of CD4 and CD8 T cells on TNFR2 for survival during Ag-driven clonal expansion *in vitro* and *in vivo*. Using the MHC class I-restricted 2C TCR and MHC class II-restricted AND TCR transgenic systems, TNFR2 was shown to regulate threshold for clonal expansion of CD4 and CD8 T cell subsets in response to cognate antigen *in vitro*. Using a novel recombinant *Listeria monocytogenes* (rLM-SIY) expressing a secreted form of the 2C agonist peptide (SIY) to investigate the role of TNFR2 for T cell immunity, TNFR2 was shown to allow larger accumulation of effector cells and conferred protection from apoptosis for a robust memory pool *in vivo*. $TNFR2^{-/-}$ CD8 T cells exhibited loss of protection from apoptosis that was correlated with diminished survivin and Bcl-2 expression. This chapter describes experiments showing a hitherto unappreciated co-stimulatory function for TNFR2 in regulating T cell survival during the differentiation program elicited by an intracellular pathogen *in vivo*.

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Kim, E.Y., J.J. Priatel, S.J. Teh, H.S. Teh. 2006. TNF Receptor Type 2 (p75) Functions as a Costimulator for Antigen-Driven T Cell Responses *In vivo*. *J. Immunol.* 176:1026-1035.

4.2 Introduction

The adaptive immune response is based on a myriad of spatially and temporally regulated interactions between receptors and ligands. These interactions are mediated by receptors on the T cell surface (TCR, adhesion molecules, cytokine receptors, co-stimulatory receptors), and their counterparts expressed on activated APCs: MHC-peptide antigen complex, cytokines (IL-2, IL-7, IL-15), and co-stimulatory ligands (B7, TNF superfamily members). The expression of these stimulatory ligands is orchestrated in a temporal sequence for effective adaptive immunity. Examination of the minimal requirements for a productive T cell response revealed that brief duration of antigenic stimulation can commit CD8 T cells to several rounds of cell division and concomitant effector/memory differentiation (1, 2). These studies demonstrated that threshold-crossing activation signals triggered by antigenic stimulation induce a differentiation program utilized by T cells for their expansion, effector and memory function. However, recent studies have shown that execution of this program for CD8 T cell differentiation also relies on external signals in addition to antigenic stimuli, indicating a dynamic process that yields flexibility for developing an appropriate adaptive response depending on all variables concerning host defense. Greater duration of antigenic stimulation leads to increased survival of primed T cells for the antigen-independent proliferative phase of the differentiation program (3), demonstrating that TCR-mediated signals provides important input for imprinting cellular survival. External factors such as IL-2 are important for enhancing antigen-independent proliferation after brief priming of CD8 T cells (2), strongly suggesting that external signals can integrate into the program to govern quantitative outcomes during clonal expansion. Given that the extent and

magnitude of clonal expansion is directly related to survival after primary activation (4), a key regulatory modality for the progression of productive antigen-driven T cell response may be derived from both internal (i.e. TCR-mediated) and external (i.e. cytokine-mediated and co-stimulatory) interactions that control survival.

Co-stimulation has been extensively studied as an integral component of the differentiation program during the primary response (5, 6). Two families of co-stimulatory receptors have been identified for T cells. CD28 and TNFR superfamilies have been shown to modulate discrete aspects of T cell function, with each member playing a role in transition points during T cell differentiation. For instance, CD28 plays a role in modulating the threshold of T cell activation, augmenting IL-2 production, and promoting T cell survival in order to sustain T cell responses (6). TNFR superfamily members such as OX40 and 4-1BB have been implicated in the effector to memory cell transition, acting temporally later than CD28 (5, 7). Mice deficient in either of these TNFR family members display a marked decrease in the memory pool due to defects in survival during and after primary expansion (8, 9). These effects have been attributed to the regulation of apoptotic molecules such as survivin and Bcl-2 mediated by co-stimulatory signals during clonal expansion (10). Several recent studies have investigated the role of co-stimulation during infection with LM. CD28-deficient mice have reduced LM-specific CD8 T cell responses compared to wild-type mice, but differentiation into effector and memory T cells appears intact (11). Mice that lack CD137L (4-1BBL) also display diminished activation of CD8 T cells compared to wild-type (12). Thus, co-stimulation mediated by members of two distinct superfamilies of receptors provides

multiple checkpoints in the differentiation program that confers flexibility in the adaptive immune response.

Listeria monocytogenes (LM) is a well-characterized model system for studying T cell-mediated immunity against intracellular bacteria (13). LM rapidly triggers an innate response that is essential for host survival; early resistance is conferred by production of IFNγ and TNFα, as mice lacking these cytokines or their cognate receptors succumb to infection (14, 15). Adaptive immunity is crucial for clearance and long-term protective immunity after LM infection. SCID mice are able to control low-dose LM infection but fail to clear it (16), and mice lacking αβ T cells rapidly succumb to high-dose LM infection within 5 days (17), demonstrating the critical role that T cells play for bacterial clearance. As an intracellular bacterium, LM induces a potent $CD8^+$ T cell response that is critical for anti-Listerial defense (18, 19). MHC class I- and II-restricted epitopes derived from LM have been characterized facilitating the tracking of both $CD4^+$ and $CD8^+$ T cell responses to intracellular bacterial challenge, and provide a powerful system for studying cellular processes and mechanisms that regulate T cell-mediated immunity *in vivo* (13).

We have recently reported that TNF receptor type-2 (TNFR2), also referred to as p75, is an important co-stimulator for T cell activation, that is functionally linked to CD28 for optimal IL-2 induction and survival during the early phase of the T cell response *in vitro* (20, 21). In these studies, we found that $TNFR2^{-/-}$ T cells are defective in IL-2 induction that was only partially rescued by CD28 co-ligation, and exhibit increased susceptibility to apoptosis during the early phase of the proliferative response, thereby reducing the accumulation of polyclonal effector cells *in vitro* (21). A study by

another group confirmed that TNFR2 functions as a co-stimulator for human T cells (22). Similarly, another study found that TNFR2$^{-/-}$ mice display a delayed clearance of replication-deficient adenovirus that correlates with decreased adenovirus-specific CTL activity of intrahepatic lymphocytes. This indicates that TNFR2 facilitates generation of CTL effector function for anti-viral immune responses in the liver *in vivo* (23). These studies show that TNFR2 functions in T cells as an important co-stimulator for the differentiation program triggered by TCR-mediated stimulation. However, the role of TNFR2 for co-stimulating antigen-driven T cell responses is poorly characterized.

To investigate the role of TNFR2 in antigen-driven CD4 and CD8 T cell responses, we utilized two well-characterized TCR transgenic systems that are restricted to either MHC class I (2C) (24, 25) or MHC class II (AND) (26). Our data establish a critical role for TNFR2 as a co-stimulator of antigen-driven T cell responses *in vivo*, and demonstrates a key modality by which progression of the T cell differentiation program can be regulated.

4.3 Materials and Methods

Mice

Breeders for C57BL/6 (B6, H-2^b) and B6-TNFR2-deficient mice were obtained from The Jackson Laboratories (Bar Harbor, ME). B6 mice deficient in TNFR2 have been previously described (27); TNFR2-deficient mice were genotyped using a PCR strategy. Mice 4 to 7 weeks of age were used for all experiments. Breeders for the H-2^b 2C TCR transgenic mice were kindly provided by Dr. D.Y. Loh (then at the Washington University, St. Louis, MO). The 2C TCR transgenic mice (24, 25) were bred onto the C57BL/6 (H-2^b) background, and crossed with TNFR2$^{-/-}$ mice to generate 2C TNFR2$^{-/-}$ mice. B10.AND TCR transgenic mice (26) were obtained from Jackson Laboratories, and subsequently crossed with TNFR2$^{-/-}$ mice to generate AND TNFR2$^{-/-}$ mice. Animal studies were approved by our institutional review board.

Cells

Lymph nodes were harvested and single cell suspensions prepared from each of the mouse lines. The CD4$^-$ CD8$^+$ (CD8$^+$) T cell and CD4$^+$ CD8$^-$ (CD4$^+$) T cell subsets were purified from whole lymph node cell suspensions using miniMACS microbeads (Miltenyi Biotec, Auburn, CA). CD4$^+$ and CD8$^+$ T cells from AND and 2C TCR Tg mice, respectively, were positively selected using a MACS MS$^+$ Separation column and MiniMACS magnet, as per manufacturer's protocol (Miltenyi Biotec), achieving > 95% purity. Splenocytes from B6 mice were irradiated for use as APCs to present the SIYRYYGL peptide (abbreviated as SIY) in complex with H-2K^b MHC class I molecules, and alone as feeder cells as indicated.

The SIY/K^b complex is a cognate ligand for the 2C TCR (25). The DCEK cell line was used as antigen presenting cells for displaying PCC peptide (amino acid residues 88-104) in complex with I-E^k MHC class II molecules and is recognized by the AND TCR (26). Cells were cultured at 37°C and 5% CO_2 in Iscove's DMEM (Life Technologies, Burlington, Ontario) supplemented with 10% (v/v) FBS (Life Technologies), 5 x 10^{-5} μM 2-ME, and antibiotics (I-media).

Antibodies and intracellular staining

Antibodies against CD4, CD8, 2C TCR (clone: 1B2 mAb), Thy1.1, Thy1.2, and IFNγ were from eBioscience, anti-survivin (NB500-201) was from Novus Biologicals, and anti-Bcl-2 was from BD Pharmingen. Cell staining and flow cytometry were performed according to standard procedures. Briefly, cells were incubated with the relevant antibodies for > 15 min at 4°C, and subsequently washed twice with FACS medium (PBS + 2% FCS). Annexin V-PE (BD Biosciences) staining was carried out at RT for 15 minutes in saline containing 10 mM HEPES (pH 7.2) and 2.5 mM $CaCl_2$. For intracellular cytokine staining, cells were incubated in a fixation/permeabilization solution (2% paraformaldehyde, 0.2% Tween20 in PBS). Anti-cytokine-, CD8-, and Thy1.2-specific antibodies were added to cells in PBC containing 0.2% Tween20. The CELLQUEST software program (Becton Dickinson, Mountain View, CA) and FACScan was used for data acquisition and analysis.

Generation of rLM-SIY and infection

A recombinant *Listeria monocytogenes* (rLM-SIY) was constructed to express a secreted form of an SIY-bearing peptide (Priatel *et al.*, manuscript in preparation). Briefly, the Ag

cassette containing the SIY-peptide, a sequence previously shown to induce strong anti-SIY responses (28), was introduced into the bacterial genome by homologous recombination as previously described (29). Mice were infected by intravenous injection of the tail vein with indicated doses in PBS. Bacterial doses were determined by plating the injected stock on brain-heart infusion agar.

In vitro re-stimulation for quantification of T cells specific for epitopes derived from LM

Splenocytes from infected and uninfected mice were harvested at various time points p.i. and re-stimulated with SIY, $LLO_{190-201}$, or anti-CD3 (plate-bound, 10 μM) for 5 hrs in the presence of golgi inhibitor (GolgiStop; BD Biosciences, San Jose CA). A total of $5x10^6$ splenocytes were cultured in 24-well flat-bottom tissue culture plates. Expression of IFNγ was determined using intracellular FACS staining (see above for details), which was used as a marker for epitope-specific T cells. Adoptive transfer experiments included the use of additional tracking markers: congenic Thy1.2 and clonotypic 2C TCR transgenic (detected by mAb 1B2).

4.4 Results

TNFR2 regulates threshold for clonal expansion of antigen-specific CD8 and CD4 T cells

2C is a well-characterized transgenic TCR that recognizes cognate antigen (SIY peptide) in the context of MHC class I (K^b) (24, 25) We used this transgenic system to investigate whether TNFR2 plays an important role for the CD8 T cell response to cognate antigen. Using an assay in which the numbers of peptide-loaded APCs (irradiated B6 splenocytes) was titrated with proportionate numbers of feeder cells (irradiated B6 splenocytes not coated with peptide) so as to keep total cell number in culture constant, we found that 2C $TNFR2^{-/-}$ CD8 T cells are hypo-responsive relative to wild-type at limiting Ag conditions. As shown in Fig. 4.1*A*, 2C CD8 T cells initiated a program of clonal expansion in response to 1 x 10^5 peptide loaded APCs (p-APCs), undergoing multiple rounds of cell division. In contrast, 2C $TNFR2^{-/-}$ CD8 T cells failed to undergo any cell division at this dose. This is consistent with our previous findings that TNFR2 lowers the threshold of T cell activation (20). Increasing antigenic stimulation (i.e. dose of peptide-loaded APCs) results in an increase in the proportion of cells that crossed this threshold to initiate a program of CD8 T cell differentiation and cell division, such that 2C $TNFR2^{-/-}$ CD8 T cells required excess p-APCs to undergo robust proliferation. Although 2C $TNFR2^{-/-}$ CD8 T cells underwent multiple rounds of cell division when stimulated with 4 x 10^5 p-APCs, we noted that the total number of cells in culture was diminished compared to wild-type (Fig. 4.1*B*) suggesting that the survival of CD8 T cells undergoing cell division may be controlled by TNFR2.

AND is a well-characterized transgenic TCR that recognizes cognate antigen (PCC_{88-104}) in the context of MHC class II (H-2 I-E^k) (26). To determine whether TNFR2 is important for co-stimulating CD4 T cell response to cognate antigen, the peptide dose was titrated in culture with APCs (DCEK cell line expressing MHC Class II-E^k) in the presence or absence of exogenous IL-2. As shown in Fig. 4.1*C*, AND $TNFR2^{-/-}$ CD4 T cells failed to undergo clonal expansion at the lowest Ag dose tested (0.01 μM), whereas AND CD4 T cells underwent multiple rounds of cell division. AND $TNFR2^{-/-}$ CD4 T cells required greater doses of cognate antigen to achieve an equivalent response as wild-type counterparts, demonstrating a role for TNFR2 in regulating threshold for clonal expansion. Interestingly, exogenous IL-2 provided only a modest increase in the clonal expansion of AND $TNFR2^{-/-}$ CD4 T cells at the lowest Ag dose, whereas AND CD4 T cells underwent robust cell division (Fig, 4.1*C*). These data demonstrate that TNFR2 is an important co-stimulatory molecule for CD4 and CD8 subsets in regulating the threshold for clonal expansion in response to cognate antigen.

CD8 T cells depend on TNFR2 for survival during the early phase of the T cell response in response to recombinant Listeria monocytogenes (rLM-SIY)

Listeria monocytogenes (LM) is a well-studied intracellular pathogen infection model for measuring T cell responses *in vivo*. We engineered a recombinant LM that expresses the 2C agonist peptide (SIY) (Priatel *et al*, manuscript in preparation) to investigate whether TNFR2 functions as a co-stimulator for T cell responses to intracellular pathogen *in vivo*. Following adoptive transfer of CFSE-labeled 2C or 2C $TNFR2^{-/-}$ CD8 Thy1.2 T cells into B6-Thy1.1 mice, rLM-SIY was used to infect hosts

(10^4 CFU) and spleens were subsequently harvested on days 3 and 7 post-infection (Figs. 4.2*A* and *B* respectively). Adoptively transferred cells were tracked using the congenic marker Thy1.2 and the transgenic 2C TCR (with mAb 1B2), analyzed for cell division using CFSE, and differentiation into effectors using intracellular cytokine staining of IFNγ. Rounds of cell division by 2C Thy1.2 CD8 T cells in the spleens in response to rLM-SIY were detected by CFSE on day 3 post-infection. The wild-type population comprised ~0.58% of the total spleen (Fig. 4.2*A*), or ~$90x10^4$ 2C CD8 T cells (Fig. 4.2*C* - total numbers of transferred cells per spleen). In contrast, 2C TNFR2$^{-/-}$ Thy1.2 CD8 T cells exhibited a marked reduction in clonal expansion, comprising only 0.13% of the total spleen of infected mice, or ~$20x10^4$ 2C TNFR2$^{-/-}$ CD8 T cells (Fig. 4.2*C*), resulting in ~4.5-fold reduction in clone size compared to wild-type. However, CFSE dilution analysis showed that 2C TNFR2$^{-/-}$ CD8 T cells were able to undergo multiple rounds of cell division at this dose of rLM-SIY infection; the diminished clone size therefore suggests that TNFR2 controls T cell survival once the program of cell division is initiated. Interestingly, the differentiation program for transiting 2C TNFR2$^{-/-}$ CD8 T cells into effectors appeared intact since surviving cells expressed IFNγ after brief re-stimulation with SIY peptide *in vitro* (Fig. 4.2*A*). These data indicate that TNFR2 regulates the accumulation of effector cells in response to intracellular pathogen, and suggest that the survival of CD8 T cells during the first rounds of cell division is dependent on TNFR2.

We next quantified the frequency of adoptively transferred cells at the peak of the T cell response to LM challenge (one week post-infection) (13). Examination of the numbers of Thy1.2$^+$ cells in infected spleens at one week post-infection revealed a

significant reduction in 2C TNFR2$^{-/-}$ CD8 T cells (1.02% vs. 0.33%; Fig. 4.2*C*). Whereas 2C CD8 T cells continued to expand to reach peak numbers at one week post-infection (~150x10^4 2C Thy1.2$^+$ CD8 T cells), the frequency of 2C TNFR2$^{-/-}$ CD8 T cells in the spleens of infected mice was reduced ~5-fold (Fig. 4.2*C*). 2C CD8 T cells underwent multiple rounds of cell division as demonstrated by complete dilution of CFSE. Strikingly, 2C TNFR2$^{-/-}$ CD8 T cells were virtually devoid of this clonally expanded population, suggesting a loss of protection from apoptosis during expansion (Fig. 4.2*C*). This dramatic defect is highlighted by the substantial reduction in the frequency of 2C TNFR2$^{-/-}$ IFNγ$^+$ CD8 effector cells in the spleens of infected mice. Furthermore, the small residual population of TNFR2$^{-/-}$ 2C CD8 T cells present at day 7 p.i. did not produce IFNγ after antigen stimulation (Fig. 4.2B), suggesting that this population is either anergic or antigen-inexperienced.

We next tested the susceptibility of TNFR2$^{-/-}$ T cells to apoptosis during clonal expansion. Annexin V staining in conjunction with CFSE dilution revealed that a large proportion of 2C TNFR2$^{-/-}$ CD8 T cells undergo cell death during the first few rounds of cell division; virtually all of the cells that were recruited to the dividing population were Annexin V$^+$, whereas wild-type counterparts contained an Annexin V^{neg} population that persisted through multiple rounds of division (Fig. 4.2*D*). Thus TNFR2 confers protection from apoptosis during the first rounds of cell division. In this staining we noted a population of Annexin V$^+$ cells from spleens of infected wild-type mice, which we believe correspond to T cells that die by attrition early during the response to LM. Adoptively transferred cells in uninfected mice remained CFSE^{hi} and Annexin V^{neg} (data not shown). This is consistent with previously reported observations of selective

depletion of T cells en masse via apoptosis in the spleens of mice infected with pro-inflammatory pathogens such as LM (30). Our results suggest that co-stimulation mediated via TNFR2 is important in rescuing Ag-specific T cells from generalized depletion that occurs during the early stages of LM infection.

We previously showed that TNFR2 lowers the threshold of T cell activation and survival (21), and so we investigated whether increasing antigenic doses of primary challenge with rLM-SIY would increase the proportion of 2C TNFR2$^{-/-}$ CD8 T cells that survive to form the effector pool. As shown in Fig. 4.3*A*, increasing doses in log increments of LM infection led to increased numbers of 2C and 2C TNFR2$^{-/-}$ CD8 T cells in the spleens of infected mice on day 4 post-infection. However, TNFR2-deficiency led to a >8-fold reduction in frequency of SIY-specific 2C CD8 T cells even at the highest dose of rLM-SIY tested. CFSE dilution revealed that 2C TNFR2$^{-/-}$ CD8 T cells were able to undergo multiple rounds of cell division (Fig. 4.3*B*), confirming that 2C TNFR2$^{-/-}$ CD8 T cells were activated by rLM-SIY infection but failed to survive during clonal expansion. These data suggest that the antigen-driven differentiation program can be aborted via apoptosis in the absence of TNFR2.

Song *et al.* (2005) recently showed that survivin expression promotes proliferation and antagonizes apoptosis, leading to enhanced accumulation of effector cells (10). T cell survival during antigen-driven response is also dependent on co-stimulation-mediated expression of anti-apoptotic Bcl-2 family members (31, 32), particularly after the phase of cell division (10). We further investigated TNFR2-mediated survival by examining the expression kinetics of anti-apoptotic molecules survivin and Bcl-2 during T cell activation *in vitro* (Fig. 4.4). TNFR2-deficiency led to a dramatic reduction in the

percentage of survivin^{+} CD8 T cells in culture: 87% for WT vs. 67% for TNFR2$^{-/-}$ on day 2; 67% for WT vs. 23% for TNFR2$^{-/-}$ on day 4. Bcl-2 expression was also regulated by TNFR2 during T cell activation, as the percentage of Bcl-2^{hi} CD8 T cells was dramatically lower in TNFR2$^{-/-}$ CD T cells on day 2 of culture: 82% for WT vs. 51% for TNFR2$^{-/-}$. TNFR2 also regulated the proportion of Bcl-2^{hi} T cells as well as Bcl-2 expression per cell on day 4: ~64% of 2C CD8 T cells were Bcl-2^{hi} (MFI of 361) whereas only ~5% of 2C TNFR2$^{-/-}$ CD8 T cells were Bcl-2^{hi} (MFI of 199). These data indicate that TNFR2 regulates the expression of anti-apoptotic molecules that confer survival during and after cell division, and correlates with its role for conferring protection against apoptosis during the antigen-driven T cell response.

TNFR2 is important for endogenous CD4 and CD8 T cell responses to rLM-SIY

The adoptive transfer-infection model above allowed tracking of the response of a clonotypic population of CD8 T cells to rLM-SIY *in vivo*. We were next interested in investigating the endogenous CD4 and CD8 T cell response to epitopes derived from rLM-SIY simultaneously in the same animal. We noted that infection of mice with rLM-SIY elicited robust endogenous responses specific for SIY (MHC class I-restricted peptide) and $LLO_{190\text{-}201}$ (MHC class II-restricted, endogenous peptide derived from listeriolysin O) peptides in host animals (33, 34). This way we could examine the importance of TNFR2 in the endogenous CD8^{+} and CD4^{+} T cell responses to MHC class I- and II-restricted epitopes of rLM-SIY in the same host. TNFR2$^{-/-}$ or wild-type animals were infected with indicated doses of rLM-SIY, and the frequency of SIY-specific CD8^{+} and LLO-specific CD4^{+} T cells were determined by intracellular cytokine staining of

IFNγ in response to brief peptide re-stimulation *in vitro*. We investigated the course of T cell response in wild-type and TNFR2$^{-/-}$ mice to assess the total numbers of CD4^{+} and CD8^{+} T cells specific for epitopes derived from rLM-SIY as a function of time after primary challenge. Examination of the kinetics of CD8^{+} and CD4^{+} T cell responses confirmed the defect in clonal expansion of TNFR2$^{-/-}$ T cells specific for epitopes derived from rLM-SIY, as indicated by dramatic reductions across the time points tested (Fig. 4.5*A* - SIY-specific CD8^{+} T cells; Fig. 4.5*B* - LLO-specific CD4^{+} T cells). Notably, TNFR2$^{-/-}$ mice exhibited very little expansion of SIY-specific CD8^{+} or LLO-specific CD4^{+} T cells between days 3 and 5 post-infection across all doses of infection tested, whereas wild-type counterparts were able undergo robust expansion. This suggests that T cell-mediated immunity may be particularly compromised around day 5 post-infection for TNFR2$^{-/-}$ mice, due to diminished expansion and survival of effectors against intracellular pathogen.

To determine whether the hypo-responsiveness of CD8^{+} and CD4^{+} T cells in TNFR2$^{-/-}$ mice was due to defective antigen presentation, we tested the response of 2C CD8^{+} T cells adoptively transferred into B6 or TNFR2$^{-/-}$ hosts and subsequently infected with rLM-SIY. Transferred cells underwent robust clonal expansion in both wild-type and TNFR2$^{-/-}$ hosts (Fig. 4.6*A* and *B*), indicating that antigen presentation was intact and that the reduction in the T cell response was not due to a T cell-extrinsic defect. Taken together, these data strongly suggest that TNFR2 can regulate the response of CD8^{+} and CD4^{+} T cell subsets to intracellular bacterial pathogen by promoting the accumulation of effector cells and lowering the threshold of antigenic stimulation required for optimal clonal expansion.

TNFR2 is important for immunity against high dose of rLM-SIY challenge

Previous reports have suggested that T cell-mediated immunity is important for clearance of LM infection when challenged with high doses of bacteria (16, 17). We therefore examined bacterial load in the spleens of infected mice to determine whether the substantial reduction in the T cell response observed for TNFR2$^{-/-}$ mice correlates with increased susceptibility to rLM-SIY infection. When challenged with lower doses of rLM-SIY, bacterial load in the spleen peaked around day 3 post-infection in wild-type and TNFR2$^{-/-}$ mice (Fig. 4.7). However, TNFR2$^{-/-}$ mice were more susceptible when challenged with high dose of rLM-SIY; bacterial load persisted in the spleens of TNFR2$^{-/-}$ mice at day 5, whereas wild-type mice were able to clear bacteria. Thus the diminished clonal expansion of CD8 and CD4 T cells specific for epitopes derived from rLM-SIY between days 3 and 5 post-infection correlates with increased susceptibility to infection when challenged with high dose. It also suggests that the bacterial burden on day 5 post-infection provided increased antigenic stimulation in driving expansion of rLM-SIY-specific T cells in TNFR2$^{-/-}$ mice observed at day 7 post-infection, which in turn coincided with clearance of rLM-SIY in the spleens of TNFR2$^{-/-}$ mice.

We were also interested in whether TNFR2-deficiency correlated with increased susceptibility to virulent LM challenge. To address this question we used the wild-type strain of LM, which possesses >10-fold greater virulence than rLM-SIY (data not shown). Consistent with rLM-SIY challenge, TNFR2$^{-/-}$ mice displayed a dramatic decrease in the frequency of LLO-specific CD4 T cell effectors (Fig. 4.8*A*) in the spleens of infected mice, corresponding to a 6-fold reduction in the number of LLO-specific CD4 T cells at the peak of the primary response against wild-type LM compared to wild-type

(Fig. 4.8*B*). Wild-type mice were able to clear bacteria by 1 week post-infection. In contrast, TNFR2$^{-/-}$ mice displayed delayed clearance of bacteria, demonstrated by titers that persisted at day 7 post-infection (data not shown). These data indicate that TNFR2 is important for mounting a strong T cell response to high dose challenge with virulent LM, which correlates with efficient clearance of bacteria from the spleens of infected mice.

Generation of memory T cells against rLM-SIY is compromised in TNFR2$^{-/-}$ mice

After expansion of antigen-specific T cells and concomitant effector function against intracellular pathogen, massive contraction by apoptosis ensues leaving a relatively small population of memory T cells (reviewed in ref. 35). Pope *et al.* (36) showed that the magnitude of the primary response to LM is correlated with the size of the resulting antigen-specific memory population (36). We therefore examined whether the diminished T cell response in TNFR2$^{-/-}$ mice against rLM-SIY would correlate with a decrease in the resulting memory pool at 3 months after primary challenge. As shown in Fig. 4.9*A* and *B* respectively, the frequency of SIY-specific CD8 and LLO-specific CD4 T cells was decreased 3-fold in the spleens of TNFR2$^{-/-}$ mice compared to wild-type. Consistent with results reported by Pope *et al.* (36), we found that increasing the dose of primary challenge led to an increase in the frequency of antigen-specific memory T cells (Fig. 4.9*A* and *B*). The percentage of SIY-specific CD8 and LLO-specific CD4 T cells that survived from the peak of the primary response to form the resulting memory pools were ~13% and 10% for wild-type mice respectively, whereas TNFR2$^{-/-}$ mice were 6% and 4% respectively (Fig. 4.9*C* and *D*, SIY- and LLO-specific memory T cells respectively). Although higher doses of primary challenge with rLM-SIY led to an

increase in frequency of the memory populations, TNFR2$^{-/-}$ mice were still compromised compared to wild-type mice suggesting that TNFR2 promotes survival during the generation of the memory population. These data indicate that TNFR2 can regulate the size of the resulting memory pool against intracellular pathogen by promoting optimal primary expansion and subsequent survival following bacterial clearance.

4.5 Discussion

The antigen-driven T cell differentiation program consists of clonal expansion and concomitant differentiation into effector and memory cells. The threshold of T cell activation is dependent on interactions in addition to those of the TCR/MHC-peptide complexes, namely co-stimulation by molecules such as CD28 (37). Indeed, CD28 has been reported to reduce the time necessary for antigenic stimuli to activate naive T cells, as well as to augment the magnitude of T-cell responses for both naive and primed T cells (38). Previous reports have suggested a molecular link between cell division and effector cytokine expression (39, 40), indicating that T cell immunity is functionally productive once a threshold has been reached for initiating a differentiation program of multiple rounds of cell division and subsequent acquisition of effector function. More recently however, members of the TNFR superfamily (such as OX40 and 4-1BB) have been shown to function at distinct phases during the T cell response (7). Notably, co-stimulation appears to confer T cell survival for driving clonal expansion of cells that progress through the differentiation program (10, 32). These studies broaden the roles for co-stimulation to include temporal and spatial regulation of distinct receptor-ligand

interactions that profoundly influence T cell fate during differentiation by controlling survival.

The T cell response is a dynamic and flexible process that depends on signals that maximize the pool of effectors and formation of memory by conferring survival through 'check points'. Members of two different families of receptors (CD28-related Ig-like superfamily and the TNFR superfamily) appear to govern specific outcomes of T cell fate: CD28 and CD27 appear to function in promoting T cell expansion during the early phase, whereas OX40 and 4-1BB control survival late in the primary response (5, 7). Our data showed that TNFR2 plays a critical role during the early phase of the T cell response by conferring survival during the first rounds of cell division, and thereby regulated the extent of clonal expansion at the peak of the response. Interestingly, the expression of anti-apoptotic molecules survivin and Bcl-2 was dependent on TNFR2 and correlated with protection from apoptosis during and after cell division. Song *et al.* (10) showed that OX40-mediated expression of survivin antagonizes apoptosis during proliferation, whereas Bcl-2 was required for the phase after cell division (10). Anti-apoptotic molecules are therefore common targets of co-stimulatory signals derived from distinct cell surface receptors for controlling survival at distinct phases of the T cell response.

The dramatic reduction in the clone size of antigen-specific TNFR2$^{-/-}$ T cells was associated with increased susceptibility to apoptosis as well as an increased requirement for greater antigenic stimulation (i.e. increased threshold of activation). In the absence of TNFR2, CD4 and CD8 T cells required greater antigenic stimulation and the clone size of effectors was thereby greatly diminished *in vitro*. However, the differentiation program as measured by activation markers such as CD44, CD69, and CD62L appeared intact in

TNFR2$^{-/-}$ T cells (data not shown), indicating that TNFR2 regulated threshold of activation, with surviving T cells retaining the ability to differentiate into effectors. Taken together, our data revealed a hitherto undefined co-stimulatory role for TNFR2 in regulating threshold of activation and early cell survival during antigen-specific T cell responses, regulating quantity rather than quality of effector cells generated.

We tested whether TNFR2-mediated co-stimulation was important for T cell-mediated immunity against LM challenge. Our data showed that TNFR2 conferred a survival advantage for CD4 and CD8 T cells during challenge with LM, whereas null mutants exhibited a dramatic reduction in the T cell response that correlated with increased susceptibility to primary challenge. These findings appear in contrast with previous studies that showed that TNFR2 did not play a significant role in the susceptibility of mice to bacterial challenge (41, 42), as bacterial loads were cleared by day 5 post-infection, and survival of TNFR2-deficient mice was comparable to wild-type control (42). Although these studies identified a critical role for TNFR1 in the inflammatory innate response to LM, they did not analyze the T cell response to LM. The end-points measured in these studies using TNFR2$^{-/-}$ mice (42) are largely attributable to innate immunity. By engineering rLM-SIY system to specifically monitor CD8 T cells specific for secreted epitope derived from intracellular bacteria as well as CD4 T cells specific for the endogenous epitope LLO, our data revealed a critical role for LM-specific CD4 and CD8 T cell expansion stemming from defective survival during the early phase of the response. The smaller pool of effector T cells that resulted from TNFR2-deficiency correlated with increased susceptibility to higher doses of primary LM challenge, which has been previously shown to be dependent on T cell-mediated immunity (16, 17). Our

results are consistent with a recent report that showed an important role for T cell-derived TNFα, which found that TNFα knocked out in the T cell lineage resulted in increased susceptibility to LM challenge, as well as inability to control bacterial load by day 4 post-infection (43). Moreover, our results support the notion that T cell-derived TNFα possesses a distinct function in host defense by providing protection against high bacterial load (43), constituting a second line of defense. We extend this model to suggest that TNF-TNFR2 interactions provide co-stimulation of the primary T cell response against LM, regulating threshold of activation and survival during the generation of effector T cells, which is important for mounting effective T cell-mediated clearance at high dose of LM infection.

The role of co-stimulation during infection has been studied using gene-targeted mice (13). The co-stimulatory role of TNFR2 during LM infection appears similar to CD28. $CD28^{-/-}$ mice possess reduced LM -specific CD8 T cell responses compared to wild-type, but differentiation into effector T cells is intact (11, 12). Moreover, $CD28^{-/-}$ mice exhibit increased susceptibility to LM, as evidenced by persistent bacterial load even up to day 7 post-infection (11, 12). This is in contrast to 4-1BBL-deficient mice that were able to clear bacteria, and the activation of the CD4 T cell compartment appeared intact while the number of Ag-specific CD8 T cells was slightly reduced compared to wild-type mice (12). The phenotypic similarity between CD28 and TNFR2 knockout mice suggests an interesting functional link during the early phase of the T cell response to infection, as we had proposed previously based on commonality of signaling intermediates used for IL-2 induction and T cell survival (21). CD28 and TNFR2 likely perform non-redundant and complementary functions since signaling defects in $TNFR2^{-/-}$

T cells were not rescued by CD28 signaling (21). Two recently characterized co-stimulatory members of the TNFR superfamily, 4-1BB and OX40, were found to have spatially and temporally segregated roles during the T cell response: OX40L-deficient mice exhibited decreased CD4 T cells late in the primary response and no detectable secondary expansion of adoptively transferred CD4 T cells, whereas 4-1BBL-deficiency had a minor effect on the primary response of CD4 T cells, but a more profound affect on the secondary response (44). OX40L/4-1BBL-double knockout mice were impaired in both the CD4 and CD8 T cell responses to protein Ag and influenza virus (44). Our data strongly suggest that TNFR2 is positioned early during the T cell response, regulating clonal expansion by promoting survival during the first rounds of cell division. This role translates into optimal clonal expansion in response to intracellular bacterial pathogens and robust generation of an antigen-specific memory T cell population.

4.6 Acknowledgements

We thank Xiaoxi Chen for assistance with intravenous injections of mice. We also thank Salim Dhanji for helpful discussions.

4.7 Figures

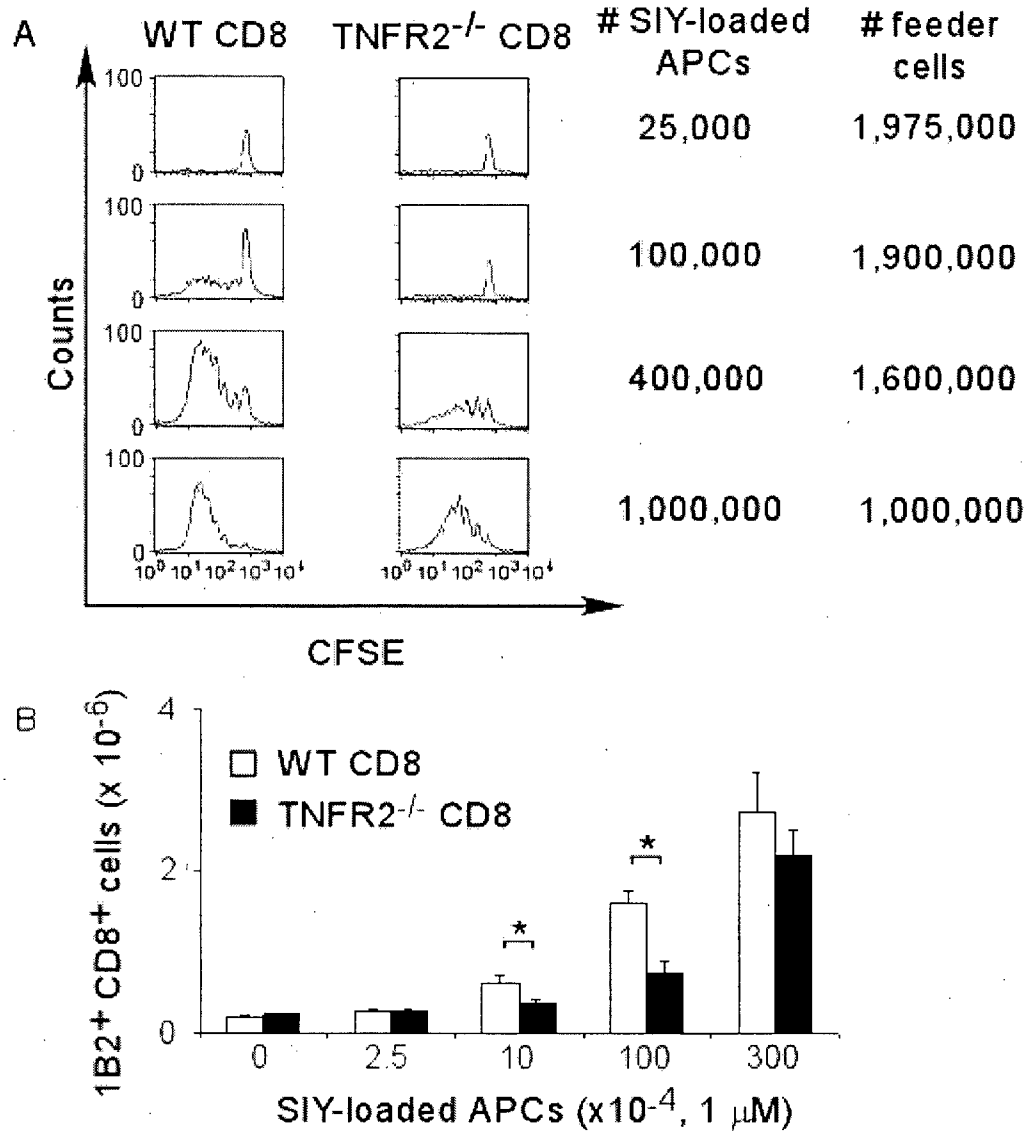

Figure 4.1 TNFR2 regulates clonal expansion of Ag-specific CD8 T cell responses *in vitro*.

A) 2C TNFR2$^{-/-}$ CD8 T cells display defective clonal expansion under antigen-limiting conditions that is associated with increased threshold for cell division and survival. 2C or 2C TNFR2$^{-/-}$ CFSE-labeled CD8 T cells ($1x10^6$) were cultured with indicated ratios of peptide-loaded APCs (irradiated B6 splenocytes pulsed with 1 μM of SIY peptide) and feeder cells (irradiated B6 splenocytes), harvested at day 3 and analyzed by FACS (see *Materials and Methods*). *B*) The total number of 2C (1B2$^+$) TNFR2$^{-/-}$ CD8 T cells in culture was significantly reduced compared to wild-type. The percentage of 1B2$^+$ CD8$^+$ cells was multiplied by total numbers of live cells in culture. Asterisk denotes statistical significance ($p < 0.05$; three independent experiments).

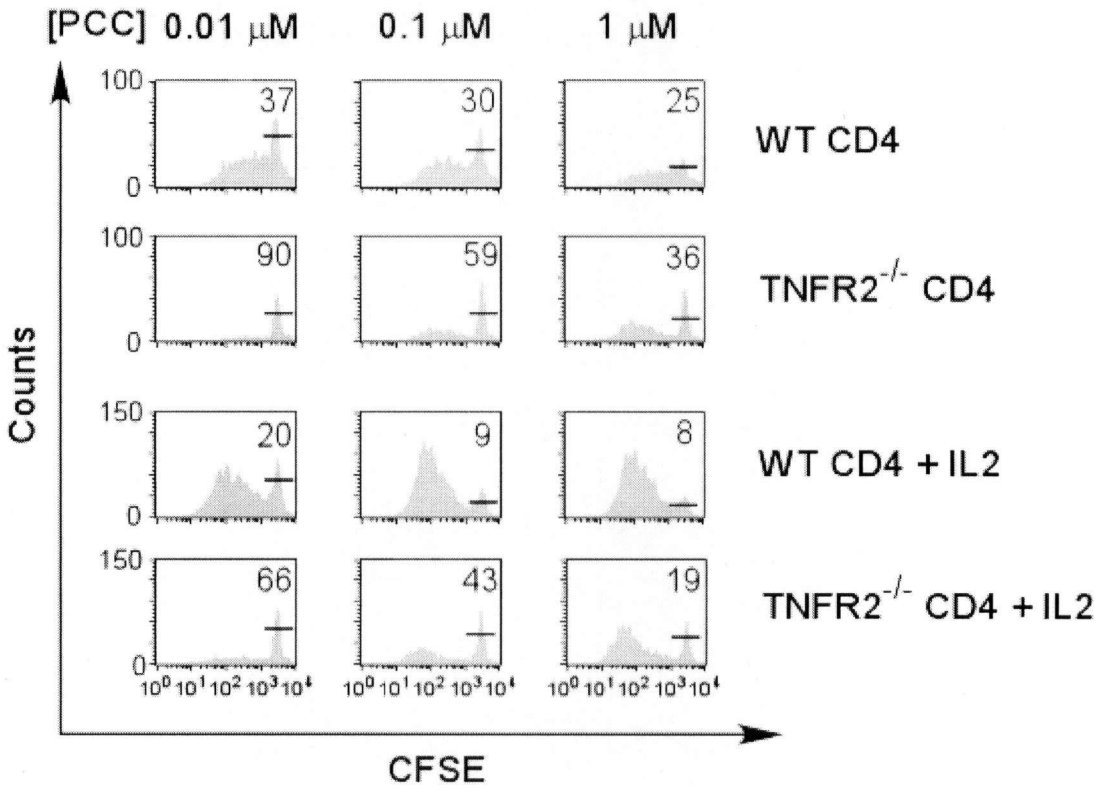

Figure 4.2 TNFR2 regulates clonal expansion of Ag-specific CD4 T cell responses *in vitro.*

AND TNFR2$^{-/-}$ CD4 T cells display defective clonal expansion that is associated with increased threshold for cell division, and only partially rescued by exogenous IL-2. AND or AND TNFR2$^{-/-}$ CFSE-labeled CD4 T cells ($1x10^6$) were cultured with peptide loaded APCs (irradiated DCEK cells pulsed with indicated concentration of PCC peptide), harvested on day 3 and analyzed by FACS (see *Materials and Methods*). Data are representative of three independent experiments.

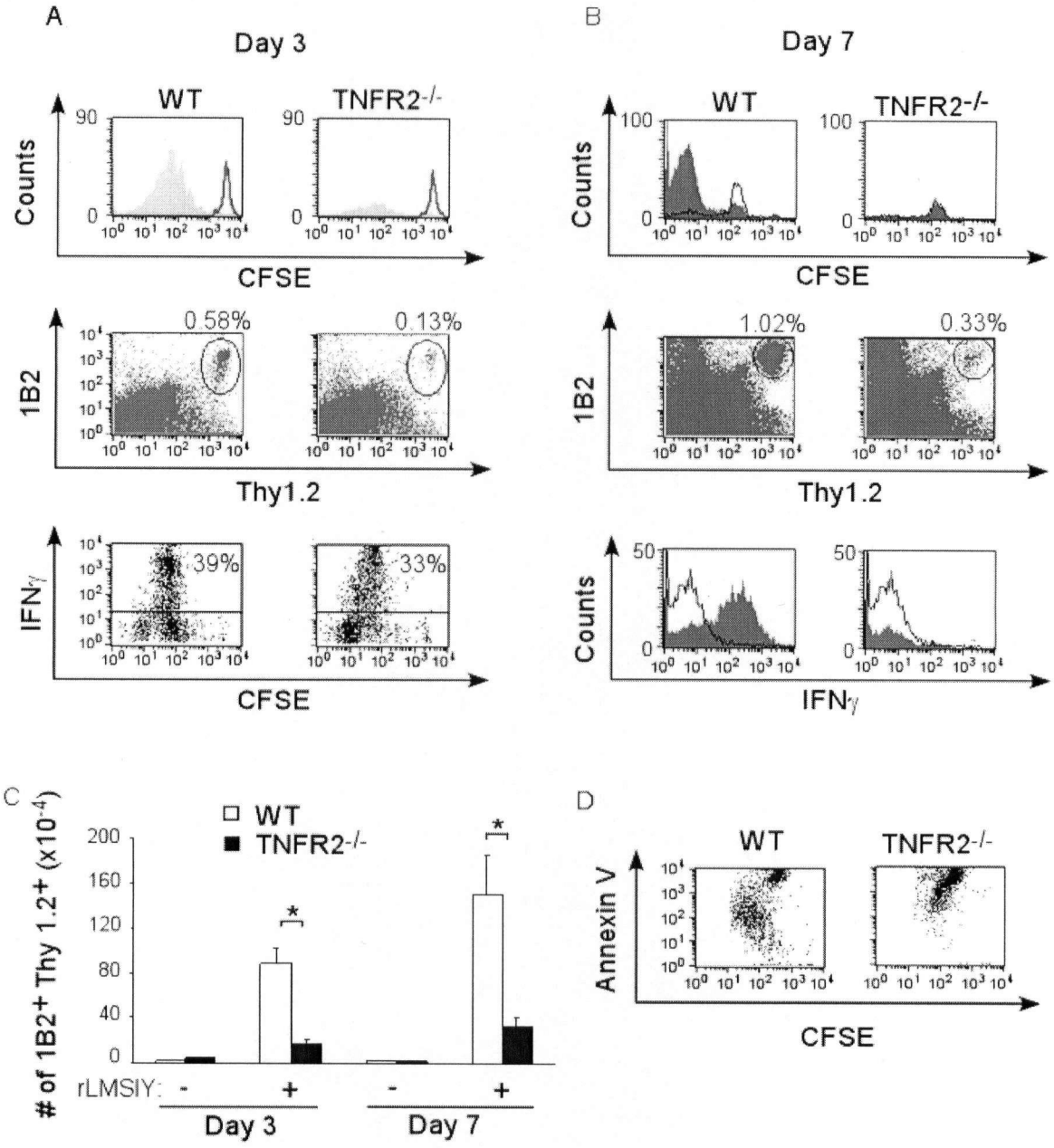

Figure 4.3 TNFR2 is critical for clonal expansion of CD8 T cells in response to secreted peptide expressed by recombinant *L. monocytogenes.*

CFSE-labeled 2C or 2C TNFR2$^{-/-}$ CD8 Thy1.2 T cells ($2x10^6$) were adoptively transferred into B6-Thy1.1 mice (transferred cells were tracked using the congenic marker Thy1.2) and analyzed for cell division using CFSE and differentiation into effectors using intracellular cytokine staining of IFNγ. One day after transfer, rLM-SIY was used to infect hosts (10^4 CFU) and spleens were subsequently harvested on days 3 (Fig. 2A) and 7 (Fig. 2B) post-infection. Uninfected mice were used as negative control, denoted as unfilled lines. *C*) Total numbers of 1B2$^+$ (2C) IFNγ$^+$ CD8$^+$ T cells

were enumerated by multiplying the percentage of the population in the spleen and the total number of viable splenocytes. Asterisk denotes statistical significance (three mice per group). *D*) Loss of protection from apoptosis during the first rounds of cell division in TNFR2$^{-/-}$ CD8 T cells. Spleens were harvested on day 3 post-infection using the adoptive transfer-infection model as above, and CFSE vs. annexin V was analyzed using flow cytometry (gated on Thy1.2^{+} cells, all of which were CD8^{+} 1B2^{+}). Data are representative of three independent experiments.

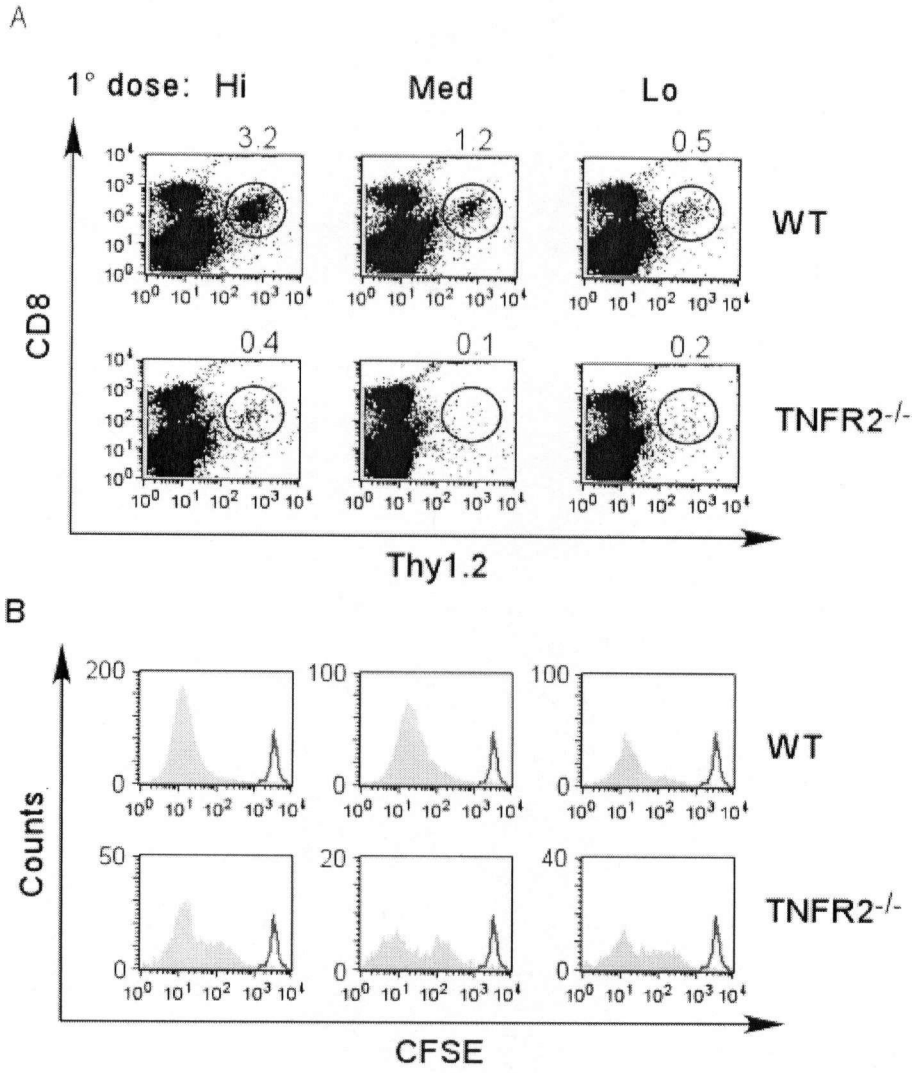

Figure 4.4 TNFR2$^{-/-}$ CD8 T cells display defective survival during the early rounds of division, leading to quantitative reduction of the T cell response to rLM-SIY.

TNFR2$^{-/-}$ CD8 T cells display defective survival during the early rounds of division, leading to quantitative reduction of the T cell response to rLM-SIY. *A*) TNFR2-deficiency in 2C CD8 T cells leads to a marked reduction in response to primary challenge with rLM-SIY. CFSE-labeled 2C or 2C TNFR2$^{-/-}$ CD8 T cells ($3x10^6$) were transferred into unmanipulated B6-Thy1.1 recipients and infected with indicated doses of rLM-SIY the next day (see *Materials and Methods*). Range of bacterial dose denoted as follows: Lo = $4x10^3$, Med = $4x10^4$, Hi = $4x10^5$ CFU. Animals were sacrificed 4 days post-infection and transferred cells were tracked using CD8 and Thy1.2 markers via flow cytometry. Numbers above gate in each dot plot refers to percentage of Thy1.2$^+$ CD8$^+$ cells in the spleen. *B*) Null mutant T cells are able to undergo cell division events in response to varying doses of rLM-SIY, but display a marked reduction in clone size. CFSE dilution was analyzed on the transferred population, as gated by Thy1.2$^+$ and CD8$^+$. Shaded line corresponds to infected mice, and bold unfilled line corresponds to uninfected control. Data are representative of three independent experiments.

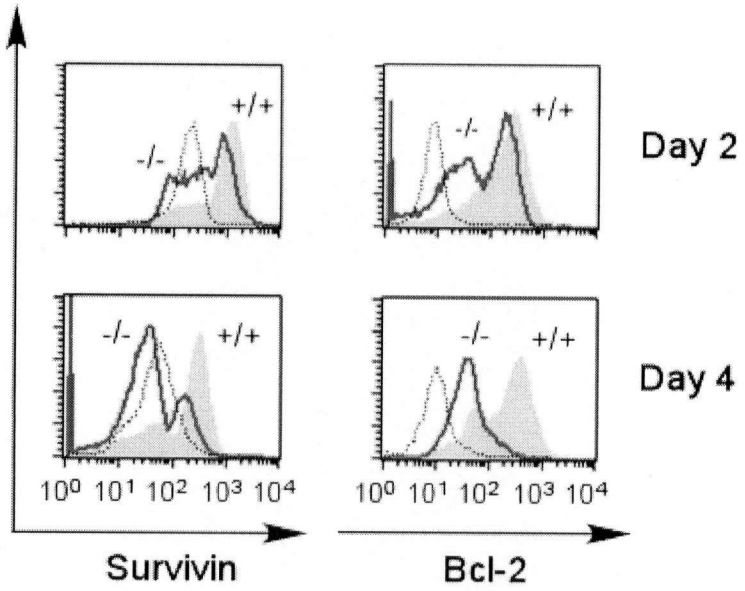

Figure 4.5 Survivin and Bcl-2 expression are dependent on TNFR2 during antigen-driven response *in vitro*.

2C or 2C TNFR2$^{-/-}$ CD8 T cells ($1x10^6$) were cultured *in vitro* with $3x10^6$ SIY peptide-loaded APCs for 4 days, and anti-apoptotic molecules were analyzed by intracellular FACS staining (see *Materials and Methods*) at indicated time points. Histograms reflect population gated on 1B2$^+$ (2C) CD8$^+$ cells. Shaded line = 2C CD8 T cells; unfilled black line = 2C TNFR2$^{-/-}$ CD8 T cells; dotted unfilled line = isotype control. Data are representative of two independent experiments.

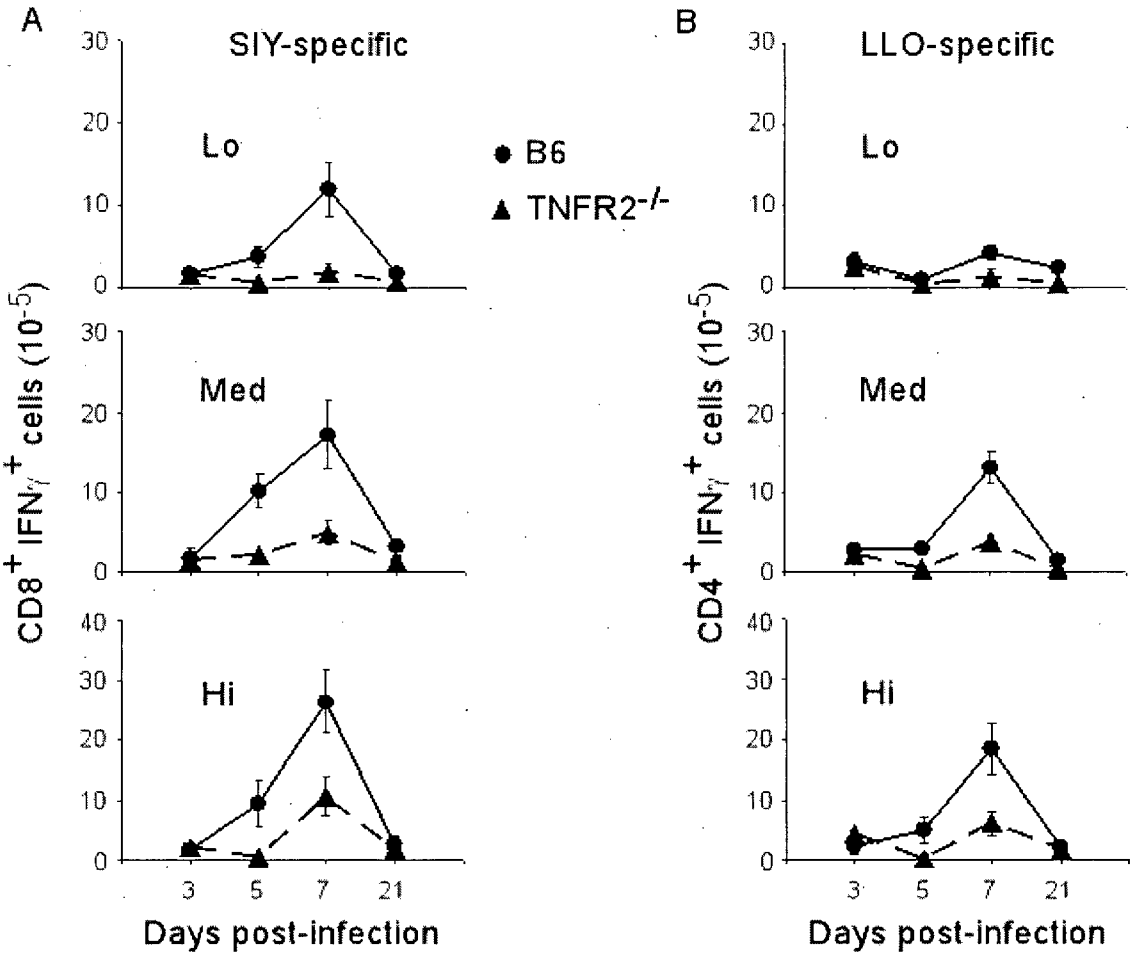

Figure 4.6 TNFR2 controls clonal expansion of CD4 and CD8 T cells against MHC class I and II-restricted epitopes of rLM-SIY.

Wild-type and TNFR2$^{-/-}$ mice were infected with doses of rLM-SIY (Lo = $1x10^3$, Med = $1x10^4$, Hi = $1x10^5$ CFU) and spleens were harvested at indicated times post-infection. To quantitate SIY- and LLO-specific cells, splenocytes were re-stimulated *in vitro* for 5 hrs in the presence of golgi inhibitor, and IFNγ expression was analyzed by intracellular FACS (see *Materials and Methods*); values correspond to number of IFNγ$^+$ cells in the spleen. Data represent mean from three animals per group. *A*) Kinetics of the T cell response to the MHC class I-restricted epitope, SIY, derived from rLM-SIY. *B*) Kinetics of the T cell response to the MHC class II-restricted epitope, LLO.

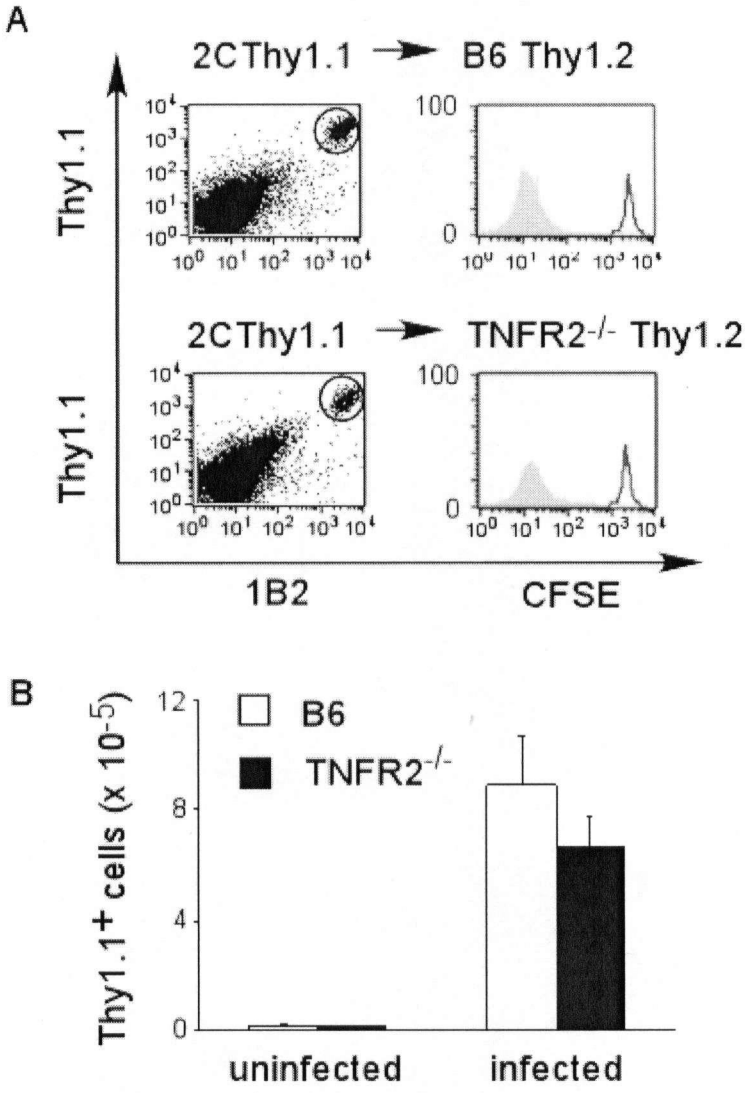

Figure 4.7 Antigen presentation in null mutant animals appears intact.

CFSE-labeled 2C Thy1.1$^+$ CD8 T cells were adoptively transferred into B6 Thy1.2$^+$ or TNFR2$^{-/-}$ Thy1.2$^+$ recipients and subsequently infected with rLM-SIY the next day. Spleens were harvested on day 4 p.i. and analyzed by FACS; transferred cells were enumerated by staining for Thy1.1 and 2C TCR (mAb: 1B2). *A*) CFSE profile was determined for transferred cells as gated by Thy1.1 and 2C TCR. *B*) Frequency of transferred cells was determined by multiplying percentage of Thy1.1$^+$ 2C$^+$ cells and total number of splenocytes (statistics determined with three mice per group). Data are representative of three independent experiments.

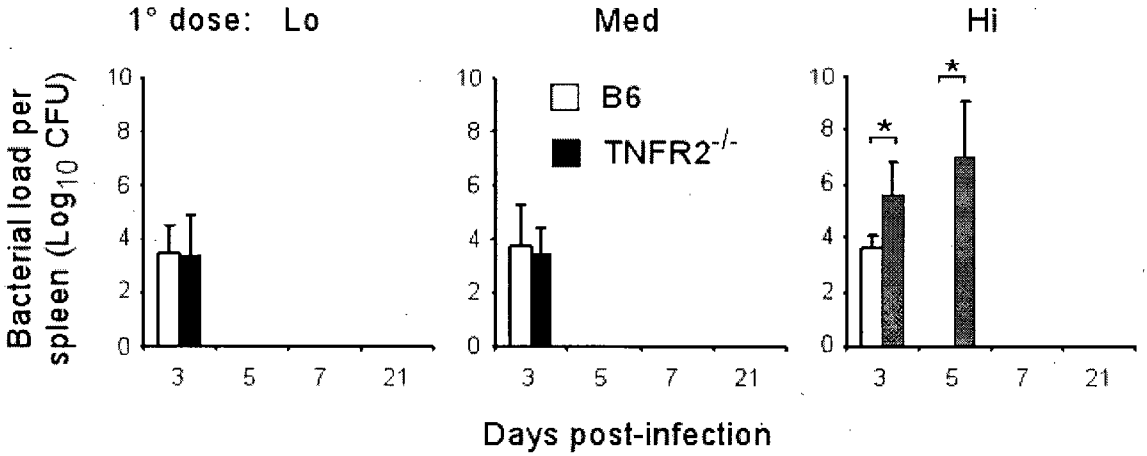

Figure 4.8 TNFR2-deficient mice display delayed bacterial clearance upon challenge with high dose of rLM-SIY.

Bacterial load in spleens of infected mice at indicated doses (Lo = $1x10^5$, Med = $1x10^4$. Hi = $1x10^5$ CFU) of primary challenge with rLM-SIY (see *Materials and Methods*). Asterisk denotes statistical significance. Data are representative of three independent experiments.

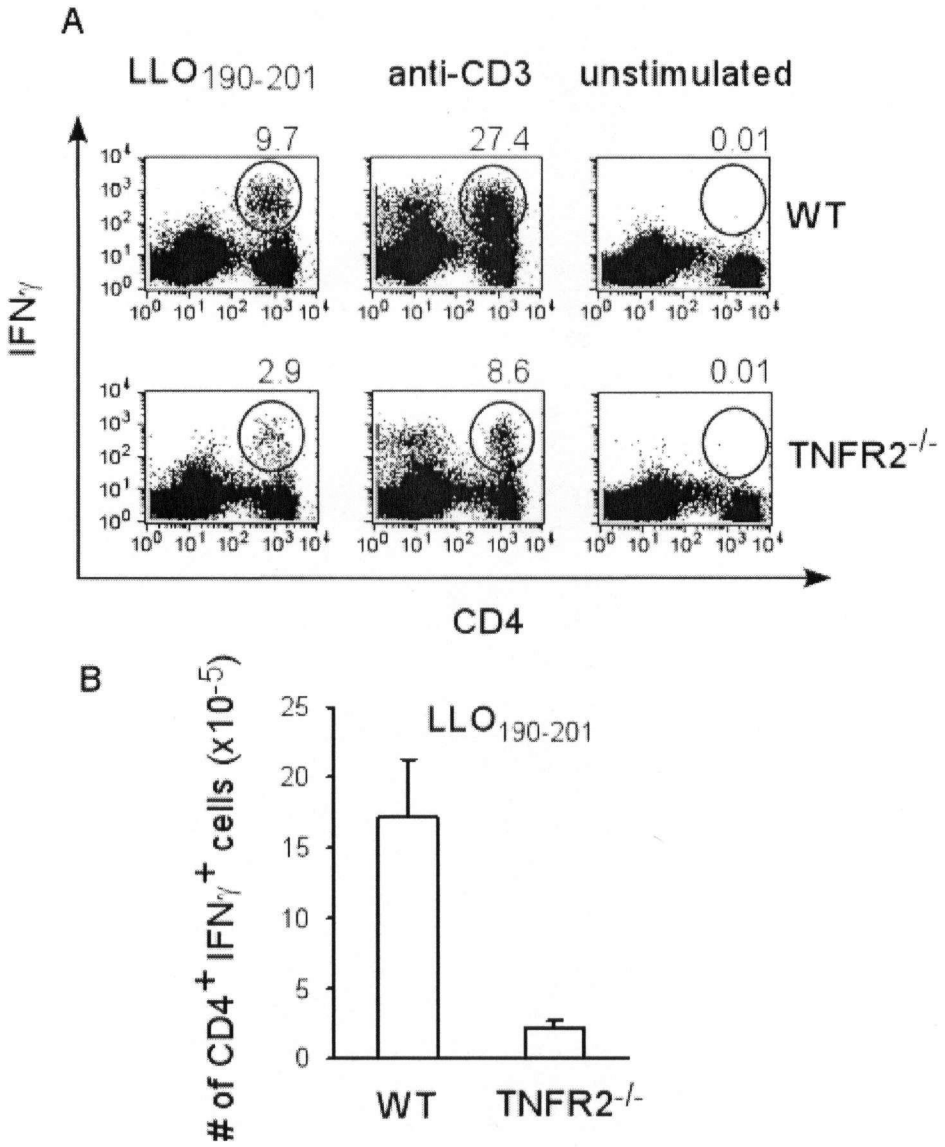

Figure 4.9 TNFR2$^{-/-}$ mice display reduced CD4 T cell response specific for wild-type strain of LM.

A) WT or TNFR2$^{-/-}$ mice were infected with wild-type LM and spleens harvested 1 week after. To quantify the frequency of CD4 T cells specific for $LLO_{190-201}$, splenocytes were re-stimulated with this peptide *in vitro* for 5 hrs and then analyzed for IFNγ expression via intracellular FACS staining (see *Materials and Methods*). As a positive control, splenocytes were also re-stimulated with 10 μM of plate-bound anti-CD3. Percentages indicated above gates in each dot plot refer to IFNγ$^+$ CD4$^+$ cells in the total spleen. *B*) Total numbers of $LLO_{190-201}$-specific CD4 T cells is diminished at the peak of the response to wild-type LM. Statistical analyses performed with 3 mice per group.

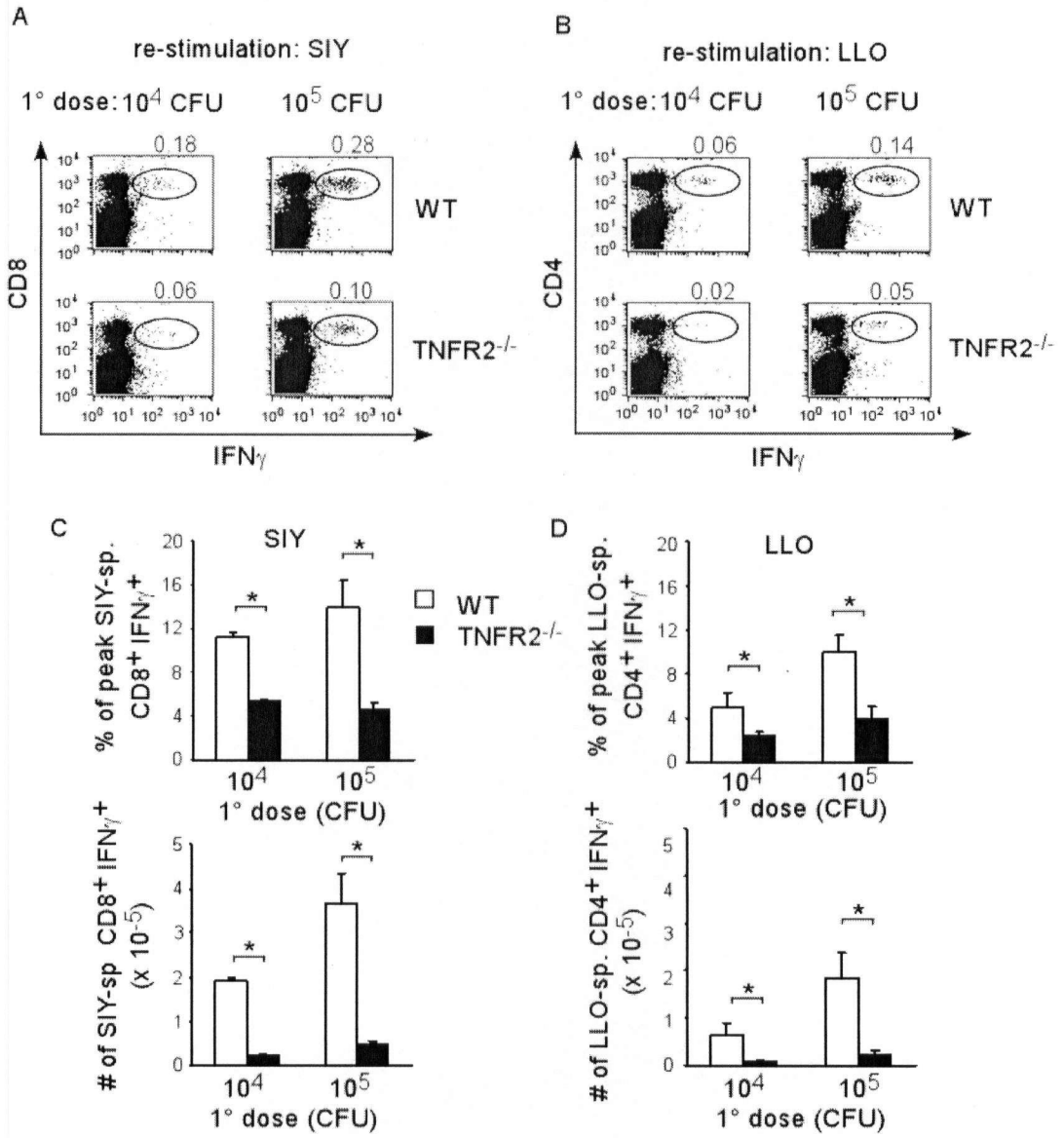

Figure 4.10 TNFR2$^{-/-}$ mice are compromised in the generation of antigen-specific memory populations against rLM-SIY.

Wild-type and TNFR2$^{-/-}$ mice were infected with indicated doses of rLM-SIY, and spleens were harvested 81 days post-infection. Splenocytes were re-stimulated for 5 hrs with SIY or $LLO_{190-201}$ peptides and IFNγ expression analyzed by intracellular FACS (*A* and *B*, respectively). *C, D*) The frequency of SIY-specific CD8 and LLO-specific CD4 memory T cells that survived from the peak of the primary response is compromised in TNFR2$^{-/-}$ mice. Percentages (average of four mice per group) were determined by dividing the total numbers of SIY-specific CD8 or LLO-specific CD4 T cells at day 81 post-infection by the total numbers at day 7 post-infection. The total numbers of SIY- and LLO-specific memory T cells at day 81 p.i. was determined by multiplying the percentage of IFNγ^{+} cells in response to the respective peptide re-stimulation and the total number of splenocytes. Asterisk denotes statistical significance (4 mice per group).

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Chapter 5 General discussion and perspectives

5.1 TNFR-mediated co-stimulation in T cell responses

5.1.1 Role of co-stimulation in T cell expansion and survival

The previous chapters showed that absence of TNFR2-mediated co-stimulatory signals results in markedly reduced IL-2 induction, increased threshold of T cell activation, and decreased survival of the dividing population of cells transiting from naive to effector T cells (1, 2). Importantly, $TNFR2^{-/-}$ mice were more susceptible to high dose of primary challenge with *Listeria monocytogenes* (LM), correlating with a marked reduction in both LM-specific CD8 and CD4 T cells that accumulated at the peak of the primary response (3). Using an engineered recombinant LM system (rLM-SIY) that expresses the secreted form of the 2C TCR-specific peptide agonist, SIY, a specific role for TNFR2 was revealed in T cell survival during the early rounds of cell division, which correlated with survivin and Bcl-2 expression (3). As the extent of antigen-specific T cell expansion has crucial bearing over the extent of adaptive immunity, TNFR2 plays a determining role in outcomes of T cell responses against primary challenge. The frequency of memory T cells is directly proportional to the number of effector T cells that accumulate at the peak of the primary response (4). Thus, the absence of TNFR2-mediated co-stimulation results in a reduced pool of effector T cells and fewer memory T cells following clearance of antigen.

From a qualitative perspective, it is noteworthy that although TNFR2 controls the absolute numbers of effector T cells that accumulate at the peak of the primary response, the relatively small pool of $TNFR2^{-/-}CD8^{+}$ T cells that do survive during cell division appear to express effector cytokines such as TNFα and IFNγ, and CTLs thus generated

are able to kill targets coated with cognate antigen. This is consistent with studies showing that cell division-dependent expression of markers of T cell differentiation such as CD44, CD45RB, and CD62L, as well as expression of effector cytokines such as IFNγ depend primarily on cell division and indirectly on receipt of co-stimulatory signals (5). That is, TNFR2 promotes the survival of CD8 T cells during early rounds of cell division but differentiation of surviving cells into effectors in TNFR2$^{-/-}$ mice depends on extent of cell division just as in wild-type mice. Thus, at least in the context of a predominantly Th1-mediated response against LM, TNFR2 appears to control the T cell response in quantitative terms to augment the numbers of cytokine-producing effector T cells.

Since TNFα is produced early upon T cell activation, the assumption for source of TNFα in T cell cultures stimulated by TCR cross-linking *in vitro* is that activated T cells produced the cytokine endogenously. Ideally, the TNF neutralization experiment described in chapter 3 would have yielded insight as to whether the hypo-proliferative response observed for TNFR2$^{-/-}$ T cells was due to blockade of TNF signaling through TNFR2 during T cell activation. However, TNFα neutralization did not reproduce the phenotype of TNFR2-deficient T cells, which may indicate interesting mechanistic properties for TNFα signaling. Since membrane-bound TNFα (memTNF) was not distinguished from soluble TNFα (solTNF) in T cell culture assays described in this thesis, it is unknown which form dominates TNFα signaling during T cell activation. If memTNF dominates, perhaps TNFα/TNFR2 interactions within cell-cell contact zones are inaccessible to neutralizing anti-TNFα antibody. Since the same T cell is capable of producing TNFα as well expressing TNFR2, perhaps their interaction and signaling occurs inside the cell rather than at outer face of the cell surface, and thus inaccessible to

neutralizing antibodies. Adding TACE inhibitors to prevent cleavage of memTNF and therefore release of solTNF could help to address their respective roles during T cell activation. To directly assess the effect of TNFα ablation on T cell proliferation/survival, TNFα-deficient T cells derived from the Lck-Cre/$TNF^{flox/flox}$ mice or $TNF^{-/-}$ mice could be used in proliferation assays.

Although this thesis identified a crucial role for TNFR2 in anti-LM T cell responses, it is important to note that the requirement for co-stimulation may be dependent on the nature of infectious agent, with parameters such as the duration and density of antigenic stimulation in acute vs. persistent infections determining this requirement. Recently, Suresh *et al.* (6) used the LCMV Armstrong strain to infect mice deficient in TNFR1, TNFR2, or both TNFR1 and TNFR2 (DKO) to examine the role of TNFRs in CD8 T cell responses to acute viral infection (6). They reported that primary CD8 T cell expansion of virus-specific CD8 T cells, which was measured using MHC class I tetramers specific for the LCMV CTL epitopes NP (D^b $NP_{396-404}$) and GP33 (D^b GP_{33-41}), was not dependent on TNFR2. The absolute numbers of $CD8^+$ Ag-specific T cells in the spleens of $TNFR2^{-/-}$ mice were not significantly different from those in wild-type mice (6). Thus, TNFR signaling does not appear to be required for optimal activation and expansion of CD8 T cells specific to the dominant CTL epitopes of LCMV. This is not surprising given the uniquely strong and prolonged signal-1 delivered in this viral infection model (7). Indeed, Suresh *et al.* (8) observed substantial activation and expansion of LCMV-specific CD8 T cells in both wild-type and $CD28^{-/-}$ mice, and blockade of CD28/B7 interactions by CTLA4-Ig only resulted in a modest reduction in the anti-LCMV CD8 T cell responses (8). Moreover, $CD28^{-/-}$ LCMV-specific memory

CD8 T cells showed normal homeostatic proliferation *in vivo* and also conferred protective immunity, indicating that CD28 signaling may be dispensable when the infectious agent delivers a potent signal-1, and thereby bypasses co-stimulatory requirements (8). This is consistent with findings by Kundig *et al.* (9) who reported that continued presence of signal-1 alone, either through prolonged viral replication or repeated injection of peptide, prevents the induction of anergy and generates a functional T cell response *in vivo* (9). Similarly, the requirement for TNFR2-mediated co-stimulation in the T cell response appears to diminish where signal 1 is potently delivered by an infectious agent.

5.1.2 Role of co-stimulation for directing effector T cell differentiation

CD28 has been shown to play a role in determining Th1/Th2 differentiation for naive CD4 T cells, in addition to its role in regulating threshold of activation and T cell survival (reviewed in ref. (10). The qualitative nature of the CD28 signal that determines this outcome is not well understood, though it may be related to its role in driving IL-2 production and thereby skewing differentiation towards a Th2 phenotype via IL-4 induction (11). Parallels may exist in the TNFR superfamily in determining Th1 versus Th2 responses. Early studies using blocking antibodies against OX40L showed that these Abs ameliorated the Th2 response that arises in BALB/c mice following *Leishmania major* infection, whereas antibodies to CD70, CD30L or 4-1BBL had no effect (12). Moreover, $OX40^{-/-}$ mice are resistant to the induction of experimental allergic lung inflammation, a Th2-mediated disease (13). This is consistent with the finding that OX40 is mainly expressed on activated $CD4^{+}$ Th2 T cells and perhaps preferentially bestows a

survival advantage to the Th2 subset. However, the outcome of OX40 signaling for Th1/Th2 differentiation appears to be dependent on the model used to study OX40/OX40L interactions, as the systemic administration of a soluble form of OX40L (which increases OX40 signaling) results in increased $CD4^+$ Th1 responses in the lung in response to infection with *Cryptococcus neoformans* (14). More studies are required to investigate a possible role for TNFR/TNF interactions in type 1 vs. type 2 T cell responses, particularly in analyses of disease states in which a predominance of one cytokine-secreting subset occurs. It would be interesting to study if differential expression patterns of TNFR2 exist between T cell subsets, or whether its role in IL-2 induction could skew responses towards Th2 as in the case of CD28. Interestingly, it has been recently shown that 4-1BB-mediated co-stimulation of $CD4^+$ T cells enhanced the survival and development of Th1 cytokine-producing T cells with a sustained increase in IFNγ-producing cells and suppression of IL-4 and TNFα production (15). This suggests that there may be differential roles for TNFR superfamily members in Th1 vs. Th2 responses, which has interesting implications in disease states associated with autoimmunity.

Lineage commitment to Th1 or Th2 by activated $CD4^+$ T cells can be traced to select transcription factors that induce differential cytokine profiles. For Th1 development, the expression of a T-box transcription factor, T-bet, plays a critical role in inducing IFN-γ and selective responsiveness to IL-12 (16, 17). GATA3 plays a critical role in Th2 differentiation, contributing to chromatin remodeling events that favor IL-4 production (18, 19). It would be interesting to investigate the possible linkage between co-stimulation and activation of these transcription factors for Th1/Th2 differentiation.

5.1.3 Role of co-stimulation in T cell memory

The generation, maintenance, and function of memory T cells, which is a hallmark of adaptive immunity, have been the subject of intense investigations in the past decade (reviewed in ref. (20). Approximately 5–10% of the clonally expanded antigen-specific T-cell pool survives and is maintained indefinitely following primary challenge (21). The T cell differentiation program during effector to memory cell transition involves epigenetic imprinting that depend on numerous cellular and molecular factors during priming such as the cytokine environment, duration of antigen stimulation, type of antigen presenting cells and co-stimulatory signals. Bourgeois *et al.* (22) reported a requirement for CD4 T cell-help in the generation of memory $CD8^+$ T cells via CD40 signals; in the absence of $CD4^+$ T-cell help, memory $CD8^+$ T cells exhibited poor proliferative capacity and cytokine production upon *in vitro* re-stimulation (22). Although mice that lack $CD4^+$ T cells mount a normal primary CD8 response to *Listeria monocytogenes*, memory $CD8^+$ T cells generated without CD4 help are defective in their ability to respond to secondary encounters with antigen (23). Thus it appears that signals provided by CD4 T cell-help during priming imprint a survival advantage on the memory population for robust recall responses.

Co-stimulation mediated by CD28 and TNFR superfamilies provides important signals during priming that control the development of memory T cells. However, in their study on the role of CD28 in memory T cell function, Suresh *et al.* (8) found that the lack of CD28/B7 interactions did not significantly affect the sensitivity of LCMV-specific memory CD8 T cells to antigenic stimulation *in vitro*, indicating that activation threshold

of memory CD8 T cells is not influenced by CD28 co-stimulation. In contrast, recent studies suggest that co-stimulatory members of the TNFR superfamily may function in secondary responses in an analogous manner to their role in primary expansion and survival (24). Upon secondary challenge, the accumulation of memory effector T cells at the peak of the recall response is markedly inhibited in CD27-deficient (25), 4-1BBL-deficient (26-28), CD30L-deficient (29), and OX40-deficient (30) mice. Given the role of TNFR2 in early T cell activation and survival in the primary response, it would be interesting to investigate the expression of TNFR2 on the memory T cell subset and whether it also may play a role in secondary challenge in an analogous manner. Along these lines, Turner *et al.* ((31)) found that TNFR2 is uniformly expressed on effector $CD8^+$ T cells specific for epitopes derived from HKx31 (H3N2) influenza A virus following secondary challenge. We have shown that the rLM-SIY infection model allows tracking of epitope-specific T cells throughout the course of the T cell response, including the visualization of the memory pool 81 days post-infection (3). Thus, this model can also be used to evaluate the role of TNFR2 for the expansion and differentiation of memory T cells upon secondary challenge. In addition, the $TNFR2^{-/-}$ can be bred onto TCR transgenic backgrounds such as OT-I and OT-II to facilitate direct tracking of clonal population of memory T cells and assess whether TNFR2 also provides an important survival signal in recall responses, as observed for OX40-deficient OT-II $CD4^+$ T cells (30). If TNFR2 is important for memory T cells, the expected result is that TNFR2-deficient memory T cells will exhibit diminished expansion upon rechallenge due to loss of protection from apoptosis.

5.2 TNFR as a potential therapeutic target of autoimmunity

The critical role of co-stimulatory molecules for the regulation of T cell responses makes these molecules attractive targets for novel therapeutic strategies. A substantial number of studies in murine models provided insight into the contribution of co-stimulation in various autoimmune diseases. Strategies utilizing a variety of approaches, such as stimulating or blocking monoclonal antibodies (mAb), fusion proteins, or gene-targeted mice, have highlighted the potential of targeting co-stimulation for controlling autoimmunity. Of the identified co-stimulatory members of the TNFR superfamily, the most compelling evidence for a role in autoimmunity and inflammation exists for OX40-OX40L interactions (24, 32). OX40-OX40L interaction has been shown to be required for the induction of EAE in mice: the addition of soluble OX40Ig to $CD11b^+$ brain microglia/macrophages inhibited T cell proliferation by 50-70%, *in vivo* administration of soluble OX-40R at the onset of EAE reduced disease symptoms, and the mice recovered more quickly from acute disease (33, 34). Moreover, blockade or deficiency in OX40/OX40L reduces the severity of collagen-induced arthritis (35), airway inflammation in mouse asthma models (13, 30, 36), and completely prevented diabetes development in NOD mice (37). The following sections highlight the therapeutic target potential of TNFR2 in several autoimmune disease models.

5.2.1 Asthma

Suppression of acute and chronic inflammation is a primary goal of asthma therapy. Novel therapeutic strategies with a more specific blockade of the inflammatory cascade are required for the significant population of patients who do not respond well to the

established anti-inflammatory therapy of corticosteroids that is normally used to control asthmatic symptoms (38). Co-stimulation is an attractive therapeutic target for controlling cellular and molecular pathways in the inflammation and autoimmunity associated with asthma.

Allergic asthma is intimately linked with airway inflammation (reviewed in (38). The initiation and progression of the inflammatory process are driven by allergen-specific T lymphocytes that attract eosinophils, mast cells, and B cells to the airways by the secretion of specific cytokines. Murine models of allergic airway disease have provided important insight into the pathophysiology of allergen-mediated lung inflammation. Although mast cells are the main effector cells of immediate hypersensitivity and anaphylaxis, it has been shown that mast cell-deficient mice (W/Wv) sensitized to ovalbumin (OVA) by intraperitoneal injection and subsequently challenged with OVA via the airways show no difference in OVA-specific IgE levels in the serum (39). Moreover, numbers of eosinophils in the bronchoalveolar lavage (BAL) fluid compared to littermate control were similar (39). This indicates that mast cells or IgE-mast cell activation are not required for the development of eosinophil-mediated inflammation in this model of allergen-induced airway hyper-responsiveness. However, there is strong evidence that allergen-induced airway inflammation is driven by Th2 cells (40), which produce proinflammatory cytokines (mainly IL-4, IL-5, and IL-13) to attract and activate other effector cells such as eosinophils, B cells, and mast cells into the airways, thus triggering the progress of asthma pathology (41).

Investigations into the role of T cell co-stimulation in airway hyper-responsiveness have yielded interesting insight on their potential as therapeutic targets.

Recently, Kimzey *et al.* (42) used adoptive transfer of Ag-specific T cells to demonstrate that CD28 has a critical role in both the induction and effector phase of allergic airway inflammation, affecting not only the priming of robust T cell expansion but also IL-4 secretion upon secondary stimulation with Ag (42). Consistent with the idea that co-stimulation plays an important role upon re-exposure to Ag *in vivo*, the transferred CD28-deficient Th2 cells were unable to support sufficient cytokine secretion to mediate an inflammatory response (42). In addition, CD28 appears to play an important role in eosinophil recruitment to both the lung and peritoneum, suggesting a generalized defect in the ability of eosinophils to accumulate at sites of inflammation *in vivo* in the absence of CD28. Memory Th2 cells secreting the cytokines IL-4, IL-5, IL-9, and IL-13 are thought to be the major driver in allergic asthma (38). Salek-Ardakani *et al.* (30) showed that co-stimulation through OX40 is critical for all aspects of lung inflammation driven by memory Th2 cells (30). Blocking OX40-OX40L interactions at the time of inhalation of aerosolized antigen suppressed memory effector accumulation in draining lymph nodes of the lung, and prevented eosinophilia, airway hyper-reactivity, mucus secretion, and Th2 cytokine production (30). The effect of OX40-OX40L interactions on recall responses regulated by memory Th2 cells is particularly relevant in the clinical setting where patients have already been sensitized. Given the co-stimulatory role of TNFR2 in regulating threshold of T cell activation and generation of a robust effector and memory T cell pool, this murine model of airway hyper-responsiveness could well be applied to $TNFR2^{-/-}$ mice to determine whether Th2 differentiation and subsequent inflammatory reactions could be altered through therapeutic blockade of TNFR2/TNF interactions.

5.2.2 Rheumatoid arthritis

The therapeutic management of patients with rheumatoid arthritis has seen a major evolution (43), with a number of biological reagents being developed for suppression of inflammation, most notably TNFα blocking agents (44, 45). However, of about 2 million people who have rheumatoid arthritis, analysts estimate that only 250,000 are on anti-TNF therapy, and 15 to 25% of these patients have an inadequate response. More recently, impressive therapeutic responses are being reported for B-cell-depleting regimens using anti-CD20 antibodies (46). Moreover, co-stimulatory blockade using CTLA4-Ig has shown remarkable therapeutic efficacy (47). Indeed, CTLA4-Ig (Orencia; generic: abatacept) was just approved by the US Food and Drug Administration as the first in a new class of drugs of co-stimulation blockers for the treatment of rheumatoid arthritis.

Interest in co-stimulatory blockade as a therapeutic modality stemmed from earlier work using the collagen type II-induced arthritis (CIA) animal model system in which immunization of genetically susceptible strains of mice, such as DBA/1, with type II collagen (CII) leads to the development of arthritis mediated by immune mechanisms involving CII-specific T cells and B cells and their products (reviewed in refs. (48, 49). Webb *et al.* (50) found that administration of CTLA-4Ig at the time of immunization prevented the development of CIA, and that this effect correlated with reduced lymphocyte expansion and abrogated production of anti-collagen IgG1 or IgG2a antibodies (50). Clinically relevant was the finding that when treatment was delayed until after the onset of clinical disease, CTLA4-Ig was still able to ameliorate CIA. This suggests an active role for T cells in the later stages of this disease and implicates both

B7-1 and B7-2-mediated co-stimulation in the pathogenesis of CIA (50). The role for CD28 in CIA was further confirmed using $CD28^{-/-}$ mice, which were found to be highly resistant to CIA (51). Similar results were also found for inducible co-stimulator (ICOS), a member of the CD28 superfamily, wherein $ICOS^{-/-}$ on the DBA/1 background were completely resistant to CIA accompanied by reduced anti-collagen IgM and IgG2a titers (52).

Co-stimulatory members of the TNFR superfamily have also been explored for a possible role in the pathogenesis of rheumatoid arthritis. Yoshioka *et al.* (35) used neutralizing anti-OX40L mAb to study the role OX40/OX40L interactions on the development of CIA, and they reported dramatic amelioration in disease severity (35). Interestingly, this treatment regimen did not inhibit the expansion of CII-reactive T cells, but suppressed IFNγ and anti-CII IgG2a production, suggesting that OX40 signaling can enhance Th1-type responses (35). A study has also suggested a role for TNFR2 in the pathogenesis of CIA (53). TNF is well-established as a potent pro-inflammatory cytokine important for the development of arthritis in human and animals. Tada *et al.* (53) showed that although $TNFR1^{-/-}$ mice developed arthritis with similar incidence and severity as $TNFR1^{+/-}$ littermates, $TNFR1^{-/-}$ mice injected with murine TNFα during the early induction phase enhanced the development of arthritis, but inhibited arthritis when administered during the late progression phase. This indicates that TNFR2 plays a role independent of TNFR1 during the early induction phase of CIA, but can also transduce an inhibitory signal against disease. It would be interesting to investigate CIA in TNFR2-deficent mice to evaluate whether type 1 responses are reduced and arthritis ameliorated. Since the $TNFR2^{-/-}$ mice are on the $H\text{-}2^{b}$ background (C57BL/6), which is relatively

resistant to a number of autoimmune conditions, these mice will need to be crossed onto susceptible H-2^d strains (e.g. DBA/1 mice) (48, 49). However, immunization protocols with collagen type II and complete Freund's adjuvant have been modified to render H-2^b mice more susceptible to CIA (54), which would allow for examination of CII-specific T cell and B cell responses in TNFR2$^{-/-}$ mice currently on the B6 background.

5.3 Concluding remarks

This thesis provided compelling evidence of a co-stimulatory role for TNFR2 in T cell responses. Naive $CD4^+$ and $CD8^+$ T cells depend on TNFR2 for survival during early rounds of cell division in response to TCR agonist and Ag-specific stimulation, particularly under antigen-limiting conditions. TNFR2 lowers the threshold of activation and promotes IL-2 production, acting as an early co-stimulatory molecule for generating a robust pool of effector T cells. Levels of antigen stimulation, as determined by strength of TCR/peptide-MHC interactions or antigenic dose, determine the degree of dependence on TNFR2-mediated co-stimulation, as stronger stimulation decreases the requirement for TNFR2 on T cell responses. Moreover, $CD4^+$ T cells appear more dependent on TNFR2 for clonal expansion than $CD8^+$ T cells, as TNFR2$^{-/-}$ CD4 T cells were hypo-proliferative in response to pAPCs with the addition of exogenous IL-2. TNFR2$^{-/-}$ mice exhibit increased susceptibility to high dose LM challenge, correlating with diminished frequency of LM-specific effector T cells. By crossing the TNFR2-null mutation onto TCR transgenic mice, an important advance was made in current understanding of T cell tolerance, identifying TNFR2 as an important co-stimulatory molecule for $CD4^+$ and $CD8^+$ T cell responses *in vivo*. The recent exciting developments in new therapeutics

designed as co-stimulatory blockers suggest that TNFR2 represents an attractive therapeutic target for modulating immune-mediated disease.

5.4 Figures

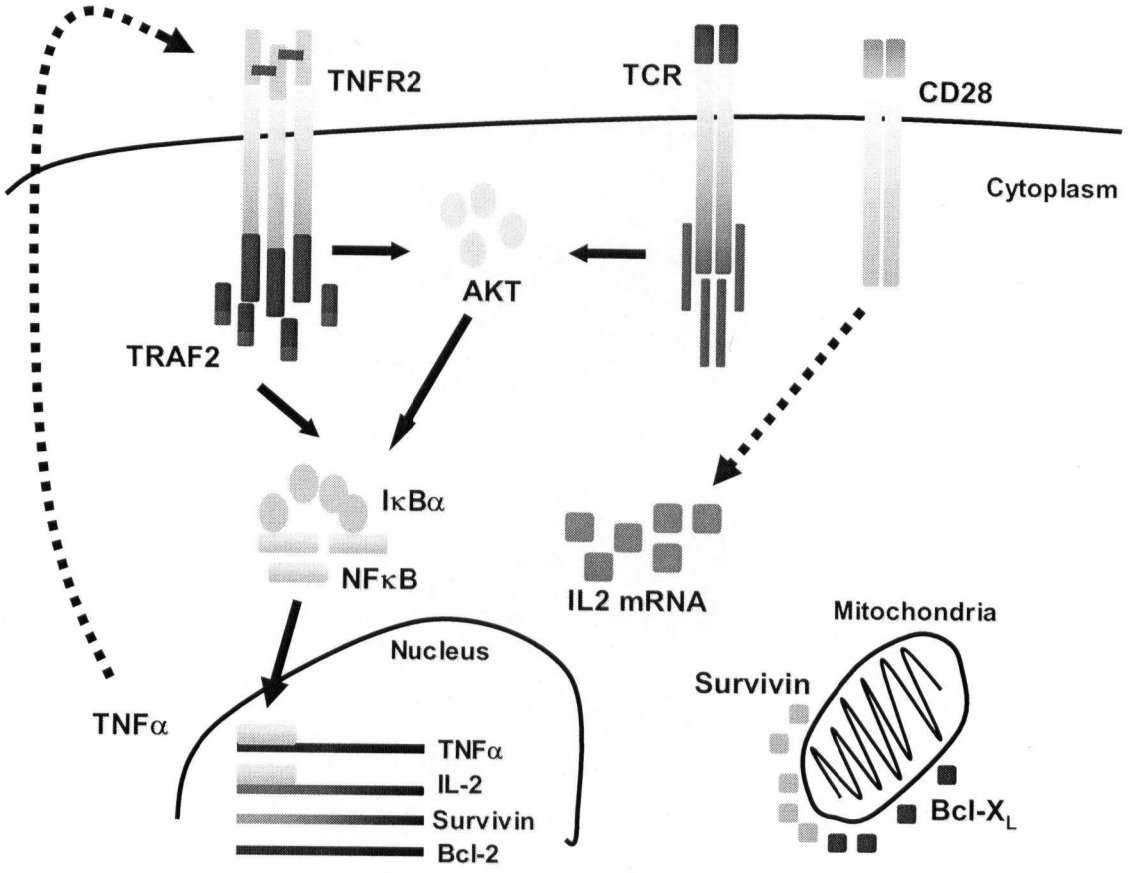

Figure 5.1 Model for TNFR2-mediated signal transduction during T cell activation.

TCR-mediated stimulation induces early synthesis of TNFα, which can subsequently act as an autocrine factor upon T cell activation. TNFR2 subsequently transduces signals to sustain AKT and NFκB activation, and thereby augments IL-2 production and expression of anti-apoptotic molecules survivin, Bcl-2 and Bcl-X_L. TNFR2 synergizes with TCR and CD28 to co-stimulate IL-2 induction, T cell proliferation and survival. Dotted lines refer to proposed signals.

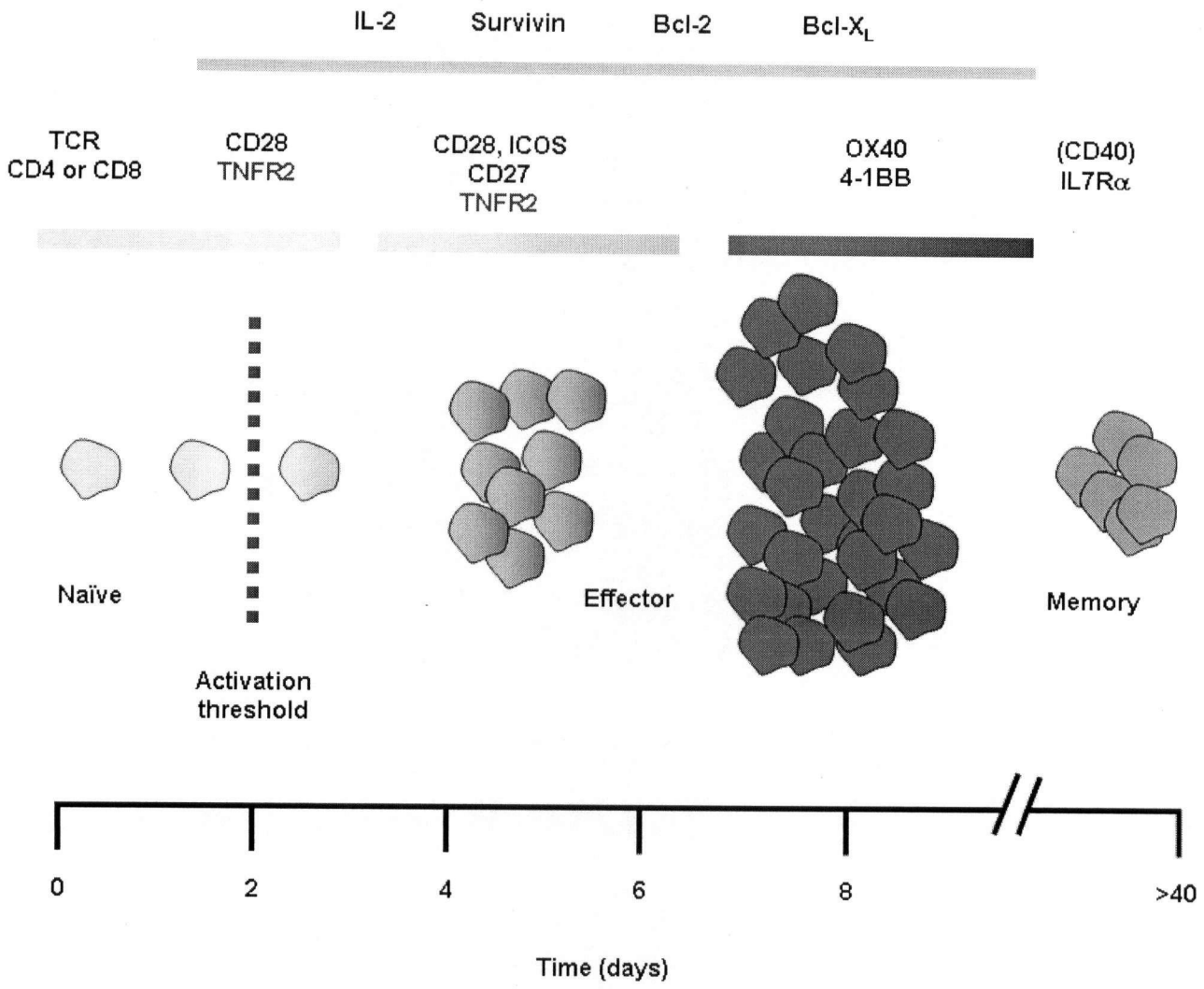

Figure 5.2 Model of TNFR2-mediated co-stimulation in the T cell response *in vivo*.

Naive T cells require signaling from TCR and CD4/CD8 co-receptor for initial activation. Co-stimulation from CD28 and TNFR2 lowers the threshold of activation and T cell survival during early expansion. If sufficient co-stimulation is provided, naive T cells undergo substantial clonal expansion with concomitant acquisition of effector function. Continued co-stimulatory signals through TNFR2, OX40, and 4-1BB are required for endowing survival on responding T cells, allowing accumulation of a massive number of effector cells at the peak of the T cell response (~ 1 week). Late acting signals from OX40 and 4-1BB are required for efficient survival of effectors to generate a robust memory population.

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