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Mechanisms of TRAFSignaling

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MECHANISMS OF TRAF SIGNALING

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Dedicated to the memory of my mother, Judith Arron

and to Sarah

The value the world sets upon motives is often grossly unjust and inaccurate. Consider, for example, two of them: mere insatiable curiosity and the desire to do good. The latter is put high above the former, and yet it is the former that moves one of the most useful men the human race has yet produced: the scientific investigator. What actually urges him on is not some brummagem idea of Service, but a boundless, almost pathological thirst to penetrate the unknown, to uncover the secret, to find out what has not been found out before. His prototype is not the liberator releasing slaves, the good Samaritan lifting up the fallen, but a dog sniffing tremendously at an infinite series of rat-holes.

H. L . Mencken

The right answer to a trivial question is also trivial, but the right question, even when insoluble in exact form, is a guide to major discovery.

Edward O. Wilson

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LIST OF ABBREVIATIONS

Ab	antibody
AP-1	activating protein-1
ATF	activating transcription factor
CD	cluster of differentiation
CMV	cytomegalovirus
CRD	cysteine-rich domain
DC	dendritic cell
DD	death domain
DMSO	dimethylsulfoxide
EBV	Epstein-Barr virus
EGFR	epidermal growth factor receptor
ERK	extracellular signal-related kinase
EST	expressed sequence tag
FADD	Fas-associated DD-containing protein
FCS	fetal calf serum
FKBP	FK506 binding protein
GC	germinal center
GM-CSF	granulocyte/macrophage-colony stimulating factor
GPI	glycophosphatidylinositol
GST	glutathione S-transferase
HA	hemagglutinin
HD	Hodgkin's Disease
HVEM	herpes virus entry mediator
IFN	interferon
IKK	I kappa B kinase
IL	interleukin
IL-1R	interleukin-1 receptor
IL-1RacP	IL-1R accessory protein
IRAK	IL-1R associated kinase
IκB	inhibitor of kappa B
JNK	c-jun N-terminal kinase
KD	kinase dead
LMP1	latent membrane protein-1
LN	lymph node
LPS	lipopolysaccharide
LT	lymphotoxin
MAPK	mitogen-activated protein kinase
M-CSF	macrophage-colony stimulating factor
MHC	major histocompatability complex
MyD88	myeloid differentiation factor-88
NF-κB	nuclear factor kappa B
NGF	nerve growth factor
NLS	nuclear localization signal
OC	osteoclast
OCIF	osteoclast inhibitory factor (OPG)
ODF	osteoclast differentiation factor (TRANCE)
OPG	osteoprotegerin
OPGL	osteoprotegerin ligand (TRANCE)
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PI3-K	phosphatidylinositol 3-kinase
PKB	protein kinase B (Akt)
PTH	parathyroid hormone
RANK	receptor activator of NF-κB (TRANCE-R)
RIP	receptor-interacting protein
SDS	sodium dodecyl sulfate
SH2	Src homology 2
SH3	Src homology 3
TCR	T cell receptor
TGF-β	tissue growth factor-beta
TLR	Toll-like receptor
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TNFRSF	TNFR superfamily
TNFSF	TNF superfamily
Tollip	Toll-interacting protein
TRADD	TNFR-associated DD-containing protein
TRAF	TNFR associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TRANCE	TNF-related activation-induced cytokine

1. ABSTRACT

This thesis describes the investigation of the mechanisms of signal transduction activated by tumor necrosis factor (TNF) superfamily proteins. Ligands of the TNF family engage TNF receptor (TNFR) family proteins, leading to a wide variety of cellular effects, and these interactions are implicated in inflammation, immune regulation, bone homeostasis, and development. TNFR proteins lack intrinsic enzymatic activity, and are coupled to intracellular signaling cascades by TNFR associated factor (TRAF) proteins, which are cytoplasmic adaptor molecules. The roles of TRAF1, TRAF2, and TRAF6 are investigated structurally and functionally in the activation of NF-κB, AP-1, and Src-family kinases. Cbl proteins are identified as positive and negative regulators of TRAF-mediated Src-family kinase signaling. The molecular structure of TRAF6 is determined and structure-function relationships between TRAF6 and the receptors to which it binds are examined. A physiological role for TRAF1 is identified in the regulation of TRAF2. This finding elucidates the role of translocation into lipid rafts in TRAF signaling and regulation. The implications of these findings are considered primarily in the reciprocal regulation of immunity and bone homeostasis by TRAF-mediated signaling pathways.

2. GENERAL INTRODUCTION

2.1 TNF/TNFR Superfamilies

Lymphotoxin (1) and tumor necrosis factor (TNF) (2) were first identified in the late 1960s and early 1970s as factors produced by immune cells that induced the death of various types of tumor cells. As molecular cloning became commonplace, it became clear that the genes that encoded these factors were part of a large family of genes, the identification of which continues to expand to this day. The TNF superfamily consists of a wide variety of proteins, some cell-bound and others secreted, that regulate a wide variety of cellular processes. In particular, TNF family proteins regulate the life and death of not only tumor cells, but of activated cells of the immune system. They modulate the activation state of immune cells, and as we are increasingly becoming aware, they mediate the signals that these cells send to each other. TNF family proteins have been implicated in areas outside of inflammation and immunity, including bone homeostasis, lymph node organogenesis, hair follicle development, and mammary gland development (3). A holistic view of the TNF family clearly demonstrates that, through TNF family proteins, these myriad biological processes are inextricably linked, and the widespread sequelae of inflammatory processes can be explained through the actions of the TNF family.

TNF family proteins mediate cellular effects by binding to their cognate cellular receptors, members of the TNF Receptor (TNFR) superfamily (3-6). TNF family proteins are expressed as type II transmembrane proteins that form trimers and may interact with their cognate receptors as cell-bound or soluble forms (7). TNFR family proteins are type I transmembrane proteins with conserved cysteine-rich domains (CRDs) that typically

consist of three conserved disulfide bridges (8). These domains form elongated receptor chains that intercalate with protomers of ligand trimers (9). It has been suggested that encounter with a ligand trimer induces the trimerization of a receptor, which leads to intracellular signaling, although recently it has been shown that TNFR proteins self-assemble in the absence of ligand, undergoing conformational changes upon ligand engagement that lead to signaling (10). While the extracellular ligand-binding domains of TNFR proteins are similar, there are three divergent subgroups of TNFR proteins that can be classified by motifs (or lack thereof) in their cytoplasmic tails: death receptors, decoy receptors, and activating receptors. Since TNFR proteins appear to lack intrinsic enzymatic activity, their intracellular signals are mediated by cytoplasmic adaptor proteins.

2.1.1 Death Receptors

Apoptosis, or programmed cell death, is a necessary feature of embryonic development, lymphocyte homeostasis, $CD8^+$ T cell-mediated killing of virus-infected or tumor cells, and the maintenance of "immune privileged" sites such as the eye (11,12). As opposed to death through necrosis, which is a response to physical injury to cells, apoptosis is a highly regulated process in which a cell "commits suicide." Morphological characteristics of apoptosis include membrane blebbing, cytoplasmic, nuclear, and chromatin condensation, and DNA fragmentation. The cell is reduced to fragments termed apoptotic bodies, which have intact membrane structures that contain surface markers that signal phagocytic cells to engulf them (13). This orderly process prevents the release of inflammatory stimuli that characterizes necrosis. During development, as tissues and structures differentiate, it is essential for certain cells to die in order to generate proper morphology. For example, in limb development, if there is a defect in apoptosis, the tissues in between digits may persist, resulting in webbing. During a cellular immune

response, there is massive proliferation of activated lymphocytes. While these activated lymphocytes are necessary to clear the infectious agent, they can cause collateral damage through nonspecific inflammation, and if they persisted, the body would eventually be overrun by lymphocytes. Indeed, mice and humans with mutations in Fas or FasL have autoimmune syndromes characterized by massive accumulation of activated lymphocytes and greatly enlarged lymph nodes (14). Thus, activated lymphocytes are extremely sensitive to apoptotic stimuli and lymphocyte homeostasis is maintained through the process of apoptosis (4). Death receptors such as TNFR1 and Fas contain a conserved intracellular "death domain" (DD) that can interact with intracellular DD containing adaptor proteins such as TRADD and FADD (15-17). FADD couples Fas and the TNFR1-TRADD complex to caspase-8 activation, which results in the orderly sequence of proteolytic events of apoptosis.

2.1.2 Decoy Receptors

Decoy receptors of the TNFR superfamily include osteoprotegerin (OPG) (18), DcR1 (19,20), DcR2 (21-24), and DcR3 (25). Their function is to negatively regulate the effects of various TNF family ligands by preventing their interaction with cell-bound receptors that mediate the dominant signals activated by those ligands. OPG is a soluble decoy receptor for TRANCE (discussed in more detail in section 2.3). DcR1 is a GPI-linked decoy receptor for the pro-apoptotic TNF family member TRAIL, DcR2 is a transmembrane decoy receptor for TRAIL, and DcR3 is a soluble decoy receptor for FasL. The expression of decoy receptors plays an important role in tempering the potent effects of TNF family members. Another mechanism of generating decoy receptors is through the proteolytic cleavage of cell-bound TNFR family proteins, rendering them soluble. This has been shown in the case of TNFR1, TNFR2, CD27, CD30, CD40, and Fas (26). A third mechanism of generating decoy receptors is through alternative splicing

of TNFR family proteins, generating membrane-bound receptors with no cytoplasmic tails, which has been shown for Fas (27) and CD40 (28).

2.1.3 Activating Receptors

Activating TNF receptors mediate their intracellular signals through a different set of adaptor proteins termed TNF receptor associated factors, or TRAFs. While DD containing receptors interact with cytoplasmic adaptor molecules that activate the pro-apoptotic caspase cascade, receptors that interact with TRAF proteins activate a variety of signal transduction pathways that mediate a large number of cellular effects, including survival, proliferation, differentiation, activation, and migration, to name but a few. TRAF proteins will be discussed in greater detail in the next section. Table 2.1 lists known TNFR family proteins (by standardized TNFRSF nomenclature), their ligands, intracellular adaptor molecules with which they interact, and known physiological functions (adapted from (3)).

Table 2.1: TNFR superfamily members

TNFR SF#	Receptor	Other names	Ligand(s)	Intracellular adaptors	Physiological Functions
1A	TNFR1	p55, CD120a	TNF-α, LT-α	TRADD, RIP, TRAF1,2,5 (indirect)	Macrophage activation, GC formation
1B	TNFR2	p75, CD120b	TNF-α, LT-α	TRAF1,2,5	Bacterial response, T cell homeostasis
3	LTβR	TNF-R-III	LT-β	TRAF3,5	LN organogenesis
4	OX40	CD134	OX40L	TRAF1,2,3,5	T cell activation
5	CD40		CD40L (CD154)	TRAF1,2,3,5,6	B cell proliferation, maturation, class switching, DC maturation, activation, survival
6	Fas	CD95, Apo1	FasL	FADD	Lymphocyte homeostasis, T cell cytotoxicity, immune privilege maintenance
7	CD27		CD27L (CD70)	TRAF2,5	T cell activation
8	CD30		CD30L	TRAF1,2,3,5	T cell regulation, expressed on Reed-Sternberg cells (HD)
9	4-1BB	CD137	4-1BBL	TRAF1,2,3	DC-T cell communication
10A	DR4	Apo2, TRAIL-R1	TRAIL	FADD	Lymphocyte homeostasis
10B	DR5	TRAIL-R2	TRAIL	FADD	Lymphocyte homeostasis
10C	DcR1	TRAIL-R3	TRAIL	None	Decoy for TRAIL
10D	DcR2	TRAIL-R4	TRAIL	None	Decoy for TRAIL
11A	TRANCE -R	RANK	TRANCE (RANKL, OPGL, ODF)	TRAF1,2,3,5,6 c-Src, c-Cbl	Osteoclastogenesis, DC survival/activation, mammary gland development, LN organogenesis
11B	OPG	OCIF	TRANCE	None	Decoy for TRANCE
12	DR3	Apo3, TRAMP	TWEAK	TRADD	
14	HVEM	ATAR	LT-α, LIGHT	TRAF1,2,3,5	HSV receptor, T cell proliferation
16	NGFR	p75	NGF	TRAF6	Neurogenesis
17	BCMA		BLyS		B cell responses
18	AITR	GITR	AITRL	TRAF1,2,3	Inhibits TCR-induced apoptosis
19	Troy	Taj			Regulator of hair follicles?
	TACI		BLyS		B cell survival
	EDAR		EDA		
	DR6				
	LMP1		Self-activating	TRAF1,2,3,5	EBV protein, activates B cells

2.2 TRAF proteins

There are six known mammalian TRAF proteins, of which TRAF1, 2, 3, 5, and 6 have been shown to interact directly or indirectly with members of the TNFR superfamily (29-31). In addition, TRAF6 mediates signaling from several other receptors that are not TNFR family proteins, including IL-1R, IL-18R, and Toll-like receptors (TLRs). The physiological role and binding partners of TRAF4 are presently unclear. TRAF proteins are characterized by a highly conserved C-terminal domain called a TRAF domain that mediates interactions of TRAFs with other proteins. The N-terminal domains of TRAFs are slightly more divergent, but each TRAF contains one or more zinc-binding domains that enable the activation of signaling cascades. TRAF proteins have been implicated in the activation of several kinase cascades, including MAP kinases, IκB kinases, and Src-family kinases, resulting in the ultimate activation of transcription factor complexes including AP-1, NF-κB, Elk-1, ATF2, and others. Thus, TRAF proteins exert their function by linking TNFR family proteins to these kinase cascades and subsequent regulation of gene expression.

2.2.1 TRAF identification, genetics, and expression

TRAF1 was first identified through the biochemical purification of intracellular factors interacting with TNFR2, and TRAF2 was cloned via a yeast two-hybrid system using TNFR2 as bait (32). TRAF3 (also called CD40bp, LAP-1, and CRAF1) was identified as an intracellular factor interacting with CD40 and LMP1 (33-35). TRAF4 was identified by differential screening of a cDNA library generated from tumor cells (36). The existence of multiple TRAFs suggested a larger family of proteins with a conserved domain (the TRAF domain), and degenerate PCR strategies were employed to clone

TRAF5 and TRAF6, which were also independently identified by yeast two-hybrid screening using CD40 as bait and the screening of an EST expression library (37-40). Since the identification of TRAF6 over 5 years ago, no other proteins strictly defined as belonging to the TRAF family have been identified, although some TRAF-like proteins that may have divergent functions have been identified recently (41). Evolutionary conservation of TRAFs is suggested by the identification of a TRAF in the nematode *C. elegans* (42) and two TRAFs in *Drosophila* (dTRAF1 and dTRAF2, (43)). To date, all six mammalian TRAFs have been deleted in mice by gene targeting, revealing various physiological roles for each (to be discussed further in section 2.2.5). Of the six mammalian TRAFs, sequence conservation analysis has shown that TRAFs 1, 2, 3, and 5 are closely related, while TRAFs 4 and 6 are more evolutionarily divergent. Of these evolutionary relations, TRAF1 and TRAF2 appear to have arisen after duplication of a common precursor, while TRAF3 and TRAF5 are derived from a different common precursor (44).

Expression patterns of TRAF proteins are widely variable. TRAF2, 3, and 6 are expressed in most cell and tissue types (32-35,39,40). The expression patterns of TRAF1, 4, and 5 are more restricted. The highest levels of endogenous TRAF1 expression have been found in tonsils, spleen, lung, and testis (32), while TRAF5 is expressed in spleen, lung, and thymus (37,38). The expression pattern of TRAF4 underscores its apparent lack of function in the arena of other TRAF proteins (inflammation and immunity), as it is predominantly expressed during embryogenesis and in certain neural tissues (36). While the expression of TRAF2, 3, 5, and 6 appears to be largely constitutive in the cells that express those TRAFs, the expression of TRAF1 is tightly regulated. Most cells with the potential to express TRAF1 express it at very low levels in a resting state. However, inflammatory stimuli including TNF family proteins, IL-1, LPS, and lymphocyte receptor

engagement induces rapid upregulation of TRAF1 expression (45). Promoter analysis of the *traf1* locus shows the presence of NF-κB and AP-1 sites (46), thus, intriguingly, transcription factors activated by TRAF-dependent signals upregulate the expression of TRAF1.

2.2.2 Structural features of TRAFs

The domain organization of TRAFs comprises a modular structure characteristic of adaptor proteins whose function is to link structurally dissimilar factors. As shown in Figure 1, the C-terminal TRAF domain (32) can be further divided into two sections, TRAF-N and TRAF-C. The TRAF-N section is helical and adopts the form of a coiled coil, mediating oligomerization of TRAF proteins. The TRAF-C domain also contributes to oligomerization as well as to interactions with TNFR family proteins and other cytoplasmic factors (47). The N-terminal halves of TRAF proteins are somewhat more divergent, but all TRAFs except for TRAF1 contain a RING finger motif at their N-termini. The RING finger comprises a set of four closely spaced Zn fingers, which, in turn, consist of two residues that can chelate a single Zn^{2+} ion (generally cysteines, but occasionally histidines or acidic residues). C-terminal to the RING finger in TRAF2, 3, 5, and 6 are five Zn fingers, while in TRAF1, there is a single Zn finger, and in TRAF4, there are seven Zn fingers. In addition to the Zn binding motifs in TRAF4 are two potential nuclear localization signals (NLS), which may direct TRAF4 localization to the nucleus (36), while TRAF1, 2, 3, 5, and 6 are chiefly cytoplasmic in their distribution. While nearly all known interactions between TRAFs and other proteins such as TNFRs and cytoplasmic factors are mediated through the TRAF domain (31), the RING and Zn fingers appear to be necessary for the ability of TRAFs to activate kinase cascades (47). Accordingly, deletion of the N-terminal domains of TRAFs renders them dominant negative for signal transduction.

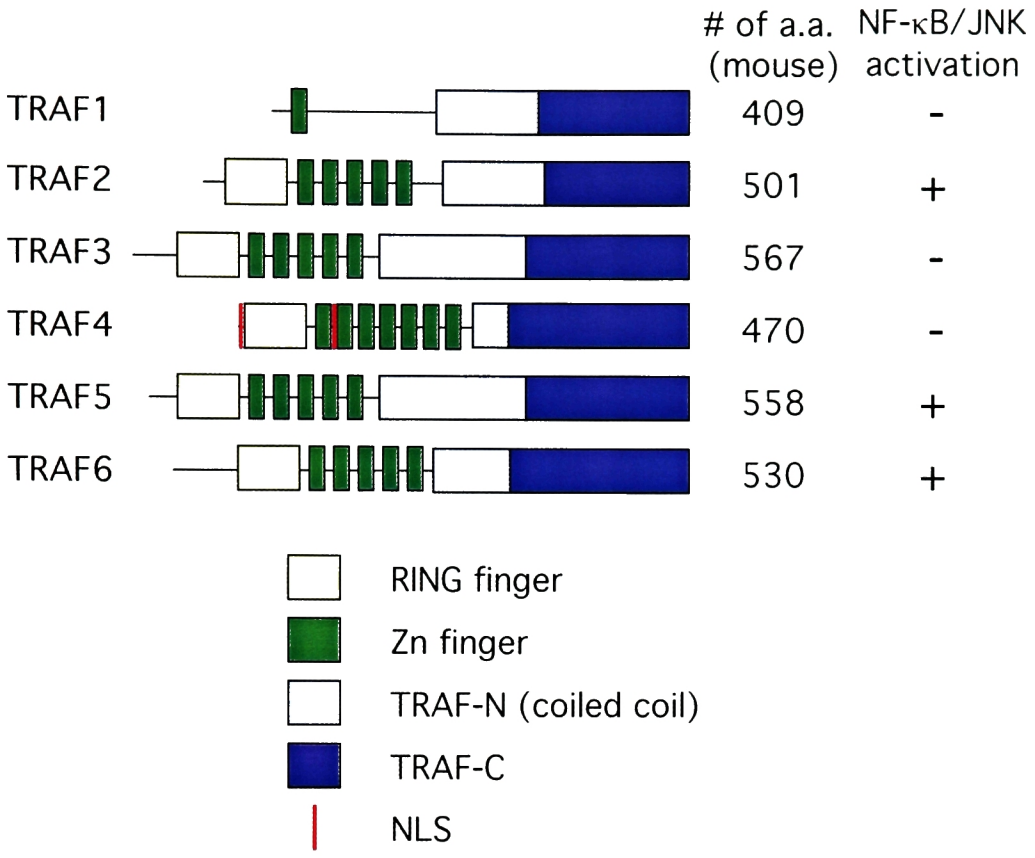

Figure 2.1. Domain organization of mammalian TRAF proteins.

The structures of the TRAF domains of TRAF2 and TRAF3 have been determined by x-ray crystallography, and have provided important insights into the mode of interaction between TRAFs and other proteins (48-51). Much as TNF family ligands are trimeric, and their binding to TNFR family receptors dictates receptor trimers, the binding of TRAF proteins to the cytoplasmic tails of TNFR proteins is also trimeric. The TRAF domains of TRAF2 and TRAF3 adopt a mushroom-shaped structure, with the coiled-coil TRAF-N domains forming the stalk and the TRAF-C domains forming the cap. The receptor-binding portion of the TRAF-C domain of TRAF2 forms a groove in which receptor sequences bind symmetrically in an extended conformation (51). The interaction of TRAF2 with TRADD, which couples TRAF2 to TNFR1, comprises a more extended binding interface, and suggests that TRADD may compete with other receptor sequences

for TRAF2 (52). The thermodynamic properties of TRAF2-receptor interactions show significantly lower affinity of TRAF2 monomers for receptor peptides than is the case with trimeric TRAF2 (53). This suggests an oligomerization-driven affinity or avidity enhancement of TRAF-receptor binding interactions, which may illuminate a mechanism of action for TRAF proteins. It has been shown that induced multimerization of the N-terminal halves of both TRAF2 and TRAF6 can activate signaling, which suggests that, *in vivo*, receptor-induced TRAF trimerization is necessary for the activation of signal cascades (54).

2.2.3 TRAF-interacting proteins

As adaptor proteins for TNFR family members, TRAFs mediate the assembly of cytoplasmic signaling complexes at the receptor. They have been shown to interact with a wide variety of cytoplasmic factors, either directly or indirectly, in addition to the TNFR family proteins listed in Table 2.1. In addition to forming self-associated homotrimers, some TRAF proteins can hetero-oligomerize with each other. Specifically, TRAF2 has been shown to form hetero-oligomers with TRAF1 and TRAF5 (32,55) while TRAF3 can form hetero-oligomers with TRAF5 (56,57). Interestingly, the hetero-oligomerization pairs of TRAF1/2 and TRAF3/5 mirror the evolutionary origin of those pairs of TRAFs (44), which suggests that hetero-oligomerization is a product of closely related structures. This may provide a further layer of regulatory roles for TRAFs – the regulation of other TRAFs. TRAF proteins bind to and mediate the function of several general types of intracellular factors, including kinases, regulators of signaling pathways, structural proteins, and other adaptor molecules (31). TRAFs are well-characterized activators of MAP kinase signaling pathways, and have been shown to interact with and activate these cascades at the level of MAP3Ks and MAP4Ks, including ASK1 (58,59), TAK1 (60), MEKK1 (54), NIK (61), and GCK3 (62). Some other kinases implicated in TRAF

signaling are Src-family kinases (63,64), IRAK (39), and PKC (65). Most of the interactions demonstrated for TRAF proteins with kinases are mediated via the TRAF domain. However, the TRAF domain alone acts as a dominant negative, thus the N-terminal RING and Zn fingers appear to play a vital role in the activation of these kinase cascades (47,66). Known interactions of TRAFs and intracellular factors are listed in Table 2.2 (adapted from (31)).

Table 2.2. TRAF-interacting cytoplasmic proteins and their functions

Cytoplasmic factor	Interacting TRAFs	Function
RIP (Receptor interacting protein)	1,2,3	Activation of IKK, assembly of TNFR1 complex
RIP2	1,2,3,5,6	Similar to RIP
IRAK (IL-1 receptor associated kinase)	6	With MyD88 or TIRAP/MAL, mediates signaling from IL-1R and TLRs to TRAF6
IRAK2, IRAK-M	6	Similar to IRAK
NIK (NF-κB inducing kinase)	1,2,3,5,6	MAP3K-like kinase upstream of IKK activation
ASK1 (apoptosis signal -regulating kinase-1	1,2,3,5,6	MAP3K upstream of JNK activation
TAK1 (TGF-β associated kinase-1)	6	MAP3K upstream of IKK, JNK
MEKK1 (MEK/ERK Kinase-1)	2,6	MAP3K upstream of JNK
IRE1	2	ER-associated stress kinase
GCK (Germinal center kinase)	2	MAP4K upstream of JNK
CDK9 (cyclin dependent kinase 9)	2	Cell cycle regulation
PKCζ (protein kinase C, ζ isoform)	6	Mitogenic signaling
TBK1/NAK	2	Upstream of IKK activation
c-Src	1,3,6	Multifunctional tyrosine kinase
cIAP-1 (cellular inhibitor of apoptosis-1)	1,2	Anti-apoptotic adaptor
cIAP-2	1,2	Similar to cIAP-1
TRIP (TRAF inhibitory protein)	1,2	Inhibitor of TRAF signaling
TANK/I-TRAF	1,2,3	Regulator of TRAF signaling
A20	2	Antiapoptotic, anti-inflammatory regulator
TRADD (TNFR associated DD containing protein)	2	Mediates assembly of TNFR1 complex, associates with RIP, FADD, A20
FLASH (FLICE-associated huge protein)	2	Assembly of apoptosis-inducing complex with caspase-8, possible role in IKK activation
ABIN (A20 binding protein)	2	Inhibits IKK activation
ECSIT	6	Enhances IKK activation
T6BP (TRAF6 binding protein)	6	Inhibits T6-IRAK interaction
TTRAP	2,3,5,6	Competes with TRAF6 for CD40 binding, inhibitor of TRAF signaling
Filamin	2	Actin-binding protein
MIP-T3	3	Binds to microtubules
Caveolin-1	1,2	Required for formation of caveolae
p62 nucleoporin	3	Mediates nuclear entry

2.2.4 Signaling pathways activated by TRAFs

TRAFs are defined by their ability to couple TNFR family proteins to signaling pathways that result in the cellular effects mediated by TNF family ligands. The most well-studied signaling pathways activated by TRAF proteins are those leading to the activation of the transcription factors NF-κB and AP-1. NF-κB describes a set of five transcription factor subunits: p50, p52, RelA, RelB, and cRel (67,68). An active complex of NF-κB consists of two of these subunits, either as a homodimer or a heterodimer. NF-κB complexes are maintained in the cytoplasm in an inactive state by an inhibitory protein, IκB, of which there are several variants (α, β, γ,and ε among them). When a cell receives an NF-κB activating stimulus, a large, multiprotein complex of IκB kinases (IKK) phosphorylates IκB on two serine residues (69). Phospho-IκB is then recognized by ubiquitin-conjugating enzymes, which quickly ubiquitinate it, resulting in its degradation. The released NF-κB subunits then translocate to the nucleus, where they bind to specific promoter sites, activating transcription of a wide variety of genes, including cytokines, adhesion molecules, transcription factors, and survival factors. Most TNF family-induced NF-κB activation has been associated with cell proliferation, survival, and differentiation signals. Oligomerization of TRAF proteins in a receptor complex results in the recruitment and activation of various kinases upstream of IKK, including RIP (70), NIK (61), TAK1 (60), MEKK1 (54), and MEKK3 (71). It is likely that no single one of these kinases is essential for TRAF-mediated NF-κB activation, but rather they may have overlapping or cell type-specific roles (72). Recently, in vitro biochemical studies have suggested that the mechanism of TRAF-mediated IKK activation involves the ability of the RING finger of TRAF6 to act as an E3 ubiquitin ligase, mediating non-classical K63-linked polyubiquitnation of TRAF6 as well as of the IKK complex (73,74).

2. General Introduction

AP-1 describes a dimeric transcription factor complex comprising combinations of Jun (v-Jun, c-Jun, JunB, or JunD), Fos (v-Fos, c-Fos, FosB, Fra1, or Fra2), or activating transcription factor (ATF2, ATF3, or B-ATF) proteins. c-Jun is activated by JNK, a stress-related MAP kinase (75). TRAF proteins, in particular TRAF2, activate JNK by activating upstream MAP3Ks including ASK1 (58,59), MEKK1 (54), TAK1 (60), and the MAP4K GCK (62). These kinases in turn activate MAPKKs such as MKK4 and MKK7, which phosphorylate JNK (76). As is the case with IKK kinases, genetic deletion of several of the MAP3Ks upstream of JNK has not revealed an absolute requirement for any one of these kinases in TRAF-mediated JNK activation, implying that there may be some redundancy in TRAF-mediated JNK activation (71,77,78). In lymphocytes, JNK activation appears to be associated with proliferation and survival signals, while in some other cell types, it is associated with apoptotic signals and stress responses (75,76). Notably, JNK is activated in response to physical and osmotic stress as well as in response to irradiation and DNA damage (79). In addition to JNK, other MAPKs are activated by TRAF signaling, including ERK and p38. ERK activates the transcription factor Elk-1, which is involved in cell activation and survival signals. p38 activates the AP-1 component ATF2, which has been implicated in stress and activation responses (76,79). Recently, we (63) and others (64) have shown that TRAF6-mediated signals can activate c-Src family tyrosine kinases. c-Src is essential for TRANCE-mediated osteoclast activation, and Src family kinase-mediated PI3-K activation results in the activation of Akt, a kinase associated with survival signals.

Some typical examples of TRAF-mediated signaling pathways are illustrated by those activated by TNF-α, TRANCE, and IL-1. TNF-α has two known transmembrane receptors, TNFR1 and TNFR2. TNFR1 is a DD-containing receptor, and does not directly bind to any TRAF proteins, while TNFR2 has no DD and can directly bind to

TRAF1 and 2 (and possibly TRAF5) (32). While TNFR1 is expressed on most cell types, the expression of TNFR2 is restricted to hematopoietic cells (80,81). Additionally, while TNFR1 can be stimulated by cell-bound or soluble TNF-α, TNFR2 is only responsive to cell-bound TNF-α (82). Upon ligand engagement, TNFR1 recruits TRADD and RIP to the receptor complex via DD interactions (17,70). There are two essential pathways that can be activated at this point – TRADD can recruit FADD, which activates the caspase cascade, resulting in apoptosis, or it can recruit TRAF2, which activates NF-κB and AP-1 signaling, which results in survival or proliferation (17). TNFR2, on the other hand, lacks a DD, and can bind to TRAF2, activating survival pathways (32). Interestingly, TNFR2 stimulation has been shown to potentiate TNFR1-mediated apoptosis, and this may be accomplished through the depletion of available TRAF2 (83). Thus, the available pool of TRAF2 may serve to regulate the balance of life and death in TNF signaling. Figure 2.2 diagrams some features of TNF signaling.

TRANCE-R, unlike TNFR1, lacks a DD, and its cytoplasmic tail can recruit TRAF1, 2, 3, 5, and 6 (84). The TRAF6 binding sites are distinct from the binding site for the other TRAFs in TRANCE-R. The TRAF1/2/3/5 sites are associated with the activation of NF-κB and JNK similar to TNFR2, as are the TRAF6 binding sites. In addition, TRAF6 has been implicated in MAPK activation and c-Src activation (63). IL-1 is not a TNF family protein, and its receptor is not a TNFR family protein, although it signals through TRAF6, but indirectly. IL-1 binding to IL-1R, which is associated with an accessory protein (IL-1RAcP), induces the formation of a receptor complex that includes the cytoplasmic factors MyD88 and Tollip. MyD88 and Tollip then associate with the cytoplasmic factor IRAK. IRAK binds to TRAF6, enabling the activation of similar downstream signals as from TRANCE-R (39,85-89). IRAK is also a key intermediate in Toll-like receptor (TLR) signaling (90-92). TLRs recognize repetitive sequences such as

bacterial and viral products, and are associated with innate immunity. Thus, by mediating signals from both TNFR family proteins and TLRs, TRAF6 serves as a molecular bridge between adaptive and innate immunity. TRAF6-dependent signaling mechanisms from TRANCE-R and IL-1R are shown in Figure 2.3.

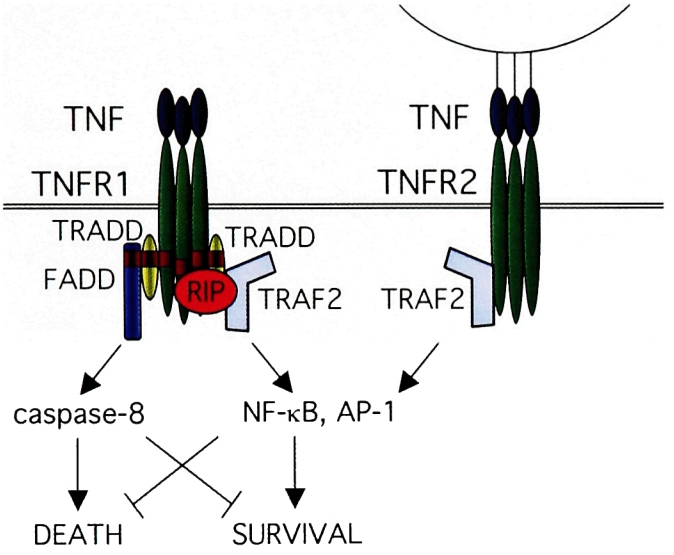

Figure 2.2. Signaling pathways activated by TNF. Soluble TNF-α acts primarily on TNFR1 while membrane-bound TNF-α acts on TNFR2. TNFR1 associates with TRAF2 through TRADD and RIP, while TRAF2 binds directly to TNFR2. Death domains (DD) are shown in red.

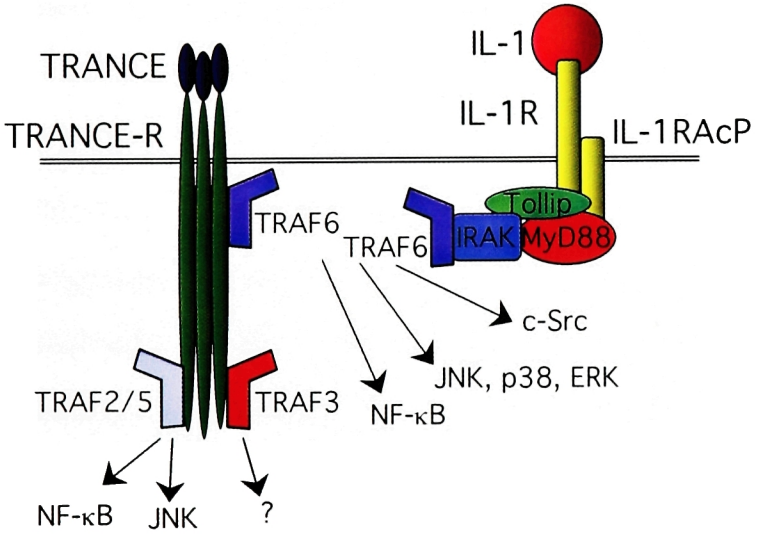

Figure 2.3. TRAF6-dependent signaling pathways. TNFR family members such as TRANCE-R and CD40 bind to TRAF6 as well as other TRAFs, activating various pathways including Src-family kinases. IL-1R and Toll-like receptors are indirectly linked to TRAF6 through MyD88, Tollip, and IRAK.

2.2.5 TRAF knockout mice

All six of the TRAF proteins have been deleted in mice by gene targeting, and the phenotypes of these mice reveal divergent roles for each TRAF. TRAF1-/- mice have no apparent developmental defects but appear to have enhanced inflammatory and T cell

responses to TNF (93). The biochemical function of TRAF1 in its regulation of TRAF2 is revealed in TRAF1-/- cells and will be discussed in detail in section 3.3. TRAF2 has been targeted by deletion as well as by overexpression of a dominant negative form of TRAF2 in lymphocytes (94,95). TRAF2-/- mice appear relatively normal at birth but become progressively runted and cachectic and generally die within several weeks. This is most likely due to a "feed-forward" response of macrophages to TNF, as TRAF2-/- macrophages produce large quantities of TNF in response to TNF stimulation, leading to systemic inflammation. TRAF2-/- cells show a lack of TNF-induced JNK activation but still have NF-κB activation, although they display an enhanced tendency toward TNF-induced apoptosis relative to wild-type cells (94). Crossing of TRAF2-/- mice to TNF-/- or TNFR1-/- mice greatly ameliorates the wasting phenotype (96). Overexpression of dominant negative TRAF2 (lacking the RING and Zn finger domains) in lymphocytes results in reduced JNK but not NF-κB activation in response to TNF and CD40L (95). The residual NF-κB activation may be explained by a redundant role of TRAF5, as TRAF2-/-TRAF5-/- cells have a complete lack of TNF-induced NF-κB activation (97). TRAF3-/- mice, like TRAF2-/- mice also appear normal at birth but become progressively runted and die within several weeks. Reconstitution of lethally irradiated mice with TRAF3-/- fetal liver cells produces lymphocyte, granulocyte, and erythroid lineages in those mice, but T cell responses are somewhat reduced, although CD40 signaling does not appear to be compromised (98). TRAF4 deletion results in tracheal malformation (99). Deletion of TRAF5 produces a relatively mild phenotype, with some defects in CD40-mediated B cell responses (100). TRAF6-/- mice have severe osteopetrosis, or thickening of bones, due to a defect in osteoclast function. Two separate deletions of TRAF6 have been reported, and one completely lacks osteoclasts (101), while the other has osteoclasts that are incapable of resorbing bone (102). In addition, both TRAF6-deficient strains have reduced responses to LPS, IL-1, and CD40. Thus,

despite the similarities in signaling pathways activated by different TRAF proteins, each appears to have a distinct physiological role.

2.3 Physiological processes regulated by TNF family proteins

TNF family proteins play vital roles in a wide variety of cellular processes, including development, inflammation, immunity, and bone homeostasis. As TNF/TNFR family proteins mediate communications between cells, they are important cellular organizers, mediating the formation of multicellular structures including inflammatory foci, lymphoid organs, bone, hair follicles, and lactating mammary glands (3). The signals transduced by TNFR family proteins that activate survival, death, differentiation, activation, and proliferation determine the dynamic organizing and reorganizing events that characterize the systems regulated by TNF/TNFR interactions. In particular, we shall consider the role of these interactions in immunity and bone homeostasis, which are both typified not by the permanence of any cellular structures but rather by the constant state of flux that ensures the robustness of each system. Additionally, it is becoming clear that the apparent divergence of the highly specialized cell types that constitute these apparently divergent systems is less obvious on a molecular level, and in fact, these systems are quite interdependent. In particular, the communication between these systems via TNF/TNFR family proteins is bridged by the processes and systemic metabolic requirements of inflammation.

2.3.1 Dendritic cells

Dendritic cells (DC) are essential organizers of immune responses. They are highly specialized cells that capture antigens in peripheral tissues, migrate to lymphoid organs, and organize T cell responses. The life cycle of the dendritic cell consists of several well-

defined stages, including differentiation, maturation, activation, and apoptosis (103). All of these processes are regulated to some extent by the action of TNF family proteins on DCs, in particular TNF, CD40L, and TRANCE (104), while DCs can stimulate T cells via expression of OX40L, CD27L, and 4-1BBL (3). Additionally, extracellular signals not originating from TNF family proteins in DCs are still TRAF-dependent, as DCs are highly sensitive to bacterial and viral products that signal through TLRs (103), as well as to IL-1, which utilize TRAF6 in their signaling pathways. DC signals that influence T cells are shown in Figure 2.4 and T cell signals that influence DCs are shown in Figure 2.5.

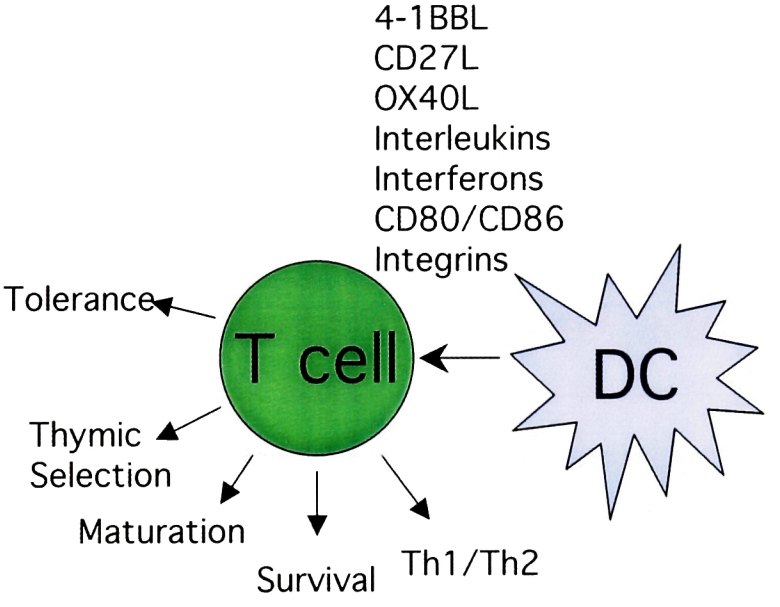

Figure 2.4. DC-T cell cross-talk I. DCs mediate a wide variety of effects on T cells, in part through the actions of the TNF family proteins 4-1BBL, CD27L, and OX40L.

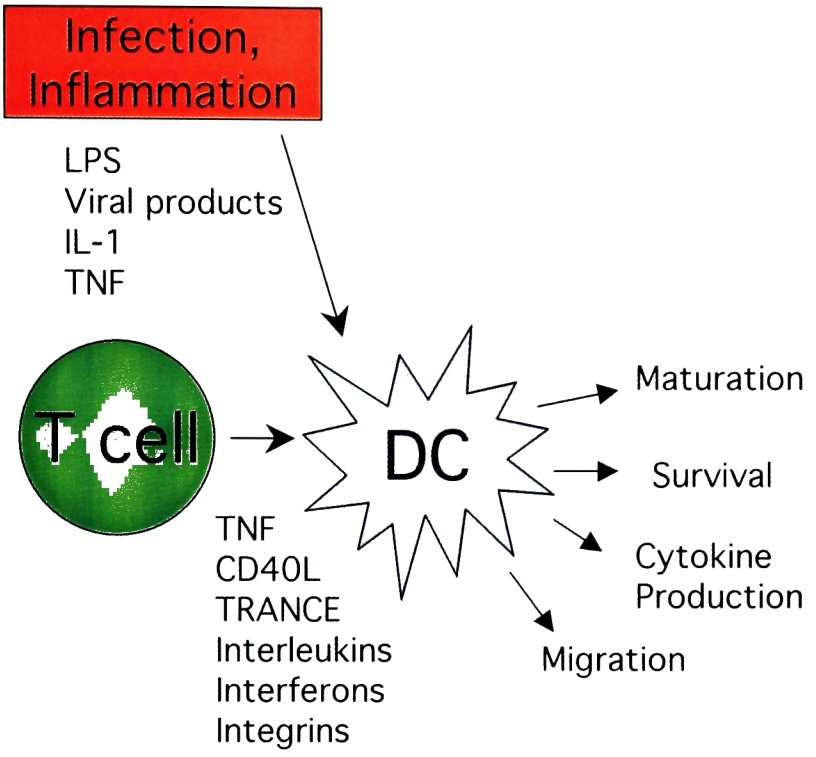

Figure 2.5. DC-T cell cross-talk II. DCs respond to a wide variety of signals from their environment and from T cells with which they interact. TRAF-dependent factors that influence DCs include LPS, viral products, IL-1, TNF, CD40L, and TRANCE.

DCs arise from multipotent precursors of the monocyte lineage. Autocrine TNF production is thought to contribute to differentiation to immature DCs (105). Immature DCs reside in peripheral tissues, sampling antigens. They are highly specialized for this task, efficiently internalizing particles from their environment. At this stage, the expression of T cell costimulatory molecules such as CD80, CD86, and MHC is low, as is the expression of TNFR family proteins such as CD40 and TRANCE-R. Many stimuli can induce the maturation of DCs, including microbial products that bind to TLRs, and inflammatory cytokines such as IL-1, TNF, and CD40L. As DCs mature, endocytosis decreases, and the surface expression of costimulatory molecules, CD40, and TRANCE-R increases (106). Maturing DCs migrate to draining lymph nodes, where they encounter activated T cells, engaging in a sophisticated cross-talk with them. In the absence of external survival stimuli, mature DCs rapidly undergo apoptosis (107), which is a likely

regulatory mechanism preventing autoimmunity. A productive interaction between an antigen-bearing DC and an antigen-specific T cell involves signals influencing both the DC and the T cell. Stimulation of the TCR and costimulatory receptors such as CD28 upregulate TNF, CD40L, and TRANCE on T cells, which provide survival and activation signals to the DC, so that it may engage more T cells in an ongoing immune response (104). Immature DCs are able to stimulate regulatory T cells leading to tolerogenic responses (108), thus the phenotype of DCs is an important regulator of the quality and intensity of a given immune response.

TNF family members are important regulators of DC function. CD40L induces DC maturation, survival, and cytokine production (109). CD40L deficiency leads to a markedly reduced ability of DCs to stimulate T cell-dependent responses (110). TRANCE- and CD40L- mediated increases in DC survival have potent effects on antigen-specific T cell responses (107,111,112), suggesting that the persistence of antigen-bearing mature DCs in the lymph node is an important factor in determining the efficiency of T cell responses. TNF-deficient mice display reduced antiviral responses, which is likely to be due to a defect in maturation (113). The spontaneous apoptosis of mature DCs appears to be at least partially due to autocrine activation of TNFR1, as TNFR1-/- DCs are highly resistant to spontaneous apoptosis (114). While the importance of TNF family proteins to DC biology is indisputable, the signaling pathways activated by TNF family proteins in DCs have not been well documented. A deeper understanding of the roles of specific signals induced by TNF family proteins in DCs may provide tools to manipulate the quality and intensity of immune responses.

2.3.2 Osteoimmunology: cross-talk between bone and the immune system

It has long been known that the cells of the immune system originate in the bone marrow. While the differentiation of lymphocytes and other cells of the hematopoietic system in bone marrow has been well described, relatively little attention has been paid to the effects that immune cells have on the bone itself. The possibility that immune cells can affect bone has been suggested in a variety of pathological conditions where normal bone growth and remodeling is disrupted, including autoimmune diseases and cancers of the hematopoietic system. The recent identification of some of the molecular mechanisms that govern bone physiology and immunity has revealed a striking amount of cross-talk between the two systems. It is rapidly becoming apparent that the fields of bone biology and immunology are inextricably intertwined.

2.3.2.1 Bone disease and osteoclasts

Rather than simply serving as a rigid, static frame on which to hang flesh and vital organs, skeletal bone is the result of a continuous, dynamic process of mineralization and resorption. These opposing actions are carried out by two cell types, osteoblasts and osteoclasts, and must be kept in balance to maintain skeletal integrity and systemic calcium metabolism. A disruption of this balance is most often observed when the rate of resorption by osteoclasts exceeds the rate of mineral deposition by osteoblasts, and bone mass is lost, as in osteoporosis (115). Increased bone resorption is observed in many inflammatory and autoimmune diseases, such as rheumatoid arthritis (116), periodontal disease (117), and Paget's disease (118). Bone destruction is also common secondary to many cancers, both those that reside in bone like leukemias and multiple myeloma, as well as breast and prostate cancers (119). A common clinical complication of cancer-mediated bone resorption is humoral hypercalcemia of malignancy (120).

Osteoclasts differentiate from multipotent bone marrow-derived cells of the monocyte/macrophage lineage (121). The life of an osteoclast can be broken down into four sequential stages: differentiation from multipotent bone marrow progenitors into mononuclear osteoclasts, fusion into multinucleated cells, activation to resorb bone, and apoptosis. These stages are regulated by the interactions of osteoclasts and their precursors with osteoblasts and other stromal cells in bone tissue, which stimulate or inhibit osteoclastogenesis in response to various stimuli, such as parathyroid hormone (PTH), vitamin D_3, and calcitonin. Deficiencies in normal osteoclast function lead to osteopetrosis, or a thickening of bone due to insufficient resorption, while hyperactivity of osteoclasts leads to osteoporosis, or loss of bone mass.

Studies in osteopetrotic mice with spontaneous and engineered mutations have revealed some of the genetic factors responsible for each of the stages in the osteoclast life cycle. Two factors crucial for the differentiation of osteoclasts are M-CSF (macrophage colony-stimulating factor) and TRANCE. Mice with a mutated M-CSF gene completely lack osteoclasts (122), as do mice with targeted deletions of TRANCE and TRANCE-R (123-125). Not all osteopetrotic mice lack osteoclasts; those with targeted deletions of TRAF6 (101,102) or the non-receptor tyrosine kinase c-Src (126,127) have osteoclasts, but they are incapable of being activated to resorb bone. Other mutant mice with osteopetrosis have demonstrated the importance of the transcription factors NF-κB (128) and AP-1 (129) in osteoclast development.

2.3.2.2 TRANCE/TRANCE-R/OPG regulation of osteoclasts

Studies of TRANCE and its two receptors TRANCE-R and OPG has suggested that cells of the immune system and the cells that remodel bone may regulate each other's function. TRANCE is normally expressed on osteoblasts and activated T cells, and its activating

receptor TRANCE-R is expressed on myelomonocytic cells, including osteoclasts and DCs (104). Although TRANCE and CD40L appear to have largely overlapping functions in stimulating the activation, survival, and adjuvant properties of DCs (107,130,131), TRANCE has been shown to be essential for certain CD40-independent anti-viral T cell-specific responses (112,132). In addition to its role in the regulation of osteoclasts and DCs, TRANCE is essential for lymph node organogenesis (133) and it regulates the function of activated T cells (134,135). OPG is a non-signaling soluble decoy receptor for TRANCE that negatively regulates its function by blocking the ability of TRANCE to bind to TRANCE-R on target cells (18,136).

TRANCE-R signaling through TRAF6 is of particular interest in osteoclasts because TRAF6 plays a role in TRANCE-mediated NF-κB and JNK activation (84,137). TRAF6 also provides a crucial link to the activation of c-Src, which activates the survival kinase Akt, prolonging the lifespan of activated osteoclasts (63,64). In addition, c-Src activation leads to the cytoskeletal changes such as membrane ruffling and actin ring formation necessary for osteoclasts to resorb bone (127).

Since TRANCE is expressed on activated T cells, and is crucial for T cell-DC communication, one might expect massive bone resorption under most inflammatory conditions. Indeed, it has been shown that TRANCE on activated T cells could activate osteoclasts in an induced arthritis model, and blocking TRANCE with OPG abrogated bone destruction but not inflammation (138). Furthermore, the bacteria that cause periodontal disease induce TRANCE expression on T cells, leading to alveolar bone destruction (139). Under conditions of estrogen withdrawal similar those seen in postmenopausal osteoporosis, it was found that increased TNF production by T cells augments TRANCE-mediated osteoclastogenesis (140). In TRANCE-deficient mice, it

has been shown that some of the osteoclast defects could be rescued by transgenic overexpression of TRANCE on T lymphocytes (124), which suggests that T cells may have a role in osteoclast activation under non-pathologic conditions. Figure 2.6 summarizes some key interactions between activated T cells, other inflammatory signals, osteoclasts, and osteoblasts. Our T cells are constantly working to fight off the universe of antigens in which we live, so what prevents them from causing extensive bone loss?

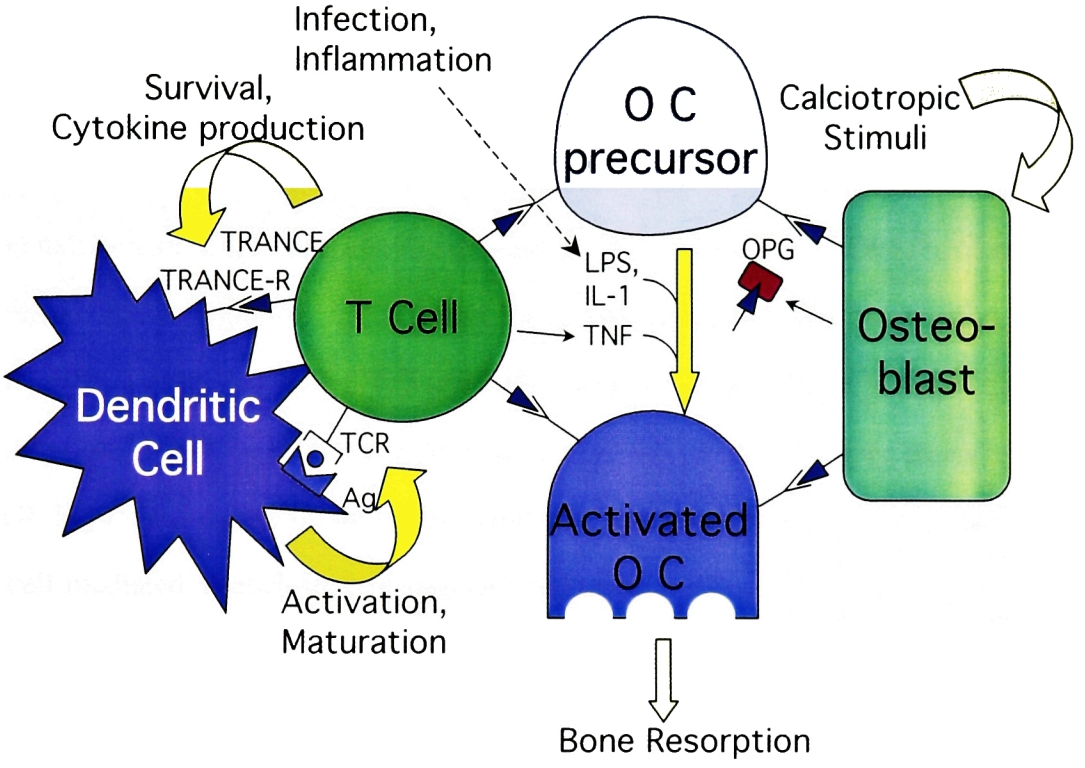

Figure 2.6. The interface between bone and the immune system. TRANCE on activated T cells stimulates DC as well as osteoclasts. Inflammatory mediators also affect OC function.

2.3.2.3 Regulatory mechanisms for inflammation-induced bone loss

Recently, a crucial counter-regulatory mechanism whereby activated T cells can inhibit TRANCE-mediated osteoclast development and activation through the action of the antiviral cytokine IFN-γ was demonstrated (141). While the type of T cells involved in inflammatory responses express TRANCE, they also secrete IFN-γ. IFN-γ can block

TRANCE-mediated osteoclastogenesis *in vitro,* and in mice deficient for the IFN-γ receptor, bone destruction in an autoimmune arthritis model is greatly exacerbated.

As is shown in Figure 2.7, the mechanism for this strong blockade of TRANCE signaling by IFN-γ in osteoclast precursors appears to be through activation of the ubiquitin-proteasome pathway for protein degradation (142), specifically targeting TRAF6 for degradation. Osteoclast formation in the presence of IFN-γ could be rescued by overexpression of TRAF6 in precursor cells, and IFN-γ's suppressive effect on osteoclastogenesis was markedly decreased in mice lacking proteasome components. Moreover, TRANCE-R is not the only receptor that signals through TRAF6. Signals downstream of CD40 are also attenuated by IFN-γ treatment. TRAF6 is an essential intermediate in signaling by LPS and IL-1, pathways associated with innate immunity (101,102). Given that IL-1 (143) and LPS (144) can induce osteoclastogenesis and bone resorption, it will be interesting to examine the effects of IFN-γ on those pathways as well. Figure 2.8 diagrams some key signaling pathways involved in the balance between T cell-mediated osteoclast activation and inhibition.

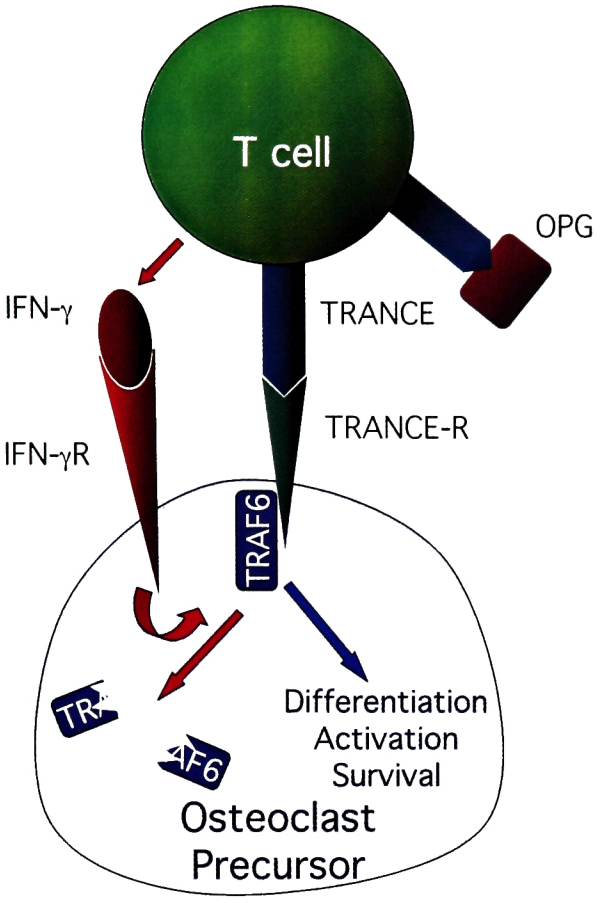

Figure 2.7. IFN-γ negatively regulates TRANCE-mediated osteoclastogenesis. Activated T cells express both TRANCE and IFN-γ. While TRANCE can activate osteoclastogenesis in a TRAF6-dependent manner, IFN-γ upregulates pathways leading to the degradation of TRAF6. This may explain why inflammatory processes do not always lead to bone loss.

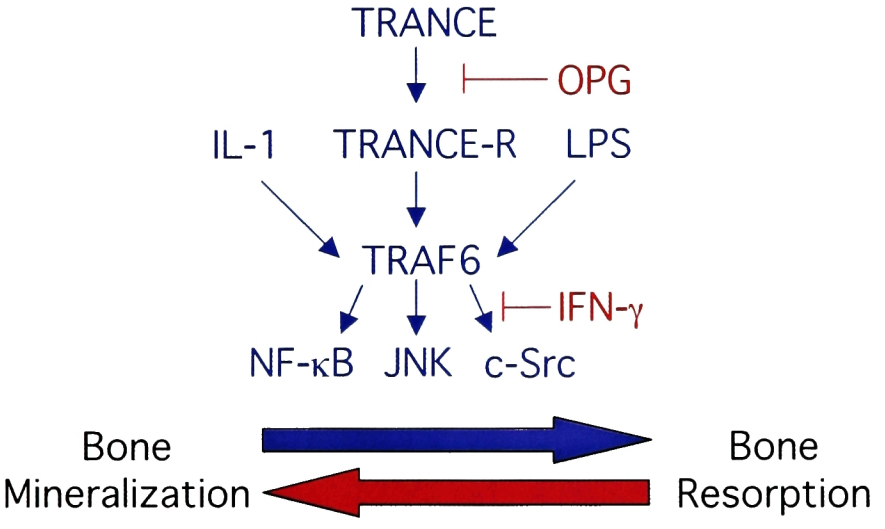

Figure 2.8. The osteoclast signaling axis. Factors promoting bone resorption by activating osteoclast function are shown in blue, and factors promoting bone mineralization by inhibiting osteoclast function are shown in red.

2.3.2.4 Other inflammatory regulators of osteoclasts

Clearly, we are only beginning to scratch the surface of the relationship between bone biology and the immune system. Other immunomodulatory factors, including TNFα (145,146), IL-6, IL-11, IL-15, and IL-17 can positively modulate osteoclast function, while IL-4, IL-10, IL-13, IL-18, and TGF-β can inhibit bone resorption (147). The clinical sequelae of bone destruction in inflammatory diseases are significant, and some treatment initiatives targeting TNF (148) have shown promising preliminary results. However, systemically targeting TNF, TRANCE, or other immune mediators to prevent chronic bone loss may result in long-term immunodeficiencies. The future design of treatments for bone loss must incorporate greater sensitivity to the potential detrimental effects of those treatments on normal immune function. Cell type-specific inhibitors of various signaling intermediates in osteoclast activation such as c-Src present a step in the right direction (149), and further advances in understanding the differences in signaling pathways activated in osteoclasts and dendritic cells should lead to more therapeutic targets.

The cells that resorb bone come from the same hematopoietic lineage as the cells that process and present antigens in the immune system. As is highlighted by the regulatory relationships between TRANCE, TRANCE-R, and OPG, immune cells and bone cells use similar molecular mechanisms in their normal functions. Increasingly, it is becoming evident that there is significant cross-talk between bone and the immune system under normal physiological conditions as well as in inflammatory pathological conditions. In light of recent advances in the treatment of both inflammatory diseases and osteoporosis, the challenge now is to carefully navigate the interface between bone (osteo-) and the

immune system. This challenge opens a new field of study, which we propose to call osteoimmunology (150).

2.4 TNF/TNFR proteins and TRAFs in health and disease

Beyond diseases of bone, there are many other clinical conditions in which dysregulation of TNF family proteins have been implicated, including Crohn's disease, stroke, multiple sclerosis, and Alzheimer's disease (151). Defects in CD40L expression results in X-linked hyper-IgM syndrome, in which B cell isotype switching is impaired, and patients are susceptible to a variety of infections (152-155). Similar clinical findings have been linked to mutations in the *CD40* gene (156). LMP1 is a viral protein expressed in Epstein-Barr virus-infected B cells, mimicking a constitutively active CD40 (35) and HVEM is a receptor for herpes simplex virus on T cells (157). While the basic underpinnings of TNFR family signaling are established, the precise mechanisms of action of TRAFs and their downstream targets are less clear. A greater understanding of TRAF-mediated signaling mechanisms, including: differentiating the mechanisms by which TRAFs activate different kinase cascades, determining the individual roles of each TRAF, elucidating structure-function relationships of TRAFs and interacting proteins, identifying modes of TRAF regulation, and dissecting the roles of combinations of TRAFs and specific receptors in a cell type-specific manner will be essential to understanding and manipulating these conditions in the future. The goal of this thesis is to address some of these issues pertaining to the mechanisms of TRAF signaling.

2.5 References

1. Granger, G. A., Shacks, S. J., Williams, T. W., and Kolb, W. P. (1969) Lymphocyte in vitro cytotoxicity: specific release of lymphotoxin-like materials from tuberculin-sensitive lymphoid cells. *Nature* **221**(5186), 1155-7.
2. Carswell, E. A., Old, L. J., Kassel, R. L., Green, S., Fiore, N., and Williamson, B. (1975) An endotoxin-induced serum factor that causes necrosis of tumors. *Proc Natl Acad Sci U S A* **72**(9), 3666-70.
3. Locksley, R. M., Killeen, N., and Lenardo, M. J. (2001) The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* **104**(4), 487-501.
4. Ashkenazi, A., and Dixit, V. M. (1998) Death receptors: signaling and modulation. *Science* **281**(5381), 1305-8.
5. Wallach, D., Varfolomeev, E. E., Malinin, N. L., Goltsev, Y. V., Kovalenko, A. V., and Boldin, M. P. (1999) Tumor necrosis factor receptor and Fas signaling mechanisms. *Annu Rev Immunol* **17**, 331-67
6. Baker, S. J., and Reddy, E. P. (1998) Modulation of life and death by the TNF receptor superfamily. *Oncogene* **17**(25), 3261-70.
7. Idriss, H. T., and Naismith, J. H. (2000) TNF alpha and the TNF receptor superfamily: structure-function relationship(s). *Microsc Res Tech* **50**(3), 184-95.
8. Smith, C. A., Farrah, T., and Goodwin, R. G. (1994) The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. *Cell* **76**(6), 959-62.
9. Banner, D. W., D'Arcy, A., Janes, W., Gentz, R., Schoenfeld, H. J., Broger, C., Loetscher, H., and Lesslauer, W. (1993) Crystal structure of the soluble human 55 kd TNF receptor-human TNF beta complex: implications for TNF receptor activation. *Cell* **73**(3), 431-45.
10. Chan, F. K., Chun, H. J., Zheng, L., Siegel, R. M., Bui, K. L., and Lenardo, M. J. (2000) A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. *Science* **288**(5475), 2351-4.
11. Thompson, C. B. (1995) Apoptosis in the pathogenesis and treatment of disease. *Science* **267**(5203), 1456-62.
12. Golstein, P. (1998) Cell death in us and others. *Science* **281**(5381), 1283.
13. Martin, S. J., Green, D. R., and Cotter, T. G. (1994) Dicing with death: dissecting the components of the apoptosis machinery. *Trends Biochem Sci* **19**(1), 26-30.
14. Nagata, S. (1997) Apoptosis by death factor. *Cell* **88**(3), 355-65.
15. Tartaglia, L. A., Ayres, T. M., Wong, G. H., and Goeddel, D. V. (1993) A novel domain within the 55 kd TNF receptor signals cell death. *Cell* **74**(5), 845-53.

16. Hsu, H., Xiong, J., and Goeddel, D. V. (1995) The TNF receptor 1-associated protein TRADD signals cell death and NF- kappa B activation. *Cell* **81**(4), 495-504.

17. Hsu, H., Shu, H. B., Pan, M. G., and Goeddel, D. V. (1996) TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell* **84**(2), 299-308.

18. Simonet, W. S., Lacey, D. L., Dunstan, C. R., Kelley, M., Chang, M. S., Luthy, R., Nguyen, H. Q., Wooden, S., Bennett, L., Boone, T., Shimamoto, G., DeRose, M., Elliott, R., Colombero, A., Tan, H. L., Trail, G., Sullivan, J., Davy, E., Bucay, N., Renshaw-Gegg, L., Hughes, T. M., Hill, D., Pattison, W., Campbell, P., Boyle, W. J., and et al. (1997) Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* **89**(2), 309-19.

19. Pan, G., Ni, J., Wei, Y. F., Yu, G., Gentz, R., and Dixit, V. M. (1997) An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science* **277**(5327), 815-8.

20. Sheridan, J. P., Marsters, S. A., Pitti, R. M., Gurney, A., Skubatch, M., Baldwin, D., Ramakrishnan, L., Gray, C. L., Baker, K., Wood, W. I., Goddard, A. D., Godowski, P., and Ashkenazi, A. (1997) Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science* **277**(5327), 818-21.

21. Marsters, S. A., Sheridan, J. P., Pitti, R. M., Huang, A., Skubatch, M., Baldwin, D., Yuan, J., Gurney, A., Goddard, A. D., Godowski, P., and Ashkenazi, A. (1997) A novel receptor for Apo2L/TRAIL contains a truncated death domain. *Curr Biol* **7**(12), 1003-6.

22. Degli-Esposti, M. A., Dougall, W. C., Smolak, P. J., Waugh, J. Y., Smith, C. A., and Goodwin, R. G. (1997) The novel receptor TRAIL-R4 induces NF-kappaB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. *Immunity* **7**(6), 813-20.

23. Pan, G., Bauer, J. H., Haridas, V., Wang, S., Liu, D., Yu, G., Vincenz, C., Aggarwal, B. B., Ni, J., and Dixit, V. M. (1998) Identification and functional characterization of DR6, a novel death domain-containing TNF receptor. *FEBS Lett* **431**(3), 351-6.

24. Pan, G., Ni, J., Yu, G., Wei, Y. F., and Dixit, V. M. (1998) TRUNDD, a new member of the TRAIL receptor family that antagonizes TRAIL signalling. *FEBS Lett* **424**(1-2), 41-5.

25. Pitti, R. M., Marsters, S. A., Lawrence, D. A., Roy, M., Kischkel, F. C., Dowd, P., Huang, A., Donahue, C. J., Sherwood, S. W., Baldwin, D. T., Godowski, P. J., Wood, W. I., Gurney, A. L., Hillan, K. J., Cohen, R. L., Goddard, A. D., Botstein, D., and Ashkenazi, A. (1998) Genomic amplification of a decoy receptor for Fas ligand in lung and colon cancer. *Nature* **396**(6712), 699-703.

26. Gruss, H. J., and Dower, S. K. (1995) Tumor necrosis factor ligand superfamily: involvement in the pathology of malignant lymphomas. *Blood* **85**(12), 3378-404.
27. Jenkins, M., Keir, M., and McCune, J. M. (2000) A membrane-bound Fas decoy receptor expressed by human thymocytes. *J Biol Chem* **275**(11), 7988-93.
28. Tone, M., Tone, Y., Fairchild, P. J., Wykes, M., and Waldmann, H. (2001) Regulation of CD40 function by its isoforms generated through alternative splicing. *Proc Natl Acad Sci U S A* **98**(4), 1751-6.
29. Arch, R. H., Gedrich, R. W., and Thompson, C. B. (1998) Tumor necrosis factor receptor-associated factors (TRAFs)--a family of adapter proteins that regulates life and death. *Genes Dev* **12**(18), 2821-30.
30. Inoue, J., Ishida, T., Tsukamoto, N., Kobayashi, N., Naito, A., Azuma, S., and Yamamoto, T. (2000) Tumor necrosis factor receptor-associated factor (TRAF) family: adapter proteins that mediate cytokine signaling. *Exp Cell Res* **254**(1), 14-24.
31. Wajant, H., Henkler, F., and Scheurich, P. (2001) The TNF-receptor-associated factor family: scaffold molecules for cytokine receptors, kinases and their regulators. *Cell Signal* **13**(6), 389-400.
32. Rothe, M., Wong, S. C., Henzel, W. J., and Goeddel, D. V. (1994) A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. *Cell* **78**(4), 681-92.
33. Hu, H. M., O'Rourke, K., Boguski, M. S., and Dixit, V. M. (1994) A novel RING finger protein interacts with the cytoplasmic domain of CD40. *J Biol Chem* **269**(48), 30069-72.
34. Cheng, G., Cleary, A. M., Ye, Z. S., Hong, D. I., Lederman, S., and Baltimore, D. (1995) Involvement of CRAF1, a relative of TRAF, in CD40 signaling. *Science* **267**(5203), 1494-8.
35. Mosialos, G., Birkenbach, M., Yalamanchili, R., VanArsdale, T., Ware, C., and Kieff, E. (1995) The Epstein-Barr virus transforming protein LMP1 engages signaling proteins for the tumor necrosis factor receptor family. *Cell* **80**(3), 389-99.
36. Regnier, C. H., Tomasetto, C., Moog-Lutz, C., Chenard, M. P., Wendling, C., Basset, P., and Rio, M. C. (1995) Presence of a new conserved domain in CART1, a novel member of the tumor necrosis factor receptor-associated protein family, which is expressed in breast carcinoma. *J Biol Chem* **270**(43), 25715-21.
37. Nakano, H., Oshima, H., Chung, W., Williams-Abbott, L., Ware, C. F., Yagita, H., and Okumura, K. (1996) TRAF5, an activator of NF-kappaB and putative signal transducer for the lymphotoxin-beta receptor. *J Biol Chem* **271**(25), 14661-4.

38. Ishida, T. K., Tojo, T., Aoki, T., Kobayashi, N., Ohishi, T., Watanabe, T., Yamamoto, T., and Inoue, J. (1996) TRAF5, a novel tumor necrosis factor receptor-associated factor family protein, mediates CD40 signaling. *Proc Natl Acad Sci U S A* **93**(18), 9437-42.

39. Cao, Z., Xiong, J., Takeuchi, M., Kurama, T., and Goeddel, D. V. (1996) TRAF6 is a signal transducer for interleukin-1. *Nature* **383**(6599), 443-6.

40. Ishida, T., Mizushima, S., Azuma, S., Kobayashi, N., Tojo, T., Suzuki, K., Aizawa, S., Watanabe, T., Mosialos, G., Kieff, E., Yamamoto, T., and Inoue, J. (1996) Identification of TRAF6, a novel tumor necrosis factor receptor-associated factor protein that mediates signaling from an amino- terminal domain of the CD40 cytoplasmic region. *J Biol Chem* **271**(46), 28745-8.

41. Zapata, J. M., Pawlowski, K., Haas, E., Ware, C. F., Godzik, A., and Reed, J. C. (2001) A diverse family of proteins containing tumor necrosis factor receptor-associated factor domains. *J Biol Chem* **276**(26), 24242-52.

42. Wajant, H., Muhlenbeck, F., and Scheurich, P (1998) Identification of a TRAF (TNF receptor-associated factor) gene in Caenorhabditis elegans. *J Mol Evol* **47**(6), 656-62.

43. Liu, H., Su, Y. C., Becker, E., Treisman, J., and Skolnik, E. Y. (1999) A Drosophila TNF-receptor-associated factor (TRAF) binds the ste20 kinase Misshapen and activates Jun kinase. *Curr Biol* **9**(2), 101-4.

44. Grech, A., Quinn, R., Srinivasan, D., Badoux, X., and Brink, R. (2000) Complete structural characterisation of the mammalian and Drosophila TRAF genes: implications for TRAF evolution and the role of RING finger splice variants. *Mol Immunol* **37**(12-13), 721-34.

45. Schwenzer, R., Siemienski, K., Liptay, S., Schubert, G., Peters, N., Scheurich, P., Schmid, R. M., and Wajant, H. (1999) The human tumor necrosis factor (TNF) receptor-associated factor 1 gene (TRAF1) is up-regulated by cytokines of the TNF ligand family and modulates TNF-induced activation of NF-kappaB and c-Jun N-terminal kinase. *J Biol Chem* **274**(27), 19368-74.

46. Dunn, I. F., Sannikova, T. Y., Geha, R. S., and Tsitsikov, E. N. (2000) Identification and characterization of two CD40-inducible enhancers in the mouse TRAF1 gene locus. *Mol Immunol* **37**(16), 961-73.

47. Takeuchi, M., Rothe, M., and Goeddel, D. V. (1996) Anatomy of TRAF2. Distinct domains for nuclear factor-kappaB activation and association with tumor necrosis factor signaling proteins. *J Biol Chem* **271**(33), 19935-42.

48. McWhirter, S. M., Pullen, S. S., Holton, J. M., Crute, J. J., Kehry, M. R., and Alber, T. (1999) Crystallographic analysis of CD40 recognition and signaling by human TRAF2. *Proc Natl Acad Sci U S A* **96**(15), 8408-13.

49. Ni, C. Z., Welsh, K., Leo, E., Chiou, C. K., Wu, H., Reed, J. C., and Ely, K. R. (2000) Molecular basis for CD40 signaling mediated by TRAF3. *Proc Natl Acad Sci U S A* **97**(19), 10395-9.
50. Park, Y. C., Burkitt, V., Villa, A. R., Tong, L., and Wu, H. (1999) Structural basis for self-association and receptor recognition of human TRAF2. *Nature* **398**(6727), 533-8.
51. Ye, H., Park, Y. C., Kreishman, M., Kieff, E., and Wu, H. (1999) The structural basis for the recognition of diverse receptor sequences by TRAF2. *Mol Cell* **4**(3), 321-30.
52. Park, Y. C., Ye, H., Hsia, C., Segal, D., Rich, R. L., Liou, H. C., Myszka, D. G., and Wu, H. (2000) A novel mechanism of TRAF signaling revealed by structural and functional analyses of the TRADD-TRAF2 interaction. *Cell* **101**(7), 777-87.
53. Ye, H., and Wu, H. (2000) Thermodynamic characterization of the interaction between TRAF2 and tumor necrosis factor receptor peptides by isothermal titration calorimetry. *Proc Natl Acad Sci U S A* **97**(16), 8961-6.
54. Baud, V., Liu, Z. G., Bennett, B., Suzuki, N., Xia, Y., and Karin, M. (1999) Signaling by proinflammatory cytokines: oligomerization of TRAF2 and TRAF6 is sufficient for JNK and IKK activation and target gene induction via an amino-terminal effector domain. *Genes Dev* **13**(10), 1297-308.
55. Aizawa, S., Nakano, H., Ishida, T., Horie, R., Nagai, M., Ito, K., Yagita, H., Okumura, K., Inoue, J., and Watanabe, T. (1997) Tumor necrosis factor receptor-associated factor (TRAF) 5 and TRAF2 are involved in CD30-mediated NFkappaB activation. *J Biol Chem* **272**(4), 2042-5.
56. Leo, E., Welsh, K., Matsuzawa, S., Zapata, J. M., Kitada, S., Mitchell, R. S., Ely, K. R., and Reed, J. C. (1999) Differential requirements for tumor necrosis factor receptor-associated factor family proteins in CD40-mediated induction of NF-kappaB and Jun N-terminal kinase activation. *J Biol Chem* **274**(32), 22414-22.
57. Pullen, S. S., Miller, H. G., Everdeen, D. S., Dang, T. T., Crute, J. J., and Kehry, M. R. (1998) CD40-tumor necrosis factor receptor-associated factor (TRAF) interactions: regulation of CD40 signaling through multiple TRAF binding sites and TRAF hetero-oligomerization. *Biochemistry* **37**(34), 11836-45.
58. Nishitoh, H., Saitoh, M., Mochida, Y., Takeda, K., Nakano, H., Rothe, M., Miyazono, K., and Ichijo, H. (1998) ASK1 is essential for JNK/SAPK activation by TRAF2. *Mol Cell* **2**(3), 389-95.
59. Hoeflich, K. P., Yeh, W. C., Yao, Z., Mak, T. W., and Woodgett, J. R. (1999) Mediation of TNF receptor-associated factor effector functions by apoptosis signal-regulating kinase-1 (ASK1). *Oncogene* **18**(42), 5814-20.
60. Takaesu, G., Kishida, S., Hiyama, A., Yamaguchi, K., Shibuya, H., Irie, K., Ninomiya-Tsuji, J., and Matsumoto, K. (2000) TAB2, a novel adaptor protein,

mediates activation of TAK1 MAPKKK by linking TAK1 to TRAF6 in the IL-1 signal transduction pathway. *Mol Cell* **5**(4), 649-58.

61. Malinin, N. L., Boldin, M. P., Kovalenko, A. V., and Wallach, D. (1997) MAP3K-related kinase involved in NF-kappaB induction by TNF, CD95 and IL-1. *Nature* **385**(6616), 540-4.
62. Yuasa, T., Ohno, S., Kehrl, J. H., and Kyriakis, J. M. (1998) Tumor necrosis factor signaling to stress-activated protein kinase (SAPK)/Jun NH2-terminal kinase (JNK) and p38. Germinal center kinase couples TRAF2 to mitogen-activated protein kinase/ERK kinase kinase 1 and SAPK while receptor interacting protein associates with a mitogen- activated protein kinase kinase kinase upstream of MKK6 and p38. *J Biol Chem* **273**(35), 22681-92.
63. Wong, B. R., Besser, D., Kim, N., Arron, J. R., Vologodskaia, M., Hanafusa, H., and Choi, Y. (1999) TRANCE, a TNF family member, activates Akt/PKB through a signaling complex involving TRAF6 and c-Src. *Mol Cell* **4**(6), 1041-9.
64. Xing, L., Venegas, A. M., Chen, A., Garrett-Beal, L., Boyce, B. F., Varmus, H. E., and Schwartzberg, P. L. (2001) Genetic evidence for a role for Src family kinases in TNF family receptor signaling and cell survival. *Genes Dev* **15**(2), 241-53.
65. Sanz, L., Diaz-Meco, M. T., Nakano, H., and Moscat, J. (2000) The atypical PKC-interacting protein p62 channels NF-kappaB activation by the IL-1-TRAF6 pathway. *Embo J* **19**(7), 1576-86.
66. Song, H. Y., Regnier, C. H., Kirschning, C. J., Goeddel, D. V., and Rothe, M. (1997) Tumor necrosis factor (TNF)-mediated kinase cascades: bifurcation of nuclear factor-kappaB and c-jun N-terminal kinase (JNK/SAPK) pathways at TNF receptor-associated factor 2. *Proc Natl Acad Sci U S A* **94**(18), 9792-6.
67. Ghosh, S., May, M. J., and Kopp, E. B. (1998) NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol* **16**, 225-60
68. Silverman, N., and Maniatis, T. (2001) NF-kappaB signaling pathways in mammalian and insect innate immunity. *Genes Dev* **15**(18), 2321-42.
69. Karin, M., and Ben-Neriah, Y. (2000) Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu Rev Immunol* **18**, 621-63
70. Hsu, H., Huang, J., Shu, H. B., Baichwal, V., and Goeddel, D. V. (1996) TNF-dependent recruitment of the protein kinase RIP to the TNF receptor- 1 signaling complex. *Immunity* **4**(4), 387-96.
71. Yang, J., Lin, Y., Guo, Z., Cheng, J., Huang, J., Deng, L., Liao, W., Chen, Z., Liu, Z., and Su, B. (2001) The essential role of MEKK3 in TNF-induced NF-kappaB activation. *Nat Immunol* **2**(7), 620-4.

72. Baud, V., and Karin, M. (2001) Signal transduction by tumor necrosis factor and its relatives. *Trends Cell Biol* **11**(9), 372-7.

73. Deng, L., Wang, C., Spencer, E., Yang, L., Braun, A., You, J., Slaughter, C., Pickart, C., and Chen, Z. J. (2000) Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell* **103**(2), 351-61.

74. Wang, C., Deng, L., Hong, M., Akkaraju, G. R., Inoue, J., and Chen, Z. J. (2001) TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature* **412**(6844), 346-51.

75. Karin, M., Liu, Z., and Zandi, E. (1997) AP-1 function and regulation. *Curr Opin Cell Biol* **9**(2), 240-6.

76. Widmann, C., Gibson, S., Jarpe, M. B., and Johnson, G. L. (1999) Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol Rev* **79**(1), 143-80.

77. Yujiri, T., Ware, M., Widmann, C., Oyer, R., Russell, D., Chan, E., Zaitsu, Y., Clarke, P., Tyler, K., Oka, Y., Fanger, G. R., Henson, P., and Johnson, G. L. (2000) MEK kinase 1 gene disruption alters cell migration and c-Jun NH2-terminal kinase regulation but does not cause a measurable defect in NF- kappa B activation. *Proc Natl Acad Sci U S A* **97**(13), 7272-7.

78. Tobiume, K., Matsuzawa, A., Takahashi, T., Nishitoh, H., Morita, K., Takeda, K., Minowa, O., Miyazono, K., Noda, T., and Ichijo, H. (2001) ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis. *EMBO Rep* **2**(3), 222-8.

79. Rao, K. M. (2001) MAP kinase activation in macrophages. *J Leukoc Biol* **69**(1), 3-10.

80. Brockhaus, M., Schoenfeld, H. J., Schlaeger, E. J., Hunziker, W., Lesslauer, W., and Loetscher, H. (1990) Identification of two types of tumor necrosis factor receptors on human cell lines by monoclonal antibodies. *Proc Natl Acad Sci U S A* **87**(8), 3127-31.

81. Hohmann, H. P., Remy, R., Brockhaus, M., and van Loon, A. P. (1989) Two different cell types have different major receptors for human tumor necrosis factor (TNF alpha). *J Biol Chem* **264**(25), 14927-34.

82. Grell, M., Douni, E., Wajant, H., Lohden, M., Clauss, M., Maxeiner, B., Georgopoulos, S., Lesslauer, W., Kollias, G., Pfizenmaier, K., and et al. (1995) The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. *Cell* **83**(5), 793-802.

83. Weiss, T., Grell, M., Siemienski, K., Muhlenbeck, F., Durkop, H., Pfizenmaier, K., Scheurich, P., and Wajant, H. (1998) TNFR80-dependent enhancement of

TNFR60-induced cell death is mediated by TNFR-associated factor 2 and is specific for TNFR60. *J Immunol* **161**(6), 3136-42.

84. Wong, B. R., Josien, R., Lee, S. Y., Vologodskaia, M., Steinman, R. M., and Choi, Y. (1998) The TRAF family of signal transducers mediates NF-kappaB activation by the TRANCE receptor. *J Biol Chem* **273**(43), 28355-9.
85. Croston, G. E., Cao, Z., and Goeddel, D. V. (1995) NF-kappa B activation by interleukin-1 (IL-1) requires an IL-1 receptor- associated protein kinase activity. *J Biol Chem* **270**(28), 16514-7.
86. Cao, Z., Henzel, W. J., and Gao, X. (1996) IRAK: a kinase associated with the interleukin-1 receptor. *Science* **271**(5252), 1128-31.
87. Wesche, H., Henzel, W. J., Shillinglaw, W., Li, S., and Cao, Z. (1997) MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. *Immunity* **7**(6), 837-47.
88. Adachi, O., Kawai, T., Takeda, K., Matsumoto, M., Tsutsui, H., Sakagami, M., Nakanishi, K., and Akira, S. (1998) Targeted disruption of the MyD88 gene results in loss of IL-1- and IL- 18-mediated function. *Immunity* **9**(1), 143-50.
89. Burns, K., Clatworthy, J., Martin, L., Martinon, F., Plumpton, C., Maschera, B., Lewis, A., Ray, K., Tschopp, J., and Volpe, F. (2000) Tollip, a new component of the IL-1RI pathway, links IRAK to the IL-1 receptor. *Nat Cell Biol* **2**(6), 346-51.
90. Medzhitov, R., and Janeway, C. A., Jr. (1998) Innate immune recognition and control of adaptive immune responses. *Semin Immunol* **10**(5), 351-3.
91. Medzhitov, R., Preston-Hurlburt, P., Kopp, E., Stadlen, A., Chen, C., Ghosh, S., and Janeway, C. A., Jr. (1998) MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. *Mol Cell* **2**(2), 253-8.
92. Medzhitov, R., and Janeway, C. A., Jr. (1998) An ancient system of host defense. *Curr Opin Immunol* **10**(1), 12-5.
93. Tsitsikov, E. N., Laouini, D., Dunn, I. F., Sannikova, T. Y., Davidson, L., Alt, F. W., and Geha, R. S. (2001) Traf1 is a negative regulator of tnf signaling. enhanced tnf signaling in traf1-deficient mice. *Immunity* **15**(4), 647-57.
94. Yeh, W. C., Shahinian, A., Speiser, D., Kraunus, J., Billia, F., Wakeham, A., de la Pompa, J. L., Ferrick, D., Hum, B., Iscove, N., Ohashi, P., Rothe, M., Goeddel, D. V., and Mak, T. W. (1997) Early lethality, functional NF-kappaB activation, and increased sensitivity to TNF-induced cell death in TRAF2-deficient mice. *Immunity* **7**(5), 715-25.
95. Lee, S. Y., Reichlin, A., Santana, A., Sokol, K. A., Nussenzweig, M. C., and Choi, Y. (1997) TRAF2 is essential for JNK but not NF-kappaB activation and regulates lymphocyte proliferation and survival. *Immunity* **7**(5), 703-13.
96. Nguyen, L. T., Duncan, G. S., Mirtsos, C., Ng, M., Speiser, D. E., Shahinian, A., Marino, M. W., Mak, T. W., Ohashi, P. S., and Yeh, W. C. (1999) TRAF2

deficiency results in hyperactivity of certain TNFR1 signals and impairment of CD40-mediated responses. *Immunity* **11**(3), 379-89.

97. Tada, K., Okazaki, T., Sakon, S., Kobarai, T., Kurosawa, K., Yamaoka, S., Hashimoto, H., Mak, T. W., Yagita, H., Okumura, K., Yeh, W. C., and Nakano, H. (2001) Critical roles of TRAF2 and TRAF5 in tumor necrosis factor-induced NF- kappa B activation and protection from cell death. *J Biol Chem* **276**(39), 36530-4.
98. Xu, Y., Cheng, G., and Baltimore, D. (1996) Targeted disruption of TRAF3 leads to postnatal lethality and defective T-dependent immune responses. *Immunity* **5**(5), 407-15.
99. Shiels, H., Li, X., Schumacker, P. T., Maltepe, E., Padrid, P. A., Sperling, A., Thompson, C. B., and Lindsten, T. (2000) TRAF4 deficiency leads to tracheal malformation with resulting alterations in air flow to the lungs. *Am J Pathol* **157**(2), 679-88.
100. Nakano, H., Sakon, S., Koseki, H., Takemori, T., Tada, K., Matsumoto, M., Munechika, E., Sakai, T., Shirasawa, T., Akiba, H., Kobata, T., Santee, S. M., Ware, C. F., Rennert, P. D., Taniguchi, M., Yagita, H., and Okumura, K. (1999) Targeted disruption of Traf5 gene causes defects in CD40- and CD27- mediated lymphocyte activation. *Proc Natl Acad Sci U S A* **96**(17), 9803-8.
101. Naito, A., Azuma, S., Tanaka, S., Miyazaki, T., Takaki, S., Takatsu, K., Nakao, K., Nakamura, K., Katsuki, M., Yamamoto, T., and Inoue, J. (1999) Severe osteopetrosis, defective interleukin-1 signalling and lymph node organogenesis in TRAF6-deficient mice. *Genes Cells* **4**(6), 353-62.
102. Lomaga, M. A., Yeh, W. C., Sarosi, I., Duncan, G. S., Furlonger, C., Ho, A., Morony, S., Capparelli, C., Van, G., Kaufman, S., van der Heiden, A., Itie, A., Wakeham, A., Khoo, W., Sasaki, T., Cao, Z., Penninger, J. M., Paige, C. J., Lacey, D. L., Dunstan, C. R., Boyle, W. J., Goeddel, D. V., and Mak, T. W. (1999) TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. *Genes Dev* **13**(8), 1015-24.
103. Banchereau, J., and Steinman, R. M. (1998) Dendritic cells and the control of immunity. *Nature* **392**(6673), 245-52.
104. Wong, B. R., Josien, R., and Choi, Y. (1999) TRANCE is a TNF family member that regulates dendritic cell and osteoclast function. *J Leukoc Biol* **65**(6), 715-24.
105. Liu, Y. J. (2001) Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. *Cell* **106**(3), 259-62.
106. Mellman, I., and Steinman, R. M. (2001) Dendritic cells: specialized and regulated antigen processing machines. *Cell* **106**(3), 255-8.
107. Josien, R., Li, H. L., Ingulli, E., Sarma, S., Wong, B. R., Vologodskaia, M., Steinman, R. M., and Choi, Y. (2000) TRANCE, a tumor necrosis factor family

member, enhances the longevity and adjuvant properties of dendritic cells in vivo. *J Exp Med* **191**(3), 495-502.

108. Dhodapkar, M. V., Steinman, R. M., Krasovsky, J., Munz, C., and Bhardwaj, N. (2001) Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. *J Exp Med* **193**(2), 233-8.
109. van Kooten, C., and Banchereau, J. (2000) CD40-CD40 ligand. *J Leukoc Biol* **67**(1), 2-17.
110. Grewal, I. S., and Flavell, R. A. (1996) The role of CD40 ligand in costimulation and T-cell activation. *Immunol Rev* **153**, 85-106.
111. Miga, A. J., Masters, S. R., Durell, B. G., Gonzalez, M., Jenkins, M. K., Maliszewski, C., Kikutani, H., Wade, W. F., and Noelle, R. J. (2001) Dendritic cell longevity and T cell persistence is controlled by CD154- CD40 interactions. *Eur J Immunol* **31**(3), 959-65.
112. Bachmann, M. F., Wong, B. R., Josien, R., Steinman, R. M., Oxenius, A., and Choi, Y. (1999) TRANCE, a tumor necrosis factor family member critical for CD40 ligand- independent T helper cell activation. *J Exp Med* **189**(7), 1025-31.
113. Trevejo, J. M., Marino, M. W., Philpott, N., Josien, R., Richards, E. C., Elkon, K. B., and Falck-Pedersen, E. (2001) TNF-alpha -dependent maturation of local dendritic cells is critical for activating the adaptive immune response to virus infection. *Proc Natl Acad Sci U S A* **98**(21), 12162-12167.
114. Funk, J. O., Walczak, H., Voigtlander, C., Berchtold, S., Baumeister, T., Rauch, P., Rossner, S., Steinkasserer, A., Schuler, G., and Lutz, M. B. (2000) Cutting edge: resistance to apoptosis and continuous proliferation of dendritic cells deficient for TNF receptor-1. *J Immunol* **165**(9), 4792-6.
115. Teitelbaum, S. L. (2000) Bone resorption by osteoclasts. *Science* **289**(5484), 1504-8.
116. Kotake, S., Udagawa, N., Hakoda, M., Mogi, M., Yano, K., Tsuda, E., Takahashi, K., Furuya, T., Ishiyama, S., Kim, K. J., Saito, S., Nishikawa, T., Takahashi, N., Togari, A., Tomatsu, T., Suda, T., and Kamatani, N. (2001) Activated human T cells directly induce osteoclastogenesis from human monocytes: possible role of T cells in bone destruction in rheumatoid arthritis patients. *Arthritis Rheum* **44**(5), 1003-12.
117. Taubman, M. A., and Kawai, T. (2001) Involvement of T-lymphocytes in periodontal disease and in direct and indirect induction of bone resorption. *Crit Rev Oral Biol Med* **12**(2), 125-35
118. Ross, F. P. (2000) RANKing the importance of measles virus in Paget's disease. *J Clin Invest* **105**(5), 555-8.
119. Rodan, G. A., and Martin, T. J. (2000) Therapeutic approaches to bone diseases. *Science* **289**(5484), 1508-14.

120. Mundy, G. R. (1991) Mechanisms of osteolytic bone destruction. *Bone* **12**(Suppl 1), S1-6.

121. Udagawa, N., Takahashi, N., Akatsu, T., Tanaka, H., Sasaki, T., Nishihara, T., Koga, T., Martin, T. J., and Suda, T. (1990) Origin of osteoclasts: mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow-derived stromal cells. *Proc Natl Acad Sci U S A* **87**(18), 7260-4.

122. Yoshida, H., Hayashi, S., Kunisada, T., Ogawa, M., Nishikawa, S., Okamura, H., Sudo, T., and Shultz, L. D. (1990) The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature* **345**(6274), 442-4.

123. Kong, Y. Y., Yoshida, H., Sarosi, I., Tan, H. L., Timms, E., Capparelli, C., Morony, S., Oliveira-dos-Santos, A. J., Van, G., Itie, A., Khoo, W., Wakeham, A., Dunstan, C. R., Lacey, D. L., Mak, T. W., Boyle, W. J., and Penninger, J. M. (1999) OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* **397**(6717), 315-23.

124. Kim, N., Odgren, P. R., Kim, D. K., Marks, S. C., Jr., and Choi, Y. (2000) Diverse roles of the tumor necrosis factor family member TRANCE in skeletal physiology revealed by TRANCE deficiency and partial rescue by a lymphocyte-expressed TRANCE transgene. *Proc Natl Acad Sci U S A* **97**(20), 10905-10.

125. Dougall, W. C., Glaccum, M., Charrier, K., Rohrbach, K., Brasel, K., De Smedt, T., Daro, E., Smith, J., Tometsko, M. E., Maliszewski, C. R., Armstrong, A., Shen, V., Bain, S., Cosman, D., Anderson, D., Morrissey, P. J., Peschon, J. J., and Schuh, J. (1999) RANK is essential for osteoclast and lymph node development. *Genes Dev* **13**(18), 2412-24.

126. Soriano, P., Montgomery, C., Geske, R., and Bradley, A. (1991) Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. *Cell* **64**(4), 693-702.

127. Boyce, B. F., Yoneda, T., Lowe, C., Soriano, P., and Mundy, G. R. (1992) Requirement of pp60c-src expression for osteoclasts to form ruffled borders and resorb bone in mice. *J Clin Invest* **90**(4), 1622-7.

128. Franzoso, G., Carlson, L., Xing, L., Poljak, L., Shores, E. W., Brown, K. D., Leonardi, A., Tran, T., Boyce, B. F., and Siebenlist, U. (1997) Requirement for NF-kappaB in osteoclast and B-cell development. *Genes Dev* **11**(24), 3482-96.

129. Grigoriadis, A. E., Wang, Z. Q., Cecchini, M. G., Hofstetter, W., Felix, R., Fleisch, H. A., and Wagner, E. F. (1994) c-Fos: a key regulator of osteoclast-macrophage lineage determination and bone remodeling. *Science* **266**(5184), 443-8.

130. Wong, B. R., Josien, R., Lee, S. Y., Sauter, B., Li, H. L., Steinman, R. M., and Choi, Y. (1997) TRANCE (tumor necrosis factor [TNF]-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor. *J Exp Med* **186**(12), 2075-80.

131. Josien, R., Wong, B. R., Li, H. L., Steinman, R. M., and Choi, Y. (1999) TRANCE, a TNF family member, is differentially expressed on T cell subsets and induces cytokine production in dendritic cells. *J Immunol* **162**(5), 2562-8.

132. Green, E. A., and Flavell, R. A. (1999) TRANCE-RANK, a new signal pathway involved in lymphocyte development and T cell activation. *J Exp Med* **189**(7), 1017-20.

133. Kim, D., Mebius, R. E., MacMicking, J. D., Jung, S., Cupedo, T., Castellanos, Y., Rho, J., Wong, B. R., Josien, R., Kim, N., Rennert, P. D., and Choi, Y. (2000) Regulation of peripheral lymph node genesis by the tumor necrosis factor family member TRANCE. *J Exp Med* **192**(10), 1467-78.

134. Wong, B. R., Rho, J., Arron, J., Robinson, E., Orlinick, J., Chao, M., Kalachikov, S., Cayani, E., Bartlett, F. S., 3rd, Frankel, W. N., Lee, S. Y., and Choi, Y. (1997) TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. *J Biol Chem* **272**(40), 25190-4.

135. Anderson, D. M., Maraskovsky, E., Billingsley, W. L., Dougall, W. C., Tometsko, M. E., Roux, E. R., Teepe, M. C., DuBose, R. F., Cosman, D., and Galibert, L. (1997) A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature* **390**(6656), 175-9.

136. Lacey, D. L., Timms, E., Tan, H. L., Kelley, M. J., Dunstan, C. R., Burgess, T., Elliott, R., Colombero, A., Elliott, G., Scully, S., Hsu, H., Sullivan, J., Hawkins, N., Davy, E., Capparelli, C., Eli, A., Qian, Y. X., Kaufman, S., Sarosi, I., Shalhoub, V., Senaldi, G., Guo, J., Delaney, J., and Boyle, W. J. (1998) Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* **93**(2), 165-76.

137. Kobayashi, N., Kadono, Y., Naito, A., Matsumoto, K., Yamamoto, T., Tanaka, S., and Inoue, J. (2001) Segregation of TRAF6-mediated signaling pathways clarifies its role in osteoclastogenesis. *Embo J* **20**(6), 1271-80.

138. Kong, Y. Y., Feige, U., Sarosi, I., Bolon, B., Tafuri, A., Morony, S., Capparelli, C., Li, J., Elliott, R., McCabe, S., Wong, T., Campagnuolo, G., Moran, E., Bogoch, E. R., Van, G., Nguyen, L. T., Ohashi, P. S., Lacey, D. L., Fish, E., Boyle, W. J., and Penninger, J. M. (1999) Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature* **402**(6759), 304-9.

139. Teng, Y. T., Nguyen, H., Gao, X., Kong, Y. Y., Gorczynski, R. M., Singh, B., Ellen, R. P., and Penninger, J. M. (2000) Functional human T-cell immunity and

osteoprotegerin ligand control alveolar bone destruction in periodontal infection. *J Clin Invest* **106**(6), R59-67.

140. Cenci, S., Weitzmann, M. N., Roggia, C., Namba, N., Novack, D., Woodring, J., and Pacifici, R. (2000) Estrogen deficiency induces bone loss by enhancing T-cell production of TNF-alpha. *J Clin Invest* **106**(10), 1229-37.
141. Takayanagi, H., Ogasawara, K., Hida, S., Chiba, T., Murata, S., Sato, K., Takaoka, A., Yokochi, T., Oda, H., Tanaka, K., Nakamura, K., and Taniguchi, T. (2000) T-cell-mediated regulation of osteoclastogenesis by signalling cross- talk between RANKL and IFN-gamma. *Nature* **408**(6812), 600-5.
142. Ciechanover, A. (1998) The ubiquitin-proteasome pathway: on protein death and cell life. *Embo J* **17**(24), 7151-60.
143. Jimi, E., Nakamura, I., Duong, L. T., Ikebe, T., Takahashi, N., Rodan, G. A., and Suda, T. (1999) Interleukin 1 induces multinucleation and bone-resorbing activity of osteoclasts in the absence of osteoblasts/stromal cells. *Exp Cell Res* **247**(1), 84-93.
144. Nair, S. P., Meghji, S., Wilson, M., Reddi, K., White, P., and Henderson, B. (1996) Bacterially induced bone destruction: mechanisms and misconceptions. *Infect Immun* **64**(7), 2371-80.
145. Kobayashi, K., Takahashi, N., Jimi, E., Udagawa, N., Takami, M., Kotake, S., Nakagawa, N., Kinosaki, M., Yamaguchi, K., Shima, N., Yasuda, H., Morinaga, T., Higashio, K., Martin, T. J., and Suda, T. (2000) Tumor necrosis factor alpha stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL-RANK interaction. *J Exp Med* **191**(2), 275-86.
146. Zhang, Y. H., Heulsmann, A., Tondravi, M. M., Mukherjee, A., and Abu-Amer, Y. (2001) Tumor necrosis factor-alpha (TNF) stimulates RANKL-induced osteoclastogenesis via coupling of TNF type 1 receptor and RANK signaling pathways. *J Biol Chem* **276**(1), 563-8.
147. Lorenzo, J. (2000) Interactions between immune and bone cells: new insights with many remaining questions. *J Clin Invest* **106**(6), 749-52.
148. Carteron, N. L. (2000) Cytokines in rheumatoid arthritis: trials and tribulations. *Mol Med Today* **6**(8), 315-23.
149. Shakespeare, W., Yang, M., Bohacek, R., Cerasoli, F., Stebbins, K., Sundaramoorthi, R., Azimioara, M., Vu, C., Pradeepan, S., Metcalf, C., 3rd, Haraldson, C., Merry, T., Dalgarno, D., Narula, S., Hatada, M., Lu, X., van Schravendijk, M. R., Adams, S., Violette, S., Smith, J., Guan, W., Bartlett, C., Herson, J., Iuliucci, J., Weigele, M., and Sawyer, T. (2000) Structure-based design of an osteoclast-selective, nonpeptide src homology 2 inhibitor with in vivo antiresorptive activity. *Proc Natl Acad Sci U S A* **97**(17), 9373-8.

150. Arron, J. R., and Choi, Y. (2000) Bone versus immune system. *Nature* **408**(6812), 535-6.

151. Balkwill, F., Foxwell, B., and Brennan, F. (2000) TNF is here to stay! *Immunol Today* **21**(10), 470-1.

152. Allen, R. C., Armitage, R. J., Conley, M. E., Rosenblatt, H., Jenkins, N. A., Copeland, N. G., Bedell, M. A., Edelhoff, S., Disteche, C. M., Simoneaux, D. K., and et al. (1993) CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome. *Science* **259**(5097), 990-3.

153. Aruffo, A., Farrington, M., Hollenbaugh, D., Li, X., Milatovich, A., Nonoyama, S., Bajorath, J., Grosmaire, L. S., Stenkamp, R., Neubauer, M., and et al. (1993) The CD40 ligand, gp39, is defective in activated T cells from patients with X-linked hyper-IgM syndrome. *Cell* **72**(2), 291-300.

154. DiSanto, J. P., Bonnefoy, J. Y., Gauchat, J. F., Fischer, A., and de Saint Basile, G. (1993) CD40 ligand mutations in x-linked immunodeficiency with hyper-IgM. *Nature* **361**(6412), 541-3.

155. Fuleihan, R., Ramesh, N., Loh, R., Jabara, H., Rosen, R. S., Chatila, T., Fu, S. M., Stamenkovic, I., and Geha, R. S. (1993) Defective expression of the CD40 ligand in X chromosome-linked immunoglobulin deficiency with normal or elevated IgM. *Proc Natl Acad Sci U S A* **90**(6), 2170-3.

156. Ferrari, S., Giliani, S., Insalaco, A., Al-Ghonaium, A., Soresina, A. R., Loubser, M., Avanzini, M. A., Marconi, M., Badolato, R., Ugazio, A. G., Levy, Y., Catalan, N., Durandy, A., Tbakhi, A., Notarangelo, L. D., and Plebani, A. (2001) Mutations of CD40 gene cause an autosomal recessive form of immunodeficiency with hyper IgM. *Proc Natl Acad Sci U S A* **98**(22), 12614-12619.

157. Montgomery, R. I., Warner, M. S., Lum, B. J., and Spear, P. G. (1996) Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell* **87**(3), 427-36.

3. RESULTS

3.1 A POSITIVE REGULATORY ROLE FOR CBL FAMILY PROTEINS IN TRANCE AND CD40L-MEDIATED AKT ACTIVATION

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3.1.1 Summary

TRANCE is a TNF family member essential for osteoclast differentiation, and it induces the activation and survival of osteoclasts and mature dendritic cells. We recently demonstrated that TRANCE activates Akt via a mechanism involving TRANCE-R/RANK, TRAF6, and c-Src. Here, we show that TRANCE-R and CD40 recruit TRAF6, Cbl family scaffolding proteins, and the phospholipid kinase PI3-K in a ligand-dependent manner. The recruitment of Cbl-b and c-Cbl to TRANCE-R is dependent upon the activity of Src-family kinases. TRANCE and CD40L-mediated Akt activation is defective in Cbl-b -/- dendritic cells and CD40L-mediated Akt activation is defective in c-Cbl -/- B cells. These findings implicate Cbl family proteins as not only negative

regulators of signaling, but as positive modulators of TNF receptor superfamily signaling as well.

3.1.2 Introduction

Tumor necrosis factor (TNF) family proteins mediate diverse effects on cells of the hematopoietic lineage via their cognate receptors, members of the TNF receptor (TNFR) family (1). TNFR family proteins lack intrinsic enzymatic activity, but are linked to intracellular signaling cascades through TNFR associated factor (TRAF) proteins, and numerous TNF family proteins have been shown to activate nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) cascades (2). TNF-related activation-induced cytokine (TRANCE; also called RANKL, ODF, and OPGL) is a TNF family member expressed on activated T cells and osteoblasts that regulates the function of dendritic cells and osteoclasts through its cognate receptor, TRANCE-R (also called RANK) (3). Recently, we demonstrated that in addition to activating NF-κB (4) and c-jun N-terminal kinase (JNK) (5), TRANCE activates Akt, a serine/threonine kinase implicated in survival signals, through a mechanism involving TRAF6 and the nonreceptor tyrosine kinase c-Src (6).

c-Cbl is a cytoplasmic adapter molecule that has been implicated in the negative regulation of signaling from a variety of receptor tyrosine kinases, including growth factor receptors and antigen receptors in lymphocytes (7,8). A highly related protein, Cbl-b, has been identified (9). The domain structure of Cbl proteins consists of several functional domains, including an SH2-like phosphotyrosine-binding domain, a RING finger, a proline-rich domain, and a leucine zipper. Originally identified as a viral proto-oncogene that acquires transforming potential with the 70z deletion (10), Cbl has been

implicated in the negative regulation of tyrosine kinase signaling by shortening the duration of activating signals (7).

c-Cbl and Cbl-b have been shown to interact with a wide variety of activated signaling molecules including phosphatidylinositol 3-kinase (PI3-K), Src-family tyrosine kinases, Syk, and adaptor proteins Grb2 and Shc (11-14). One mechanism by which they may negatively regulate signaling is by acting as an E3 ubiquitin ligase, which results in the degradation of activated molecules by the proteasome (15-17). This E3 ubiquitin ligase activity has been localized to the RING finger of c-Cbl, and the RING finger has been associated with the negative regulation of a number of tyrosine kinases, including the epidermal growth factor receptor (EGFR) (18,19), syk (20), and CSF-1R (21), among others. Cbl-b has been associated with the negative regulation of Vav-mediated JNK activation (22) and EGFR signaling (23).

Mice with targeted deletions in c-Cbl (24), and recently, Cbl-b (25,26) have been described; each displays a phenotype of decreased thresholds for lymphocyte activation and development of autoimmunity. Mice with a targeted deletion in c-Cbl display enhanced thymic positive selection, likely due to the persistence of activated costimulatory molecules that are ordinarily targeted for degradation by c-Cbl (24). Cbl-b-/- mice demonstrate T cell hyperactivation and hyperproliferation in response to antigen receptor stimulation, uncoupling of T cell receptor (TCR) and CD28 stimulation, and develop spontaneous autoimmunity (25,26).

In this report, we identify a mechanism by which the TNF family members TRANCE and CD40L activate Akt through their cognate receptors TRANCE-R and CD40. We previously demonstrated that, upon ligand engagement, TRANCE-R forms a complex

with c-Src and TRAF6 (6). We now show that PI3-K and Cbl proteins are also components of this signaling complex. Examination of the mechanism of the interaction of c-Cbl and Cbl-b with TRANCE-R shows a requirement for c-Src kinase activity, which may be regulated by TRAF6. Furthermore, a stable complex of TRANCE-R and Cbl-b is observable only in presence of a proteasome inhibitor, suggesting that Cbl-b may negatively regulate TRANCE signaling by downregulating one or more components of the TRANCE-R signaling complex. Finally, using cells derived from Cbl-b -/- and c-Cbl -/- mice, we show that Akt activation by TRANCE and CD40L in dendritic cells is dependent on Cbl-b, while Akt activation in B cells by CD40L is dependent on c-Cbl, suggesting a novel positive regulatory role for Cbl proteins in signaling.

3.1.3 Experimental Procedures

3.1.3.1 Reagents

MG-132 was from Calbiochem; SAM68 was from Santa Cruz Biotechnology; PP1 was from Alexis Biochemicals, soluble hCD8-TRANCE (TRANCE) was purified from insect cells as described (27); and soluble mCD8-CD40L (CD40L) was generated in insect cells and supernatant was used at a 1:100 dilution as described (28).

Antibodies (Abs) specific for phospho-Akt (Ser-473), Akt, and IκB-α were from New England Biolabs; c-Src (N-16), TRAF6 (H-274), and Cbl-b (N-19) were from Santa Cruz Biotechnology; phosphotyrosine (4G10-HRP) and the p85 subunit of PI3-K (rabbit antiserum) were from Upstate Biotechnology Inc; c-Cbl (17) from Transduction Laboratories; HA (12CA5) from Boehringer Mannheim, and the Flag epitope (M2) were from Sigma. Anti-TRANCE-R (1E6.66) was previously described (29) and anti-CD40

(FGK-115) was purified from supernatants of hybridoma cells provided by Dr. Randolph Noelle (Dartmouth University, Hanover, NH).

3.1.3.2 Primary Cells

Mature dendritic cells were generated from bone marrow precursors as described (30). Osteoclasts were generated from bone marrow precursors as described (31). Lymphocytes were prepared from whole spleens by making single-cell suspensions followed by erythrocyte lysis and plating for 1h on tissue culture plates to deplete adherent cells.

3.1.3.3 Plasmids

Expression constructs encoding FLAG-tagged wild-type mouse TRANCE-R (TR-wt), chicken c-Src, c-SrcKD (K295M), and HA-tagged human c-Cbl in pcDNA3.1 (Invitrogen) have been described (6). TR-Y345F, Y440F, and Y468F were generated by the QuickChange method of site directed mutagenesis (Stratagene). HA-tagged human Cbl-b (WT and ΔN) constructs in the pCEFL vector were kindly provided by Dr. Stan Lipkowitz (National Cancer Institute, Bethesda, MD) and have been described (32).

3.1.3.4 Cell Stimulation, Transfection, and Analysis

In vitro differentiated mature dendritic cells and osteoclasts, and freshly isolated splenocytes were extensively washed to remove exogenous growth factors, cultured in medium with low serum (0.5% FCS, 2-4 h), then stimulated by adding TRANCE or CD40L as indicated. After stimulation, cells were washed with ice-cold PBS, lysed, and subjected to immunoprecipitation and western blotting as described (6). In order to control for equal loading of each timepoint, the protein concentration of each sample was

determined and samples were normalized for total protein content prior to further processing.

293T cells were transfected by calcium phosphate precipitation as described (4). The amount of transfected DNA was held constant to 1 µg by addition of empty vector DNA where necessary. Cells were processed for analysis 24 h after transfection. Where indicated, MG-132 or an equivalent amount of vehicle (DMSO) was added to a final concentration of 10 µM 4 h prior to processing. Cells were processed and subject to immunoprecipitation and western blotting as described (6). All transfection experiments were repeated at least three times and representative results are shown.

3.1.4 Results

3.1.4.1 TRANCE-R and CD40 Interact with PI3-K and c-Cbl Upon Ligand Stimulation

Since TRANCE activates Akt in dendritic cells (DC) and osteoclasts, and Akt activation is dependent on the activity of PI3-K, we investigated whether PI3-K was associated with TRANCE-R. In order to determine whether PI3-K is part of the TRANCE-R signaling complex in primary cells, we immunoprecipitated TRANCE-R from TRANCE-treated DC. The p85 regulatory subunit of PI3-K was recruited to TRANCE-R in a ligand-dependent manner (Figure 3.1.1A, top), which correlates with Akt phosphorylation in the whole cell extract (6). Since PI3-K has been shown to associate with the cytoplasmic scaffolding protein c-Cbl in a variety of cell types, we probed the TRANCE-R immunoprecipitates for c-Cbl and found that it associates with TRANCE-R in a TRANCE-dependent fashion. This correlates with a ligand-dependent increase in TRANCE-R-associated TRAF6 (Figure 3.1.1A, top). Immunoprecipitation of c-Cbl from

dendritic cell lysates confirmed that TRAF6 inducibly associates with c-Cbl upon ligand stimulation (Figure 3.1.1A, bottom).

In order to determine whether other TNFR family members known to signal through TRAF6 behave similarly to TRANCE-R, we treated DC with CD40L, which, like TRANCE, promotes the survival and activation of myeloid dendritic cells (33). Immunoprecipitation of CD40 and western blotting showed that there was a ligand-dependent increase in the p85 subunit of PI3-K and c-Cbl associated with CD40 (Figure 3.1.1B, top). As we have previously found with TRANCE-R (6), Src-family kinase activity co-precipitates with ligand-stimulated CD40 as assayed by the ability of the immunoprecipitates to phosphorylate recombinant SAM68, a known Src-family kinase substrate, *in vitro* (Figure 3.1.1B, bottom).

We previously observed a peak of TRANCE-induced Akt phosphorylation after 20 minutes of stimulation (6). In order to determine if this activation correlates kinetically with PI3-K recruitment to TRANCE-R, we stimulated DC with TRANCE for up to 60 minutes. Surprisingly, although PI3-K and c-Cbl continue to accumulate in the TRANCE-R complex in increasing amounts (Figure 3.1.1C, top), Akt activation decreases after 20 minutes of stimulation (Figure 3.1.1C, bottom. See also Figure 3.1.4A).

Since TRANCE-mediated Akt activation in DCs is dependent on the activity of Src-family kinases and can be inhibited by the Src-family kinase inhibitor PP1 (6), we endeavored to determine whether the association of c-Cbl and PI3-K is dependent on Src-family kinase activity. We pretreated DCs with PP1 or vehicle (DMSO), stimulated the DCs with TRANCE, and immunoprecipitated c-Cbl. In the absence of PP1, c-Cbl was

constitutively phosphorylated on tyrosine in unstimulated DCs, and its phosphorylation state was unaffected by TRANCE treatment. Pretreatment with PP1 completely blocked all c-Cbl phosphorylation (Figure 3.1.1D, top). However, the phosphorylation state of c-Cbl did not affect its binding to PI3-K, as PI3-K was constitutively associated with c-Cbl regardless of PP1 treatment or TRANCE stimulation (Figure 3.1.1D, bottom). To ensure that TRANCE stimulation activated signaling by TRANCE-R, we probed whole cell extracts with antibodies to IκB-α and observed equivalent TRANCE-dependent degradation of IκB-α in both DMSO- and PP1-pretreated cells (not shown).

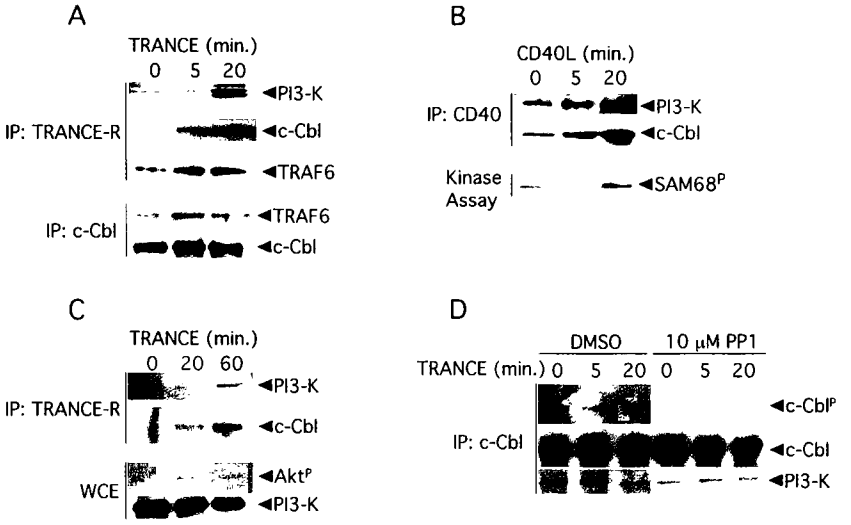

Figure 3.1.1. PI3-K and c-Cbl are recruited to TRANCE-R and CD40 upon ligand stimulation in dendritic cells.

A. Dendritic cells were treated for the indicated number of minutes with TRANCE (2 μg/ml) and lysed. TRANCE-R (top) and c-Cbl (bottom) were immunoprecipitated and the immunoprecipitates were probed with antibodies to PI3-K, c-Cbl, and TRAF6 as indicated.

B. DC were treated with soluble CD40L (1:100) for the indicated number of minutes and CD40 was immunoprecipitated. The immunoprecipitates were probed with antibodies to c-Cbl and PI3-K (top and middle). An in vitro Src-family kinase assay was performed on the CD40 immunoprecipitates (bottom) with recombinant SAM68 as a substrate.

C. DC were treated as in (A), TRANCE-R was immunoprecipitated, and the immunoprecipitates were probed with antibodies to PI3-K and c-Cbl as indicated (top). Whole cell extracts (WCE) were immunoblotted with antibodies to phospho-Akt (Akt^P) and PI3-K as indicated.

D. DC were pretreated with vehicle (DMSO) or PP1 (10 μM) for 90 minutes, then stimulated and lysed as in (A). c-Cbl was immunoprecipitated and the immunoprecipitates were probed with antibodies to phosphotyrosine, c-Cbl, and PI3-K as indicated.

3.1.4.2 TRANCE-R and Cbl Proteins Interact only in the Presence of Active Src

To further elucidate the mechanism of c-Cbl's interaction with TRANCE-R, we transiently transfected HEK 293T cells with constructs driving the expression of c-Cbl, c-Src, or Flag-epitope tagged TRANCE-R from a CMV promoter. In the presence of overexpressed c-Src, an anti-c-Cbl antibody co-immunoprecipitated TRANCE-R (Figure 3.1.2A, lane a). In the absence of overexpressed c-Cbl, endogenous c-Cbl was sufficient to demonstrate a c-Src dependent interaction with TRANCE-R (Figure 3.1.2A, lane c). Conversely, in the absence of overexpressed c-Src, neither overexpressed nor endogenous c-Cbl could co-precipitate TRANCE-R (Figure 3.1.2A, lanes b and d). To differentiate between catalytic and structural roles for c-Src in the TRANCE-R/c-Cbl complex, we cotransfected a kinase-inactive mutant of c-Src (c-SrcKD) with TRANCE-R and c-Cbl. While the wild-type c-Src construct used has been shown to phosphorylate c-Cbl in overexpression systems, c-SrcKD does not (34). Immunoprecipitation of TRANCE-R with the Flag antibody and western blotting revealed that the kinase-active form of c-Src was able to promote a strong interaction between c-Cbl and TRANCE-R (Figure 3.1.2B, lane a). The kinase-inactive form of c-Src, however, could not promote a strong interaction between c-Cbl and TRANCE-R (Figure 3.1.2B, lane b).

To determine if Cbl-b, another Cbl family protein, could also interact with TRANCE-R, we cotransfected Cbl-b, TRANCE-R, and c-Src or c-SrcKD. Immunoprecipitation of TRANCE-R with the Flag antibody did not reveal the presence of Cbl-b protein in either case (Figure 3.1.2C, lanes a and b). However, in the presence of MG-132, a proteasome inhibitor (35), we were able to coprecipitate Cbl-b with TRANCE-R in a c-Src kinase-dependent manner (Figure 3.1.2C, lanes c and d). This suggests that Cbl-b may downregulate one or more of the essential components of the TRANCE-R complex by ubiquitination, thus creating a transient interaction between TRANCE-R and Cbl-b.

3. Results

Indeed, when we expressed TRANCE-R in the presence of c-Src and full-length Cbl-b, we observed a marked decrease in the amount of TRANCE-R protein in the cell extract (Figure 3.1.2D, lane a). However, when we either substituted a truncated form of Cbl-b with a deletion of the N-terminal SH2 domain (Cbl-bΔN, Figure 3.1.2D, lane b), omitted c-Src (lane c), or both (lane d), TRANCE-R expression was normal, which suggests that Cbl-b may mediate TRANCE-R downregulation in a c-Src dependent manner.

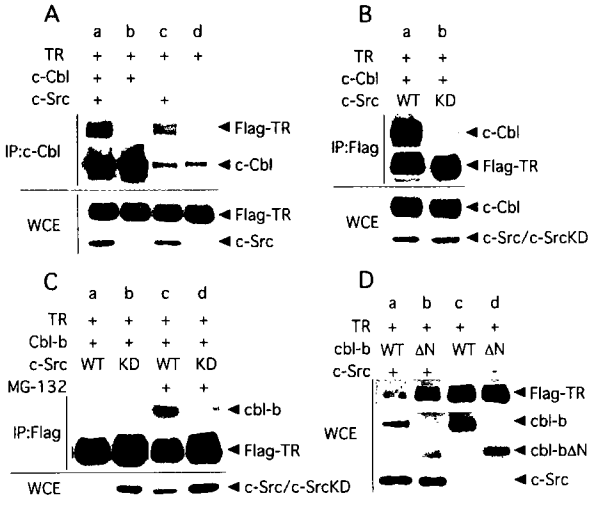

Figure 3.1.2. c-Cbl and Cbl-b interact with TRANCE-R in a c-Src kinase dependent manner.

A. 293T HEK cells were transiently transfected with Flag-epitope tagged TRANCE-R (TR, 0.5 μg), c-Cbl (0.3 μg), and c-Src (0.05 μg) as indicated. c-Cbl was immunoprecipitated and the immunoprecipitates (IP) were probed with antibodies to Flag and c-Cbl as indicated. The whole cell extracts (WCE) were probed with antibodies to Flag and c-Src as indicated.

B. As in (A), with either wild-type (WT, 0.05 μg, lane a) or a kinase dead (KD, 0.1 μg, lane b) mutant of c-Src (K295M) transfected as indicated. TRANCE-R was immunoprecipitated with an anti-Flag antibody.

C. As in (B), but with transfection of Cbl-b (0.3 μg) instead of c-Cbl. MG-132, a proteasome inhibitor was added 4 h prior to cell lysis to a concentration of 10 μM where indicated (+, c and d) and an equivalent amount of DMSO (vehicle) was added to the other samples (-, a and b).

D. 293T cells were transfected with TR, c-Src, and either wild-type Cbl-b (WT, lanes a and c) or a truncation mutant in which the N-terminal SH2 domain of Cbl-b has been deleted (ΔN, 0.3 μg, lanes b and d) as indicated. Whole cell extracts (WCE) were probed with antibodies to Flag, HA (Cbl-b and Cbl-bΔN), and c-Src. The relative positions of Cbl-b and Cbl-bΔN are indicated.

3.1.4.3 c-Src Phosphorylates TRANCE-R

Since c-Cbl and Cbl-b interact with TRANCE-R only in the presence of active c-Src, we investigated whether this interaction is dependent on tyrosine phosphorylation of TRANCE-R. Sequence analysis of the cytoplasmic domain of mouse TRANCE-R revealed the presence of three tyrosine residues that could potentially serve as targets of c-Src: Y345, Y440, and Y468. Alignment with human TRANCE-R shows that while Y345 and Y468 are conserved, Y440 is not. There is an additional tyrosine in human TRANCE-R at position 422, corresponding to position 418 in mouse TRANCE-R (Figure 3.1.3A). In order to determine if any of the tyrosine residues in mouse TRANCE-R are phosphorylated by c-Src, we employed site-directed mutagenesis to change each of the tyrosine residues to phenylalanine. We then cotransfected the tyrosine mutants of TRANCE-R with c-Src or c-SrcKD and immunoprecipitated TRANCE-R. Western blotting of the immunoprecipitates with an anti-phosphotyrosine antibody revealed that wild-type TRANCE-R is phosphorylated by or downstream of c-Src on Y468, as only the Y468F mutant was not phosphorylated in the presence of c-Src. Neither wild-type TRANCE-R nor any of its tyrosine mutants was phosphorylated on tyrosine when cotransfected with c-SrcKD, suggesting that Y468 is a specific target of c-Src activity (Figure 3.1.3B). We then cotransfected TRANCE-R constructs containing tyrosine mutations with c-Src and Cbl-b or c-Cbl and found that Cbl-b and c-Cbl co-precipitated with all of the Y-F mutants of TRANCE-R, which suggests that the interaction between TRANCE-R and Cbl is not dependent on the tyrosine phosphorylation of TRANCE-R (Figure 3.1.3C-D). There was no interaction between a mutant of TRANCE-R with the cytoplasmic tail deleted and c-Cbl or Cbl-b, indicating that the interaction is specific to the cytoplasmic tail of TRANCE-R (data not shown).

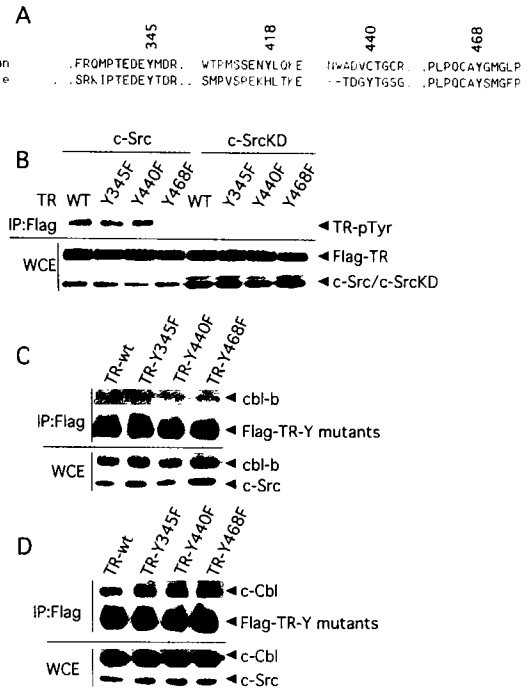

Figure 3.1.3. TRANCE-R is phosphorylated on tyrosine 468 by c-Src, but tyrosine phosphorylation of TRANCE-R is not necessary for association with Cbl-b and c-Cbl.

A. Alignment of amino acid sequences of a portion of the cytoplasmic tails of human and mouse TRANCE-R. Mouse residues are numbered, and tyrosine residues are indicated in boldface.

B. 293T cells were transfected with Flag-tagged mouse TRANCE-R constructs with the indicated point mutations (WT, wild-type) and either c-Src or c-SrcKD. TRANCE-R was immunoprecipitated with an anti-Flag antibody and the immunoprecipitates were probed with an antibody to phosphotyrosine (4G10).

C. 293T cells were transfected with TRANCE-R or its tyrosine mutants as indicated, Cbl-b, and c-Src. MG-132 (10 μM) was added 4 h prior to cell lysis. TRANCE-R (WT or tyrosine mutants) was immunoprecipitated and the immunoprecipitates were probed with antibodies to Cbl-b or Flag as indicated. The whole cell extracts were probed with Cbl-b or c-Src as indicated.

D. As in (C), but with c-Cbl substituted for Cbl-b.

3.1.4.4 Cbl Proteins Regulate TRANCE- and CD40-Mediated Akt Activation

Gene-targeted mice with deletions in c-Cbl (24) and Cbl-b (25,26) have been described recently. In order to determine the role of Cbl proteins in TRANCE and CD40L-mediated activation of Akt, we used B lymphocytes, osteoclasts, and DC derived from mice deficient in c-Cbl or Cbl-b. In cells derived from Cbl-b-deficient mice, we observed that neither TRANCE (Figure 3.1.4A) nor CD40L (Figure 3.1.4B) was able to strongly activate Akt in DC within 20 minutes of stimulation, as opposed to what we observed in wild-type cells. In all cases, NF-κB activation as measured by IκB-α degradation was identical in wild-type and knockout cells. Interestingly, at later time points (>3h),

3. Results

TRANCE and CD40L treatment did result in Akt activation in DC, which was similar to a second wave of Akt activation observed in wild-type cells, suggesting that other gene products that activate Akt via a Cbl-b-independent mechanism are upregulated over this time period. Since Akt has been widely characterized as a survival factor, we investigated whether there was a defect in TRANCE- or CD40L-mediated survival in Cbl-b -/- DC. Perhaps due to the intact secondary wave of Akt activation, there was no difference observed in TRANCE or CD40L-mediated survival in DC derived from Cbl-b-/- mice over a 72h period (data not shown). In DC derived from c-Cbl-/- mice, we did not observe any differences in TRANCE or CD40L-induced Akt activation or survival (data not shown).

In contrast to the results obtained in DC, in B lymphocytes from c-Cbl-/- mice, there was a marked deficiency in CD40L-induced Akt activation but Akt activation was intact in Cbl-b-/- B lymphocytes (Figure 3.1.4C). In osteoclasts derived from c-Cbl-/- and Cbl-b-/- mice, we did not observe any defects in TRANCE-induced Akt activation (Figure 3.1.4D). Consistent with intact TRANCE-mediated Akt activation in c-Cbl-/- and Cbl-b-/- osteoclasts, we did not observe any defects in the differentiation or survival of osteoclasts derived from these mice as determined by TRAP assay (data not shown).

Taken together, these results suggest that Cbl-b and c-Cbl may have cell type-dependent, overlapping roles in Akt activation. It appears that c-Cbl is required for CD40L-dependent Akt activation in B cells while Cbl-b is required for TRANCE and CD40L-dependent Akt activation in DC. In osteoclasts, c-Cbl and Cbl-b appear to be able to substitute for one another in TRANCE-dependent Akt activation. In whole cell extracts, protein expression levels of c-Cbl and Cbl-b in dendritic cells, B lymphocytes, and osteoclasts do not account for these cell type specific differences in function (data not

shown). It has been reported recently that, in c-Cbl-/- osteoclasts, Cbl-b is compensatorily overexpressed (36). However, it is possible that the availability of c-Cbl and Cbl-b to the various receptor signaling complexes differs in a cell type dependent manner due to other, as yet unidentified components of the signaling complexes.

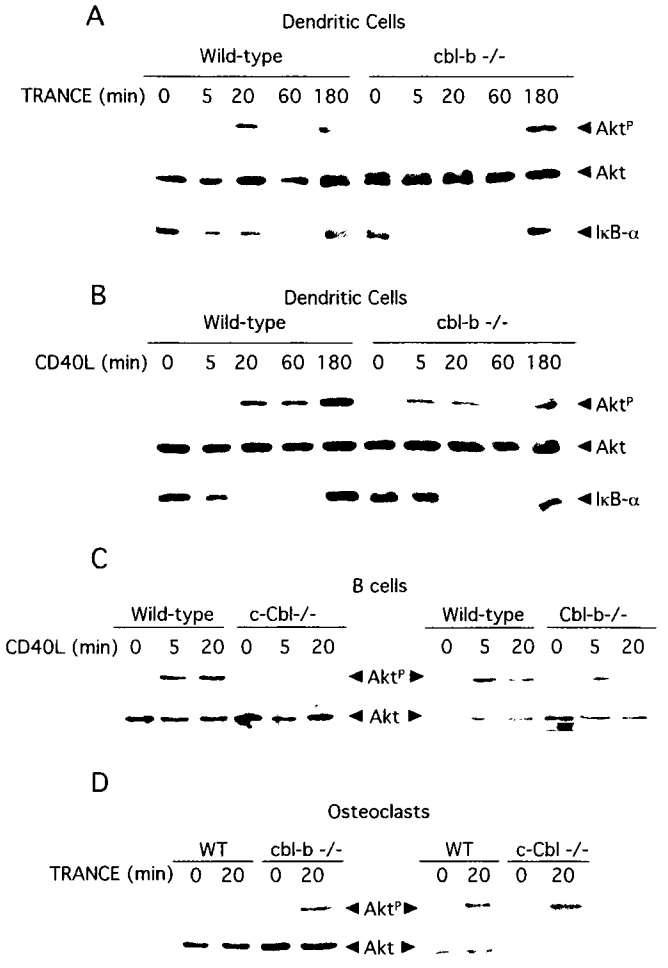

Figure 3.1.4. Cbl proteins are required for cell type-specific TRANCE and CD40L-mediated Akt activation.

A. DC were derived from wild-type or Cbl-b deficient (-/-) mice, serum starved, and treated with TRANCE (2 μg/ml) for the indicated time. Lysates (50 μg) were immunoblotted with a phospho-specific Akt antibody to indicate activation of Akt (Akt^P). Membranes were stripped and reprobed with antibodies to total Akt to normalize for protein loading, and IκB-α to demonstrate activation of the NF-κB signaling pathway as indicated. Note degradation of IκB-α after 5 minutes and appearance of newly synthesized protein by 180 minutes.

B. As in (A), but cells were treated with CD40L (1:100) instead of TRANCE.

C. B lymphocytes were isolated from the spleens of wild-type, c-Cbl, or Cbl-b deficient (-/-) mice, serum starved, and treated with CD40L for the indicated time. Lysates were immunoblotted as in (A) and (B).

D. Osteoclasts were derived from bone marrow of wild-type, Cbl-b, or c-Cbl deficient (-/-) mice, serum starved, and treated with TRANCE for the indicated time. Lysates were immunoblotted as in (A), (B), and (C).

3.1.5 Discussion

3.1.5.1 A Positive Signaling Role for Cbl

While Cbl family proteins have been widely held to play a negative role in tyrosine kinase signaling, our results suggest a positive role as well. c-Cbl and Cbl-b have been demonstrated to associate with the p85 subunit of PI3-K both constitutively and in response to ligand stimulation in a number of cell types and receptor/ligand pairs (12-14,37). In dendritic cells, we observed constitutive association between c-Cbl and PI3-K, which is independent of Src-family kinase activity. Overexpression of Cbl-b has been shown to abrogate Akt activation downstream of EGFR in response to ligand stimulation (23), and the hyperactivation and increased survival of T cells in Cbl-b -/- mice suggests that Cbl-b negatively regulates TCR and CD28-mediated signaling (25,26). However, using cells derived from gene-targeted mice, we found that in dendritic cells, Cbl-b is required for TRANCE and CD40L-induced Akt activation, and in B lymphocytes, c-Cbl is required for CD40L-induced Akt activation. In osteoclasts, c-Cbl and Cbl-b appear to substitute for one another in TRANCE-induced Akt activation. Cbl proteins, therefore, may positively regulate PI3-K activation via TNFR family proteins in a receptor- and cell type-specific manner by recruiting PI3-K to the receptor complex, where it is phosphorylated by Src family kinases.

3.1.5.2 Potential Negative Roles for Cbl in TRANCE Signaling

This positive role, however, appears to be short-lived, as Akt activation by TRANCE and CD40L declines in dendritic cells after approximately 20 minutes of stimulation. It is possible that Cbl proteins are responsible for the termination of signaling by downregulating Src kinases, PI3-K, TRAF6, or TRANCE-R and CD40 via internalization

and/or ubiquitination. EGFR, a receptor tyrosine kinase, is rapidly autophosphorylated within several minutes of ligand binding and its major signaling events take place rapidly (38). Cbl-mediated ubiquitination of EGFR becomes evident on the order of 20-30 minutes after ligand binding, and quenches the activation signal over the next 20-30 minutes (15). Since c-Cbl binds exclusively to phosphorylated EGFR, only activated EGFR is ubiquitinated and targeted for destruction. For productive signaling to occur, there is necessarily a time lag between the activation of the kinase and its destruction. It is therefore likely that, by acting as a scaffold for the assembly of the PI3-K signaling complex and the TRANCE-R signaling complex, Cbl can make a short-lived positive contribution to signaling before downregulating activated proteins. Since the TRANCE-R-c-Cbl-PI3-K complex is observed in DCs long after Akt activation is quenched (Figure 3.1.1C), it is possible that Akt downregulation is independent of Cbl in the receptor complex.

In support of the notion that Cbl indeed has a role in the negative regulation of TRANCE signaling, we were only able to observe Cbl-b binding to TRANCE-R in the presence of MG-132, a proteasome inhibitor. Additionally, we found that overexpression of full-length Cbl-b and c-Src resulted in a marked decrease in TRANCE-R protein in cell lysates, while eliminating either the N-terminal domain of Cbl-b or c-Src overexpression did not reduce TRANCE-R protein levels. This suggests that TRANCE-R and/or other essential activated components of the TRANCE-R signaling complex are targeted for proteasome-mediated degradation by Cbl-b. Three likely candidates are TRAF6, c-Src, and PI3-K. Recently, Takayanagi et al. (38) demonstrated that TRAF6 is ubiquitinated and subsequently degraded by the proteasome in response to TRANCE stimulation in osteoclast precursor cells. Harris et al. (39) reported that active c-Src is ubiquitinated and subsequently degraded while the steady-state level of c-SrcKD is consistently higher than

that of active c-Src. This may explain the slight increase in c-Src observed in the whole cell extract in the presence of MG-132 in Figure 3.1.2C. Fang et al. (41) reported that Cbl-b binds to and induces ubiquitination of the p85 subunit of PI3-K.

3.1.5.3 Roles of TRAF6 and c-Src in Receptor Assembly

We have shown that the C-terminal receptor binding domain of TRAF6 can interact with TRANCE-R (4) and c-Src (6). We have also found that the C-terminal half of TRAF6 interacts with c-Cbl and Cbl-b. This interaction promotes the activation of c-Src to tyrosine phosphorylate c-Cbl and Cbl-b, but phosphorylation is dependent on the N-terminal half of TRAF6 (6 and data not shown). Since catalytically active c-Src is necessary to promote an interaction between TRANCE-R and Cbl proteins, but phosphorylation of TRANCE-R on a specific tyrosine residue does not affect binding, it is likely that phosphorylation of Cbl proteins ultimately promotes this interaction. Nevertheless, the possibility remains that a component of the complex that has yet to be identified is the true target of c-Src that facilitates Cbl-TRANCE-R binding. Therefore, in addition to activating the PI3-K cascade, c-Src appears to play a vital role in the assembly of the signaling complex.

3.1.5.4 Physiological Consequences of Cbl in TRANCE and CD40L Signaling

If Cbl-b is essential for Akt activation by TRANCE and CD40L in dendritic cells, why is there no apparent defect in DC survival in Cbl-b-/- mice? When bone marrow-derived DC reach maturity after 8 days in GM-CSF culture, they begin to undergo apoptosis in the absence of survival stimuli (40). However, this process is observable on the order of many hours to days, and is most likely due to high levels of pre-existing bcl-2 protein (27). In Cbl-b-/- DC, we observed Akt activation after several hours of TRANCE or CD40L stimulation, consistent with a second wave of activation seen in wild-type DC. It

is possible that this Cbl-b independent Akt activation is due to the expression of new gene products induced by TRANCE or CD40L in DC, since other signaling pathways activated by these cytokines appear to be intact in Cbl-b deficient cells. NF-κB activation, as measured by decreasing and subequently increasing IκB levels, follows identical kinetics in Cbl-b-/- and wild-type DC. Furthermore, the upregulation of IκB observed in these cells at the 3 h time point suggests that the expression of other proteins that could potentially activate Akt is upregulated. Attempts to inhibit this second wave of Akt activation by blocking new gene transcription via the addition of cycloheximide were unsuccessful, as even extremely low doses of cycloheximide (<50 ng/ml) completely abrogated even the first wave of Akt activation (data not shown). Therefore, the contribution of Akt activation to TRANCE and CD40L-mediated DC survival remains to be determined.

While Akt is principally known as a regulator of cell survival, it is possible that it may serve other roles as well. Meili *et al.* have shown that Akt plays an essential role in cell motility in chemoattractant responses in *Dictyostelium* (41). In particular, Akt has effects on actin-mediated cytoskeletal rearrangements. Since dendritic cells, when activated, are quickly mobilized to migrate from outer tissues to draining lymph nodes, it is possible that Akt activation by TNF family proteins or other inflammatory mediators such as IL-1 and LPS (6) plays a role in DC migration. Indeed, CD40L-CD40 (42) and LPS-Toll-like Receptor (43) interactions appear to be required for dendritic cell migration *in vivo*. We did not observe significant differences in DC migration in Cbl-b deficient mice (data not shown), but again, the later wave of Akt activation in Cbl-b -/- DC could be sufficient to allow DC migration within the experimental time frame. Given the importance of Akt in a variety of cell functions, further study is warranted to determine its role in DC biology.

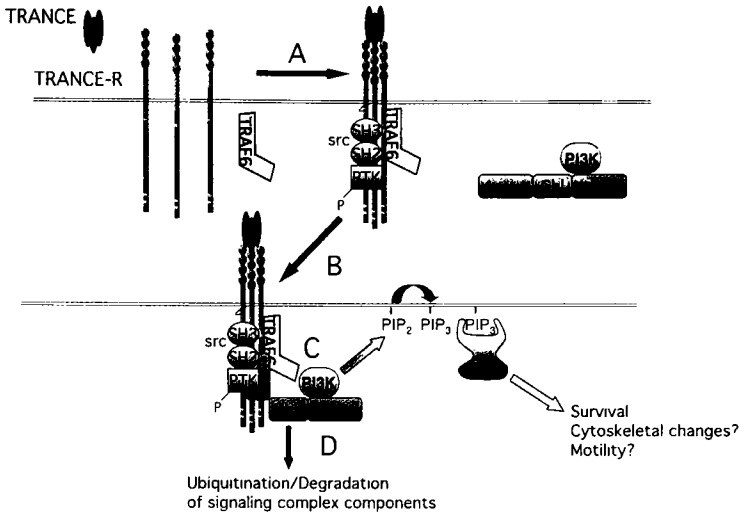

Figure 3.1.5. Model of the proposed mechanism of Akt activation by TRANCE.

A. Soluble TRANCE binds to membrane-bound TRANCE-R, leading to its aggregation. TRAF6 and c-Src are recruited to the TRANCE-R complex.

B. Cbl recruits PI3-K to the TRANCE-R complex.

C. c-Src, activated by its association with TRAF6, phosphorylates PI3-K, activating it to phosphorylate membrane phosphatidyl inositides. Akt is recruited to these phosphatidyl inositides via its pleckstrin homology domain and is activated.

D. Cbl acts as an E3 ubiquitin ligase, leading to the ubiquitination and subsequent degradation of one or more components of the TRANCE-R signaling complex, quenching the activating signal.

3.1.5.5 Conclusion

TRANCE and CD40L activate Akt in a variety of cell types. In dendritic cells, the TRANCE-R and CD40 signaling complexes recruit TRAF6, Src family kinases, PI3-K, and Cbl in a ligand-dependent manner. The association of TRANCE-R and c-Cbl and Cbl-b is dependent on Src kinase activity, and TRAF6 can enhance Src-mediated Cbl phosphorylation. Cbl-b appears to downregulate TRANCE-R expression in a Src-dependent manner. In c-Cbl and Cbl-b deficient mice, there are cell type-specific defects in Akt activation downstream of TRANCE-R and CD40, indicating that Cbl proteins may be required for TRANCE and CD40L-dependent PI3-K activation. In Figure 3.1.5, we propose a model in which Cbl brings PI3-K to the receptor complex, where it is activated by c-Src with rapid kinetics. Concurrently, but with slightly slower kinetics, Cbl acts as a ubiquitin ligase, leading to the degradation of one or more of the essential components of

the signaling complex, quenching the activating signal. Cbl proteins thereby may act as both positive and negative regulators of TRANCE and CD40L signaling in a kinetically controlled manner.

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3.1.7 References

1. Baker, S. J., and Reddy, E. P. (1998) Modulation of life and death by the TNF receptor superfamily. *Oncogene* **17**(25), 3261-70.
2. Arch, R. H., Gedrich, R. W., and Thompson, C. B. (1998) Tumor necrosis factor receptor-associated factors (TRAFs)--a family of adapter proteins that regulates life and death. *Genes Dev* **12**(18), 2821-30.
3. Wong, B. R., Josien, R., and Choi, Y. (1999) TRANCE is a TNF family member that regulates dendritic cell and osteoclast function. *J Leukoc Biol* **65**(6), 715-24.
4. Wong, B. R., Josien, R., Lee, S. Y., Vologodskaia, M., Steinman, R. M., and Choi, Y. (1998) The TRAF family of signal transducers mediates NF-kappaB activation by the TRANCE receptor. *J Biol Chem* **273**(43), 28355-9.
5. Wong, B. R., Rho, J., Arron, J., Robinson, E., Orlinick, J., Chao, M., Kalachikov, S., Cayani, E., Bartlett, F. S., 3rd, Frankel, W. N., Lee, S. Y., and Choi, Y. (1997) TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. *J Biol Chem* **272**(40), 25190-4.
6. Wong, B. R., Besser, D., Kim, N., Arron, J. R., Vologodskaia, M., Hanafusa, H., and Choi, Y. (1999) TRANCE, a TNF family member, activates Akt/PKB through a signaling complex involving TRAF6 and c-Src. *Mol Cell* **4**(6), 1041-9.
7. Lupher, M. L., Jr., Rao, N., Eck, M. J., and Band, H. (1999) The Cbl protooncoprotein: a negative regulator of immune receptor signal transduction. *Immunol Today* **20**(8), 375-82.

8. Rudd, C. E., and Schneider, H. (2000) Lymphocyte signaling: Cbl sets the threshold for autoimmunity. *Curr Biol* **10**(9), R344-7.
9. Keane, M. M., Rivero-Lezcano, O. M., Mitchell, J. A., Robbins, K. C., and Lipkowitz, S. (1995) Cloning and characterization of cbl-b: a SH3 binding protein with homology to the c-cbl proto-oncogene. *Oncogene* **10**(12), 2367-77.
10. Blake, T. J., Shapiro, M., Morse, H. C., 3rd, and Langdon, W. Y. (1991) The sequences of the human and mouse c-cbl proto-oncogenes show v-cbl was generated by a large truncation encompassing a proline-rich domain and a leucine zipper-like motif. *Oncogene* **6**(4), 653-7.
11. Elly, C., Witte, S., Zhang, Z., Rosnet, O., Lipkowitz, S., Altman, A., and Liu, Y. C. (1999) Tyrosine phosphorylation and complex formation of Cbl-b upon T cell receptor stimulation. *Oncogene* **18**(5), 1147-56.
12. Hunter, S., Burton, E. A., Wu, S. C., and Anderson, S. M. (1999) Fyn associates with Cbl and phosphorylates tyrosine 731 in Cbl, a binding site for phosphatidylinositol 3-kinase. *J Biol Chem* **274**(4), 2097-106.
13. Hartley, D., Meisner, H., and Corvera, S. (1995) Specific association of the beta isoform of the p85 subunit of phosphatidylinositol-3 kinase with the proto-oncogene c-cbl. *J Biol Chem* **270**(31), 18260-3.
14. Panchamoorthy, G., Fukazawa, T., Miyake, S., Soltoff, S., Reedquist, K., Druker, B., Shoelson, S., Cantley, L., and Band, H. (1996) p120cbl is a major substrate of tyrosine phosphorylation upon B cell antigen receptor stimulation and interacts in vivo with Fyn and Syk tyrosine kinases, Grb2 and Shc adaptors, and the p85 subunit of phosphatidylinositol 3-kinase. *J Biol Chem* **271**(6), 3187-94.
15. Levkowitz, G., Waterman, H., Zamir, E.. Kam, Z., Oved, S., Langdon, W. Y., Beguinot, L., Geiger, B., and Yarden, Y. (1998) c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. *Genes Dev* **12**(23), 3663-74.
16. Levkowitz, G., Waterman, H., Ettenberg, S. A., Katz, M., Tsygankov, A. Y., Alroy, I., Lavi, S., Iwai, K., Reiss, Y., Ciechanover, A., Lipkowitz, S., and Yarden, Y. (1999) Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. *Mol Cell* **4**(6), 1029-40.
17. Yokouchi, M., Kondo, T., Houghton, A., Bartkiewicz, M., Horne, W. C., Zhang, H., Yoshimura, A., and Baron, R. (1999) Ligand-induced ubiquitination of the epidermal growth factor receptor involves the interaction of the c-Cbl RING finger and UbcH7. *J Biol Chem* **274**(44), 31707-12.
18. Biscardi, J. S., Maa, M. C., Tice, D. A., Cox, M. E., Leu, T. H., and Parsons, S. J. (1999) c-Src-mediated phosphorylation of the epidermal growth factor receptor on Tyr845 and Tyr1101 is associated with modulation of receptor function. *J Biol Chem* **274**(12), 8335-43.

19. Waterman, H., Levkowitz, G., Alroy, I., and Yarden, Y. (1999) The RING finger of c-Cbl mediates desensitization of the epidermal growth factor receptor. *J Biol Chem* **274**(32), 22151-4.

20. Ota, S., Hazeki, K., Rao, N., Lupher, M. L., Jr., Andoniou, C. E., Druker, B., and Band, H. (2000) The RING finger domain of Cbl is essential for negative regulation of the Syk tyrosine kinase. *J Biol Chem* **275**(1), 414-22.

21. Lee, P. S., Wang, Y., Dominguez, M. G., Yeung, Y. G., Murphy, M. A., Bowtell, D. D., and Stanley, E. R. (1999) The Cbl protooncoprotein stimulates CSF-1 receptor multiubiquitination and endocytosis, and attenuates macrophage proliferation. *Embo J* **18**(13), 3616-28.

22. Bustelo, X. R., Crespo, P., Lopez-Barahona, M., Gutkind, J. S., and Barbacid, M. (1997) Cbl-b, a member of the Sli-1/c-Cbl protein family, inhibits Vav- mediated c-Jun N-terminal kinase activation. *Oncogene* **15**(21), 2511-20.

23. Ettenberg, S. A., Keane, M. M., Nau, M. M., Frankel, M., Wang, L. M., Pierce, J. H., and Lipkowitz, S. (1999) cbl-b inhibits epidermal growth factor receptor signaling. *Oncogene* **18**(10), 1855-66.

24. Naramura, M., Kole, H. K., Hu, R. J., and Gu, H. (1998) Altered thymic positive selection and intracellular signals in Cbl- deficient mice. *Proc Natl Acad Sci U S A* **95**(26), 15547-52.

25. Chiang, Y. J., Kole, H. K., Brown, K., Naramura, M., Fukuhara, S., Hu, R. J., Jang, I. K., Gutkind, J. S., Shevach, E., and Gu, H. (2000) Cbl-b regulates the CD28 dependence of T-cell activation. *Nature* **403**(6766), 216-20.

26. Bachmaier, K., Krawczyk, C., Kozieradzki, I., Kong, Y. Y., Sasaki, T., Oliveira-dos-Santos, A., Mariathasan, S., Bouchard, D., Wakeham, A., Itie, A., Le, J., Ohashi, P. S., Sarosi, I., Nishina, H., Lipkowitz, S., and Penninger, J. M. (2000) Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-b. *Nature* **403**(6766), 211-6.

27. Wong, B. R., Josien, R., Lee, S. Y., Sauter, B., Li, H. L., Steinman, R. M., and Choi, Y. (1997) TRANCE (tumor necrosis factor [TNF]-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor. *J Exp Med* **186**(12), 2075-80.

28. Lee, S. Y., Reichlin, A., Santana, A., Sokol, K. A., Nussenzweig, M. C., and Choi, Y. (1997) TRAF2 is essential for JNK but not NF-kappaB activation and regulates lymphocyte proliferation and survival. *Immunity* **7**(5), 703-13.

29. Kim, D., Mebius, R. E., MacMicking, J. D., Jung, S., Cupedo, T., Castellanos, Y., Rho, J., Wong, B. R., Josien, R., Kim, N., Rennert, P. D., and Choi, Y. (2000) Regulation of peripheral lymph node genesis by the tumor necrosis factor family member TRANCE. *J Exp Med* **192**(10), 1467-78.

30. Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., and Steinman, R. M. (1992) Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* **176**(6), 1693-702.
31. Wani, M. R., Fuller, K., Kim, N. S., Choi, Y., and Chambers, T. (1999) Prostaglandin E2 cooperates with TRANCE in osteoclast induction from hemopoietic precursors: synergistic activation of differentiation, cell spreading, and fusion. *Endocrinology* **140**(4), 1927-35.
32. Liu, Y. C., Liu, Y., Elly, C., Yoshida, H., Lipkowitz, S., and Altman, A. (1997) Serine phosphorylation of Cbl induced by phorbol ester enhances its association with 14-3-3 proteins in T cells via a novel serine-rich 14- 3-3-binding motif. *J Biol Chem* **272**(15), 9979-85.
33. Josien, R., Wong, B. R., Li, H. L., Steinman, R. M., and Choi, Y. (1999) TRANCE, a TNF family member, is differentially expressed on T cell subsets and induces cytokine production in dendritic cells. *J Immunol* **162**(5), 2562-8.
34. Shishido, T., Akagi, T., Ouchi, T., Georgescu, M. M., Langdon, W. Y., and Hanafusa, H. (2000) The kinase-deficient Src acts as a suppressor of the Abl kinase for Cbl phosphorylation. *Proc Natl Acad Sci U S A* **97**(12), 6439-44.
35. Jensen, T. J., Loo, M. A., Pind, S., Williams, D. B., Goldberg, A. L., and Riordan, J. R. (1995) Multiple proteolytic systems, including the proteasome, contribute to CFTR processing. *Cell* **83**(1), 129-35.
36. Sanjay, A., Houghton, A., Neff, L., DiDomenico, E., Bardelay, C., Antoine, E., Levy, J., Gailit, J., Bowtell, D., Horne, W. C., and Baron, R. (2001) Cbl associates with Pyk2 and Src to regulate Src kinase activity, alpha(v)beta(3) integrin-mediated signaling, cell adhesion, and osteoclast motility. *J Cell Biol* **152**(1), 181-95.
37. Kanagasundaram, V., Jaworowski, A., and Hamilton, J. A. (1996) Association between phosphatidylinositol-3 kinase, Cbl and other tyrosine phosphorylated proteins in colony-stimulating factor-1- stimulated macrophages. *Biochem J* **320**(Pt 1), 69-77.
38. Takayanagi, H., Ogasawara, K., Hida, S., Chiba, T., Murata, S., Sato, K., Takaoka, A., Yokochi, T., Oda, H., Tanaka, K., Nakamura, K., and Taniguchi, T. (2000) T-cell-mediated regulation of osteoclastogenesis by signalling cross- talk between RANKL and IFN-gamma. *Nature* **408**(6812), 600-5.
39. Harris, K. F., Shoji, I., Cooper, E. M., Kumar, S., Oda, H., and Howley, P. M. (1999) Ubiquitin-mediated degradation of active Src tyrosine kinase. *Proc Natl Acad Sci U S A* **96**(24), 13738-43.
40. Josien, R., Li, H. L., Ingulli, E., Sarma, S., Wong, B. R., Vologodskaia, M., Steinman, R. M., and Choi, Y. (2000) TRANCE, a tumor necrosis factor family

member, enhances the longevity and adjuvant properties of dendritic cells in vivo. *J Exp Med* **191**(3), 495-502.

41. Meili, R., Ellsworth, C., Lee, S., Reddy, T. B., Ma, H., and Firtel, R. A. (1999) Chemoattractant-mediated transient activation and membrane localization of Akt/PKB is required for efficient chemotaxis to cAMP in Dictyostelium. *Embo J* **18**(8), 2092-105.
42. Moodycliffe, A. M., Shreedhar, V., Ullrich, S. E., Walterscheid, J., Bucana, C., Kripke, M. L., and Flores-Romo, L. (2000) CD40-CD40 ligand interactions in vivo regulate migration of antigen- bearing dendritic cells from the skin to draining lymph nodes. *J Exp Med* **191**(11), 2011-20.
43. De Smedt, T., Pajak, B., Muraille, E., Lespagnard, L., Heinen, E., De Baetselier, P., Urbain, J., Leo, O., and Moser, M. (1996) Regulation of dendritic cell numbers and maturation by lipopolysaccharide in vivo. *J Exp Med* **184**(4), 1413-24.

3.2 STRUCTURAL BASIS OF TRAF6 SIGNALING BY THE TNF RECEPTOR AND IL-1/TOLL-LIKE RECEPTOR SUPERFAMILIES

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3.2.1 Summary

We determined crystal structures of TRAF6, alone and in complex with TRAF6-binding sites from CD40 and TRANCE-R/RANK at 2.5, 1.8 and 2.0Å resolution. The structures reveal a distinct receptor-binding groove of TRAF6, the key structural determinant of the interaction. The structural information allows the identification and further confirmation of TRAF6-binding sequences in the IRAK proteins using quantitative affinity determinations. This leads to a proposed TRAF6-binding motif, whose structural requirement was further investigated using site-directed mutagenesis. The mutual recognition of TRAF6 with CD40, TRANCE-R, and the IL-1 receptor (via IRAK) was further demonstrated *in vivo* by correlating the mutational effects of receptors and of a

dominant negative form of TRAF6 on downstream NF-κB, JNK, and p38 signaling. The structures of the TRAF6 complexes also provide a physical model for the connection to Src signaling through cooperative ternary complex formation. These studies jointly establish the structural basis of TRAF6 as the direct convergence point for the TNF receptor and the IL-1 receptor superfamilies.

3.2.2 Introduction

Tumor necrosis factor receptor (TNFR) family proteins are important regulators of immune and inflammatory responses. TNFR family proteins lack intrinsic signaling activity, but they are coupled to downstream signaling molecules through TRAF (TNFR Associated Factor) proteins, of which six have been identified (1). Of the known TRAF proteins, TRAF6 is of particular interest because, while it functions similarly to other TRAFs in mediating signaling by TNFR family proteins, it is also the only TRAF known to mediate signaling from receptors other than TNFR family members. Specifically, TRAF6 has been implicated as a key mediator of signals originating from the interleukin (IL)-1 receptor (IL-1R) (2) and Toll-like receptors (TLR), which bind to the bacterial product lipopolysaccharide (LPS) (3). Thus, TRAF6 represents a central point of convergence for signaling by TNFR and IL-1R/TLR family proteins, and it plays a critical role in bone homeostasis and both adaptive and innate immunity.

Two TNFR family proteins that bind directly to TRAF6 are CD40 and TRANCE-R. CD40 is a key regulator of B cell proliferation, survival, and isotype switching, as well as a mediator of dendritic cell (DC) maturation, survival, and costimulatory ability (4). TRANCE-R is also important for DC survival and costimulation, and it is essential for osteoclast differentiation, maturation, and survival (5). Additionally, TRANCE has been

shown to be a critical regulator of mammary gland development and lactation (6). TRAF6 does not directly interact with IL-1R or TLR proteins. IL-1R and TLRs bind to the cytoplasmic protein MyD88 (7-9), which interacts with the N-terminal portion of IL-1R associated kinase (IRAK). TRAF6 binds to the C-terminal domain of activated IRAK in a similar fashion to its interaction with TRANCE-R and CD40 (10).

Studies from knockout mice have shown that TRAF6 is necessary for normal bone metabolism, as mice deficient in TRAF6 have severe osteopetrosis due to defects in osteoclast differentiation and maturation (11,12). This phenotype is remarkably similar to that found in mice deficient for TRANCE (13,14), TRANCE-R (15), and c-Src (16), which has suggested that TRAF6 may serve to link TNF signaling pathways to Src-family tyrosine kinase pathways (17). Additionally, TRAF6-deficient mice have defects in signaling by IL-1, CD40L, and LPS, which results in deficiencies in nitric oxide production by macrophages as well as in B lymphocyte proliferation and isotype switching (11). Furthermore, TRAF6-/- mice display defects in lymph node organogenesis (12), which has also been observed in TRANCE-deficient mice (13,14).

TRAF6 has the characteristic domain structure of TRAF proteins, with an N-terminal half containing a RING finger and several zinc finger domains, which have been shown to be essential for the activation of downstream signaling pathways, including NF-κB, MAPK, and Src-family kinases (2,17,18). The C-terminal half of TRAF6 consists of the highly conserved TRAF domain (19), with a coiled-coil domain required for TRAF oligomerization and a receptor-binding domain. The C-terminal half of TRAF6 acts as a dominant negative inhibitor of signaling by binding to the receptor but preventing the activation of downstream kinases (2).

3. Results

Of the six TRAF proteins, TRAFs 2, 5, and 6 can activate the NF-κB and MAPK signaling pathways including ERK, p38, and JNK (1). Although TRANCE-R and CD40 have been shown to activate NF-κB and MAPK via TRAF2 and TRAF5 in overexpression experiments (20,21), there is evidence that in primary cells, TRAF6 may be the key physiological mediator of signaling by these receptors. In TRAF6-/- osteoclast precursor cells, TRANCE stimulation fails to activate NF-κB, p38, or JNK (22). In the cytoplasmic tails of both TRANCE-R and CD40, the binding site for TRAF6 is proximal to the membrane and is distinct from the binding site for TRAFs 1, 2, 3, and and 5, which bind to more C-terminal sites on each receptor. Furthermore, the sequence of the receptor-binding TRAF domain of TRAF6 is the least conserved of all the TRAFs (2), suggesting that TRAF6 may have a distinct mode of receptor binding from the other TRAF proteins.

Although the RING and zinc fingers in the N-terminal half of TRAF6 are necessary for the activation of downstream kinases, the receptor-binding domain is important structurally because oligomerization of TRAF6 at the receptor in response to ligand stimulation is a physiological prerequisite for signaling (23). A detailed structural understanding of the receptor binding mode of TRAF6 will help to elucidate the mechanism of TRAF6 signaling and will provide a framework for the rational design of immunomodulatory and anti-osteoporotic therapeutics.

3.2.3 Results and Discussion

3.2.3.1 Identification of the core receptor binding site for TRAF6

As a first step toward understanding the molecular basis of the signaling specificity of TRAF6, we used CD40 as a prototype since it contains a rather short cytoplasmic domain

(residues 216-277). The C-terminal region of this domain (residues 246-277) contains the known TRAF2-binding site, while the N-terminal region (residues 216-245) has been implicated in TRAF6 binding (24,25). To pinpoint the TRAF6-binding site of CD40, we generated a series of deletions within this region and measured the binding affinity of these peptides to the TRAF domain of TRAF6 (residue 333-508), using isothermal titration calorimetry (ITC) (Figure 3.2.1A) (26). The interaction of TRAF6 with the entire N-terminal region of the CD40 intracellular domain exhibited a dissociation constant of ~60 μM. A short peptide of CD40 (residues 230-238) conferred close to 90% of the binding energy to TRAF6, while further deletion abolished the interaction. This short region of CD40 formed the biochemical basis for the structural studies on TRAF6 interactions.

3.2.3.2 Crystal structures of TRAF6

We determined three crystal structures of the TRAF-C domain of TRAF6 (residues 346-504), in its free form and in complex with the minimal TRAF6-binding site from human CD40 (230-KQEPQEIDF-238) and the homologous sequence of human TRANCE-R (342-QMPTEDEY-349), at 2.5Å, 1.8Å and 2.0Å respectively. To facilitate crystallization, the CD40 sequence in the complex contains a mutation (N237D) that has been shown previously to enhance affinity to TRAF6 (Figure 3.2.1A) (21). The overall architectures of the three TRAF6 structures are rather similar, with rms distances of 0.4Å among the peptide-bound and 0.6Å between the free and bound forms. Previous structural and biochemical studies have established that TRAF proteins are able to form trimers (23,27-29), as a way to sense receptor trimerization and oligomerization by trimeric extracellular ligands (30). Even though the current TRAF6 structures are monomeric due to the deletion of their coiled-coil regions, the putative 3-fold axis of the structures can be located. When this is aligned in the vertical orientation, the receptor

peptides lie along a surface ridge from the top (near the cellular membrane) to the bottom (towards the cytoplasm) ends of the molecule (Figure 3.2.1B). This directionality of the peptides makes possible the direct docking of the receptors onto TRAF6 after exiting from their transmembrane regions. The peptides extend the total length of 25Å along this side of TRAF6 and bury ~580Å^2 surface area.

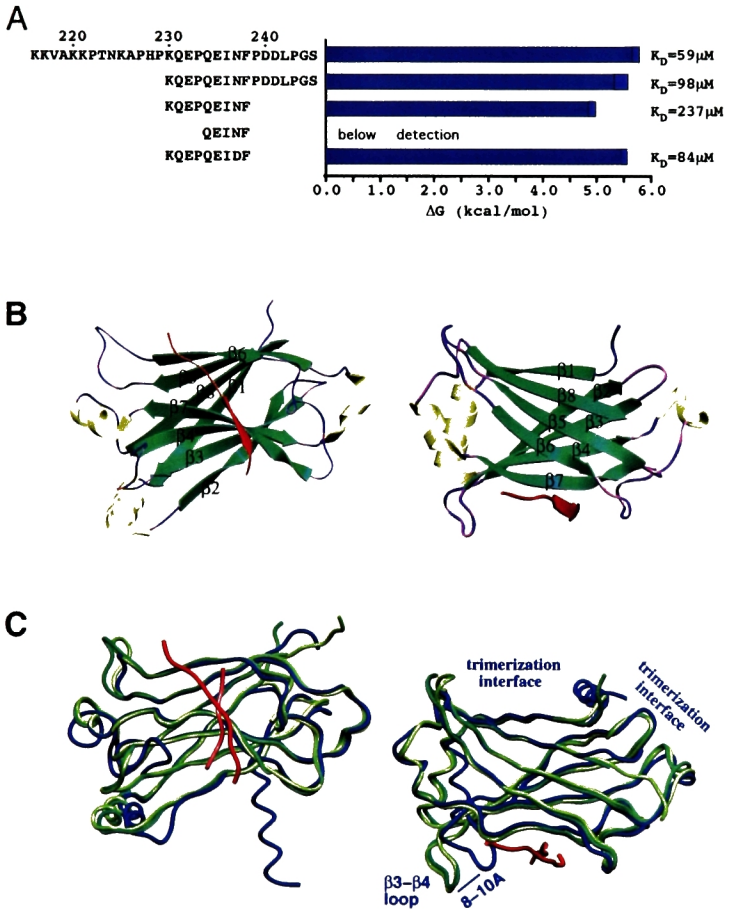

Figure 3.2.1. Overview of the structures.
A. Mapping of TRAF6-binding site on CD40, showing a representative isothermal titration curve (left) and the ΔG and K_D of the interaction of TRAF6 with CD40 deletion series (right).
B. Ribbon drawings of the structure of TRAF6 in complex with CD40, viewing down the putative three-fold axis of the TRAF6 molecule (left) and with the three-fold axis in a vertical orientation (right).
C. Structural superposition of the TRAF6/CD40 complex with a TRAF2/CD40 complex, showing the distinct peptide directions in the two complexes. Same orientations as in (B).

Substantial structural differences between TRAF6 and TRAF2 (27-29) result in dramatic differences in the bound peptides, yielding a 40° cross in the peptide directions in their respective complexes (Figure 3.2.1C). The most prominent conformational difference resides at the β3–β4 loop of TRAF6, which exhibits up to 11.5Å in C_α distances relative to TRAF2. This loop is displaced away from the receptor-binding site so that it no longer

interacts with the peptides in the TRAF6 complexes. The conformational change of the β3– β4loop is most likely brought about by the replacement of a 398-GxGxGxG-404 sequence in TRAF2 and other TRAFs with non-glycine residues in TRAF6. In TRAF2, the β3– β4 loop is highly twisted. The glycine residues are crucial for maintaining two successive tight β-turns of type II' (residues 399-DGTG-402) and type II (residues 402-GAGT-405) respectively, which require glycines at specific positions. These two turns are further proceeded by a type I turn (395-YLNG-398) within the same loop. In TRAF6, the analogous residues continue further along the β3 strand, followed by a sharp type I turn at P398 (i+1 residue) to create a 90° bend in the loop. Most other connections between β strands show modest but significant differences in C_α positions (2-5Å). A superposition of the TRAF6 structure in complex with CD40 with a TRAF2 structure in complex with CD40 shows an rms distance of 1.2Å for 127 aligned C_α positions within 3.0Å.

Both main chain and side chain hydrogen bonding interactions appear to play key anchoring roles in receptor recognition by TRAF6. A large portion of the receptor peptides (Q234-F238 for CD40 and T345-Y349 for TRANCE-R) assumes a typical anti-parallel β conformation and makes main chain hydrogen bonds with residues P468-G472 in the β7 strand of TRAF6 (Figure 3.2.2A and 3.2.2B). The side chains of E235 of CD40 and E346 of TRANCE-R fit snugly into a surface groove and form hydrogen bonds to the main chain amide nitrogens of L457 and A458. In CD40, the carboxylate of D237 makes a hydrogen bond and a salt bridge to K469 of TRAF6. This salt bridge may explain the higher affinity of the N237D mutant of CD40 to TRAF6. In TRANCE-R, the carboxylate of D347 forms hydrogen bonds and a salt bridge to the guanidinium group of R392. The TRAF6 surface is in general rather basic, formed, among others, by the side chains of

R392 and K469, and is complementary to these acidic residues in the CD40 and TRANCE-R sequences (Figure 3.2.2C).

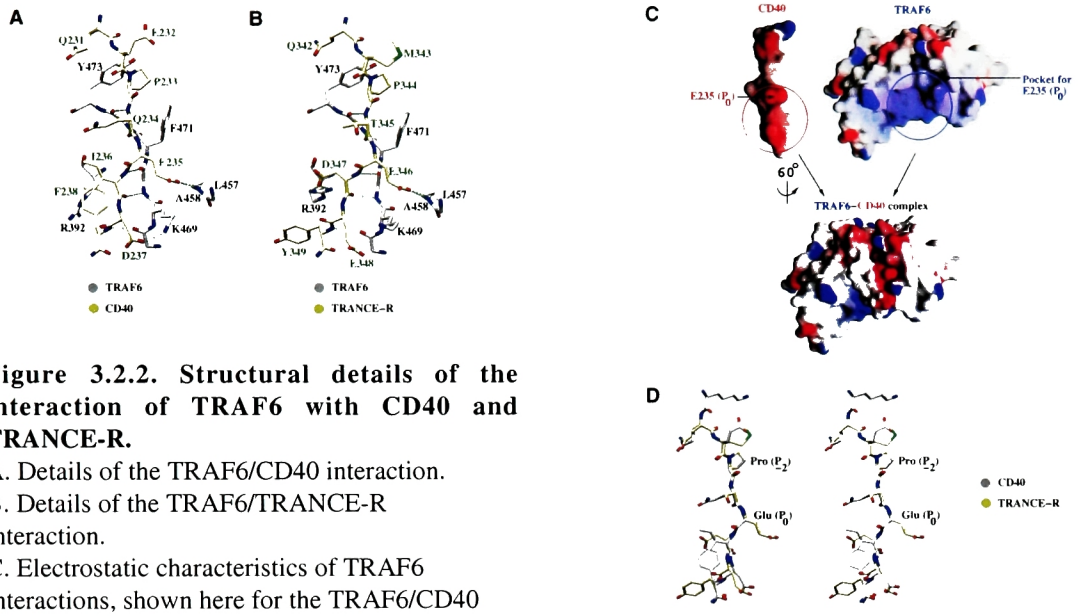

Figure 3.2.2. Structural details of the interaction of TRAF6 with CD40 and TRANCE-R.
A. Details of the TRAF6/CD40 interaction.
B. Details of the TRAF6/TRANCE-R interaction.
C. Electrostatic characteristics of TRAF6 interactions, shown here for the TRAF6/CD40 interaction.
D. Stereo view of the superposition of CD40 with TRANCE-R peptides.

Among the eight residues that directly contact TRAF6 (Q231-F238 of CD40 and Q342-Y349 of TRANCE-R), there are other important hydrophobic and hydrophilic interactions. Residue K230 of CD40 does not make any van der Waals contacts to TRAF6, in contrast with the previous notion that K230 is important for TRAF6 interaction (21). Residues Q231-P233 of CD40 and Q342-P344 of TRANCE-R interact with hydrophobic TRAF6 residues F471 and Y473 to close the partially exposed hydrophobic core. In particular, the proline residue in both structures is completely buried. The side chains of Q231 of CD40 and Q342 of TRANCE-R are relatively less well defined and highly exposed to solvent, even though they appear to form several potential hydrogen bonds (Table 3.2.2). A dramatic difference exists between the

conformation of residue F238 of CD40 and the corresponding residue Y349 of TRANCE-R. While F238 inserts in between a series of aromatic and positively charged side chains including F410, R392, H394 and H412 of TRAF6, Y349 binds at the surface of this highly basic and aromatic region of TRAF6. A model building excise shows that the extra hydroxyl in Y349 appears to expel its insertion into the same pocket.

Comparison of the CD40 and the TRANCE-R conformations by aligning the corresponding TRAF6 structures reveals the structural conservation and variation between the two sequences (Figure 3.2.2D). As residues E235 of CD40 and E346 of TRANCE-R superimpose extremely well and form crucial hydrogen bonding interactions with TRAF6, we denote this residue as the P_0 position of the TRAF6-binding sequence. Residues P233 of CD40 and P344 of TRANCE-R are therefore at the P_{-2} position, while residues F238 of CD40 and Y349 of TRANCE-R reside at the P_3 position. Main chain conformations are identical between the P_{-4} to the P_0 positions and slight deviations occur at the P_1-P_3 positions. Asp residues at both P_1 and P_2 are able to form hydrogen bonds (Figure 3.2.2A and 3.2.2B), while a Glu at P_2 is no longer able to preserve the same interaction. There are significant conformational adjustments in the side chains of the hydrogen bonding partners in TRAF6 (R392 and K469) in the presence and absence of these specific interactions. The difference in the side chain positions of the P_3 residues is not a consequence of the main chain difference, but rather a change in the side chain torsion.

Table 3.2.1. Crystallographic statistics.

	NATIVE	CD40 COMPLEX	TRANCE-R COMPLEX
CRYSTAL			
PEPTIDE	NONE	HCD40	HTRANCE-R
PROTEIN CONSTRUCT	346-504	346-504	346-504
SPACE GROUP	$P2_1$	$P2_12_12_1$	$P2_12_12_1$
CELL DIMENSIONS (Å)	A=32.2, B=55.6, C=47.7, _=101.0°	A=39.9, B=43.8, C=101.4	A=38.0, B=45.0, C=106.5
DIFFRACTION DATA			
RESOLUTION (Å)	40-2.5	40-1.8	40-2.0
R_{SYM} (LAST SHELL) (%)	9.2 (24.1)	5.6 (22.1)	5.5 (13.9)
COMPLETENESS (LAST SHELL) (%)	92.8 (90.4%)	99.1 (93.0)	96.0 (86.9)
REFINEMENT			
RESOLUTION (Å)	20-2.5	20-1.8	20-2.0
SIGMA CUTOFF	2.0	2.0	2.0
NUMBER OF PROTEIN RESIDUES	155	164	161
NUMBER OF PROTEIN ATOMS	1269	1340	1331
NUMBER OF SOLVENT ATOMS	45	122	80
NUMBER OF REFLECTIONS USED	5538	14644	12396
RMSD BOND LENGTH (Å)			
RMSD BOND ANGLE (°)			
R (R_{FREE}) (%)	20.4 (27.4)	20.3 (25.8)	21.3 (24.2)

3.2.3.3 Affinities of various TRAF6-binding sequences for TRAF6 and identification of a consensus binding site

To determine whether the observed interaction of TRAF6 with CD40 and TRANCE-R can be applied to the signal transduction of the IL-1R/TLR superfamily, we utilized the structural information to identify and align putative TRAF6-binding sequences from IRAK and its homologues IRAK2 and IRAKm (Figure 3.2.3). We used isothermal titration calorimetry to measure the affinities of these sequences for TRAF6. These ITC experiments showed that all the identified sequences in IRAK proteins produced binding affinities similar to that of the CD40 peptide (Figure 3.2.3), clearly demonstrating the

generality of the TRAF6 interaction and the mechanism of convergence of TRAF6 signaling by the two superfamilies of receptors. In contrast, two sequences from NGFR and TACI, which were previously implicated in TRAF6 binding (31,32) but do not bear any recognizable structure-based sequence homology, failed to interact with our TRAF6 construct. It is possible that these sequences bind to a different region of TRAF6.

		K_D(μM)	K_A(10^3M^{-1})
hCD40	230-KQEPQEINF-238	237	4.2±0.9
hCD40 (N237D)	230-KQEPQEIDF-238	84	11.9±2.2
mCD40	234-RQDPQEMED-242		
hTRANCE-R	341-RQMPTEDEY-349	78	12.8±4.1
mTRANCE-R	337-RKIPTEDEY-345		
hIRAK	701-RQGPEESDE-709	56	17.9±2.3
mIRAK	666-SQGPEESDE-674		
hIRAK-2	523-SNTPEETDD-531	64	15.7±3.0
hIRAK-M	475-PSIPVEDDE-483	143	7.0±1.3
Motif	xxPxEdd(F,Y,D,E)		
Position	$P_{-4} \leftarrow P_0 \rightarrow P_3$		
Other sequences			
hTACI	SPEPVETCSFCFPEC	below detection	
hNGF receptor	EGEKLHSDSGISVDS	below detection	

Figure 3.2.3. Structure-based identification and alignment of TRAF6-binding sequences, showing the proposed TRAF6-binding motif and the measured K_D of the interactions.

The structural and sequence information led to a putative TRAF6-binding motif of xxPxEdd(F/Y/D/E) (positions P_{-4} to P_3) (Figure 3.2.3), which is distinct from what was proposed earlier (21,24). The inclusion of the P_{-4} and P_{-3} residues in the motif is based on the role of their main chain atoms in closing the hydrophobic core of TRAF6. The Asp residues at positions P_1 and P_2 are shown in lower case, as these may be the most favorable residues due to the specific hydrogen bonds, but other residues, especially acidic residues are tolerated at these positions due to the general charge complementarity with TRAF6. Based on the current structural information, we suspect that the Asp and Glu residues at the P_3 position may adopt the Y349 conformation in TRANCE-R, rather than the inserted F238 conformation, due to a potential unfavorable charge burial.

To establish the importance of the peptide sequence in the context of full-length CD40 intracellular domain, we used native gel shift and size exclusion chromatography to determine the mutational effects of CD40 and TRAF6 on this mutual recognition *in vitro* (Table 3.2.2). We performed mutagenesis for five out of the eight CD40 residues in contact with TRAF6. These residues exhibit either substantial surface area burials and/or potential hydrogen bonding interactions. Mutations E235A (P_0) and F238A (P_3) resulted in complete abrogation of binding, demonstrating the importance of these residues in TRAF6 recognition. While P233A (P_{-2}) did not show qualitative difference in TRAF6 binding, a P233Q mutation abolished the interaction, confirming that the buried binding pocket for P233 could not accommodate large side chains. On the TRAF6 side, we individually mutated all contacting residues involved in CD40 interaction to alanines to determine the energetic contributions of their side chains. Two residues, F471 and Y473, were singled out as the most crucial, as they produced drastic effects in the qualitative gel-shift and size exclusion assays. Other individual mutations did not produce qualitative differences in CD40 binding, suggesting that these residues contribute collectively, rather than singly, to the interaction. In addition, it is possible that the relative importance of TRAF6 residues may be somewhat different for different interacting sequences.

Our structural observation raises the question whether the conserved Glu at the P_0 position of the TRAF6-binding motif is analogous to the P_0 position (a Glu or a Gln) of the TRAF2-binding motif (29). Interestingly, even though the binding peptides for TRAF6 and TRAF2 take their own courses on the surface of the TRAF proteins, they intercept near the P_0 residues (Figure 3.2.1C). In TRAF2 complexes, three Ser residues (S453-S455) appear to form a tight grip at the side chain of the P_0 Glu or Gln. In TRAF6, these three residues are replaced by L456-L457-A458. Reciprocal movements in both

TRAF6 and the peptides are apparent to accommodate the P_0 Glu at this position. The side chains of L456 and L457 move away from E235 to create an optimal cavity for E235 to approach in the main chain amides of L457 and A458 for hydrogen bonding interactions. In addition, an E235Q mutation of CD40 did not produce qualitative difference in TRAF6 binding (Table 3.2.2). These observations led us to propose a model of co-evolution between TRAF proteins and their binding sequences, in which the P_0 residue serves as a primary conserved feature or anchoring point in this process.

Table 3.2.2. Structural characteristics and mutational effects of the TRAF6/CD40 interaction as assessed by native gel shift and size exclusion chromatography.

Mutations	Effects	Surface area burial	Side chain exposure in the complex
Human CD40 mutations			
Q231A (P_{-4})	+	109Å	0.28
P233A (P_{-2})	+	105Å	0.10
P233Q			
E235A (P_0)		123Å	0.20
E235Q	+		
N237A (P_2)	+	56Å	0.55
F238A (P_3)		129Å	0.31
Human TRAF6 mutations			
R392A (R400)*	+	31Å	0.10
F410A (F418)*	+	36Å	0.01
E448A	+	36Å	0.20
L456A	+	26Å	0.24
P468A	+	46Å	0.43
K469A (K477)*	+	62Å	0.27
F471A (F479)*		46Å	0.01
Y473A (Y481)*		56Å	0.01
V474A	+	37Å	0.26
T475A	+	25Å	0.33

+: binding preserved, no or minor effects; -: binding disrupted, major effects
*shown in parentheses are corresponding residues in mouse TRAF6 used in the dominant negative experiments (see below)

3.2.3.4 Mapping of potential TRAF6-binding sites in CD40, TRANCE-R, and IRAK

Previous studies have localized a single TRAF6 binding site in CD40 to a membrane proximal region (21). In TRANCE-R, we (20) and others (24) have identified at least two regions of potential TRAF6 interaction: one in what we have defined as the membrane proximal "N domain" of the cytoplasmic tail of TRANCE-R, and another in the "M domain," which is C-terminal to the N domain. The binding site(s) for TRAFs 1, 2, 3, and 5 is in the extreme C-terminal portion of TRANCE-R, the "C domain," and this domain does not appear to interact with TRAF6 (20). In IRAK, similar to TRANCE-R, two potential TRAF6-interacting regions have been identified in the C-terminal portion of the molecule, defined as "C1" and "C2" (10). With our structural information about the consensus binding sequence for TRAF6 (xxPxEdd[F/Y/E/D], Figure 3.2.3), we identified one TRAF6 binding site in CD40 and three potential TRAF6 binding sites each in TRANCE-R and IRAK (Figure 3.2.4A). Two of the predicted binding sites in TRANCE-R are located in the "M" region and two of the predicted binding sites in IRAK are located in the "C1" region defined by previous mapping studies (Figure 3.2.4B).

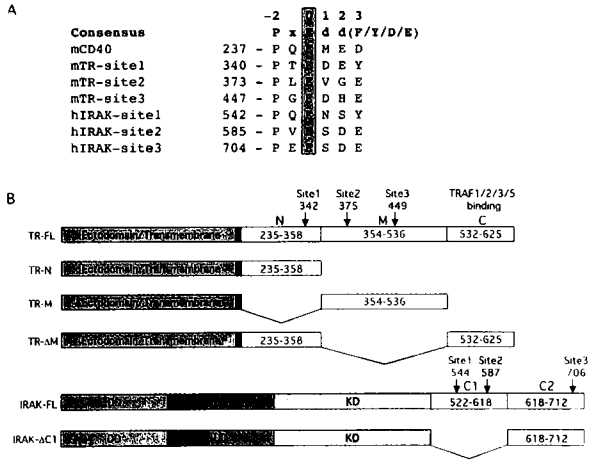

Figure 3.2.4. Identification of consensus TRAF6-binding sequences in mCD40, mTRANCE-R, and hIRAK.

A. Alignment of consensus TRAF6-binding sequences. P_0 position is boxed in gray.

B. Schematic domain structure of TRANCE-R and IRAK. FL denotes full-length; N, M, and C in TRANCE-R follow convention in (20). DD denotes death domain, UD unknown domain, and KD kinase domain (IRAK). TRAF6-binding sites are marked with arrows and the P_0 residue number is noted.

3.2.3.5 Functional mapping of predicted TRAF6-binding residues on CD40, TRANCE-R, and IRAK

In order to determine the relative *in vivo* contributions of each residue in the predicted TRAF6-binding consensus sequences of mCD40, mTRANCE-R (site 1), and hIRAK (site 3), we changed potential TRAF6-interacting residues by site-directed mutagenesis and analyzed the ability of these mutants to activate NF-κB in a luciferase reporter assay. Mutation of positions P_{-4}, P_{-2}, P_0, P_2, and P_3 revealed that each of the residues from P_{-2} to P_3 contributed to TRAF6-mediated NF-κB activation, while the side chain of postion P_{-4} was unimportant for TRAF6-mediated NF-κB activation, in agreement with the structural observation that the contribution of the P_{-4} position to TRAF6 binding is from the main-chain C_α. In CD40, there was a variable degree of inhibition in each of the mutants, with E239A mutation, predicted by our structural observations to be at the center of the TRAF6 binding groove (postion P_0), producing the most significant block in NF-κB activation (Figure 3.2.5A). The residual NF-κB activity in the CD40 mutants is due to an incomplete block of TRAF6 binding and/or normal binding to other TRAFs including TRAF2 and TRAF5 through CD40's C-terminal TRAF-binding site, shown to interact with TRAFs 1, 2, 3, and 5 but not TRAF6 (21). In order to map similar sites on TRANCE-R and IRAK and rule out the possibility of other TRAFs contributing to NF-κB activation, we examined deletion constructs of TRANCE-R and IRAK predicted to have only a single TRAF6 binding site (TR-N and IRAK-ΔC1, Figure 3.2.4B). Without the potential for residual activity mediated by other TRAFs, it is clear that single mutations of P_{-2}, P_0, P_2, and P_3 are all capable of substantial inhbition of TRAF6-mediated NF-κB activation by TR-N and IRAK-ΔC1 (Figure 3.2.5, B and C). To examine whether other TRAF6-mediated signaling pathways are blocked by these mutations, we performed an in vitro JNK assay on overexpressed TR-N and its mutants, obtaining similar results (Figure 3.2.5D). Taken together, these data suggest that mutation of any of

the predicted TRAF6-interacting residues of the consensus binding site is sufficient to block TRAF6-mediated signaling by CD40, TRANCE-R, or IRAK.

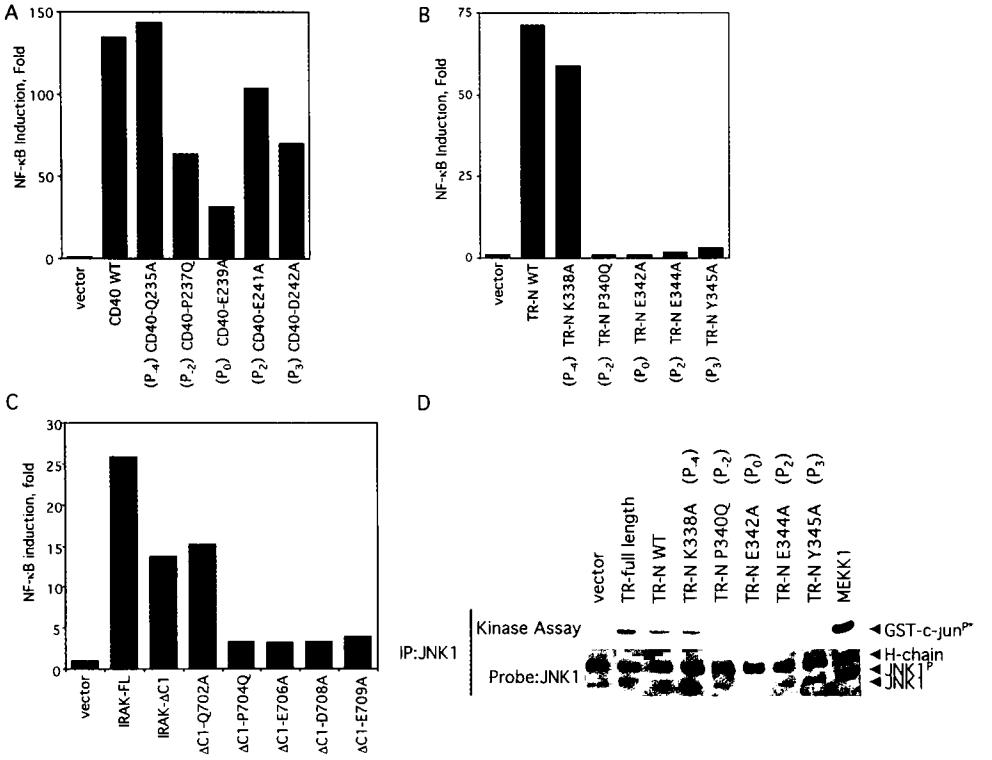

Figure 3.2.5. Functional importance of predicted TRAF6-binding residues in CD40, TRANCE-R, and IRAK.

A. Site-directed mutagenesis was employed to generate point mutations in residues predicted to bind to TRAF6 in mCD40. CD40 (wild-type and mutants, 100 ng) were transfected in 293T cells with an NF-κB-responsive luciferase reporter plasmid (75 ng) and β-galactosidase (25 ng). Relative luciferase activity was normalized for β-galactosidases activity. Representative results of at lest 3 separate transfections are shown.

B. Mouse TRANCE-R consisting of the extracellular domain and residues 235-368 of the cytoplasmic tail (TR-N, wild-type and mutants, 50 ng) was transfected as in A.

C. Human IRAK (full-length, FL; and with a deletion of residues 522-618, ΔC1; wild-type and mutants, 100 ng) was transfected as in A.

D. As in B, but TR-N constructs were transfected with JNK1 and the lysates were subjected to an *in vitro* c-Jun N-terminal kinase assay. MEKK1 was included as a positive control.

3.2.3.6 Functional mapping of predicted receptor-binding residues on TRAF6

We next determined the importance of the observed TRAF6 interface for signaling by CD40, TRANCE-R, and IRAK using a dominant negative assay. The receptor-binding

TRAF domain of TRAF6 has been shown to exert a strong dominant negative phenotype on endogenous signaling by these receptors, presumably due its ability to bind to receptors and its lack of the N-terminal domains necessary for downstream signaling (2,33,34). Mutant TRAF domain constructs of TRAF6 that are defective in receptor or IRAK binding, on the other hand, should then be incapable of or exhibit reduced ability for dominant negative interference.

We created the following point mutants of dominant negative mouse TRAF6 (T6.DN, residues 289-530) by site-directed mutagenesis: R400A, F418A, K477A, F479A, and Y481A (see Table 3.2.2 for the corresponding human TRAF6 residues). When co-expressed with CD40, R400A, F479A and Y481A exhibited significantly reduced ability to inhibit NF–κB activation, while the F418A and K477A mutants retained the ability to reduce NF–κB activation nearly as well as wild-type TRAF6.DN (Figure 3.2.6A). When co-expressed with TR-N, all of the TRAF6.DN mutants could inhibit NF–κB activation to varying degrees, but none of the mutants could block activation as strongly as wild-type TRAF6.DN (Figure 3.2.6B). Taken together, these functional data are consistent with the TRAF6 interface defined by structural studies.

To test the effect of the TRAF6.DN mutants on IL-1 mediated NF–κB activation, we transfected 293T cells with the various TRAF6.DN constructs and NF–κB responsive reporter elements, and treated them for 6 hours with IL-1. The F418A and K477A mutants could block NF–κB induction as effectively as wild-type TRAF6.DN, while the others demonstrated virtually no blockade (Figure 3.2.6C), confirming the functional significance of the observed structural information on IL-1 signaling as well. As there are several known IRAK isoforms, the complete inability of some of the TRAF6 mutants to exert dominant negative interference on NF–κB activation confirmed our structural

observation on the conserved recognition of TRAF6 for all three IRAKs including IRAK, IRAK2 and IRAKm.

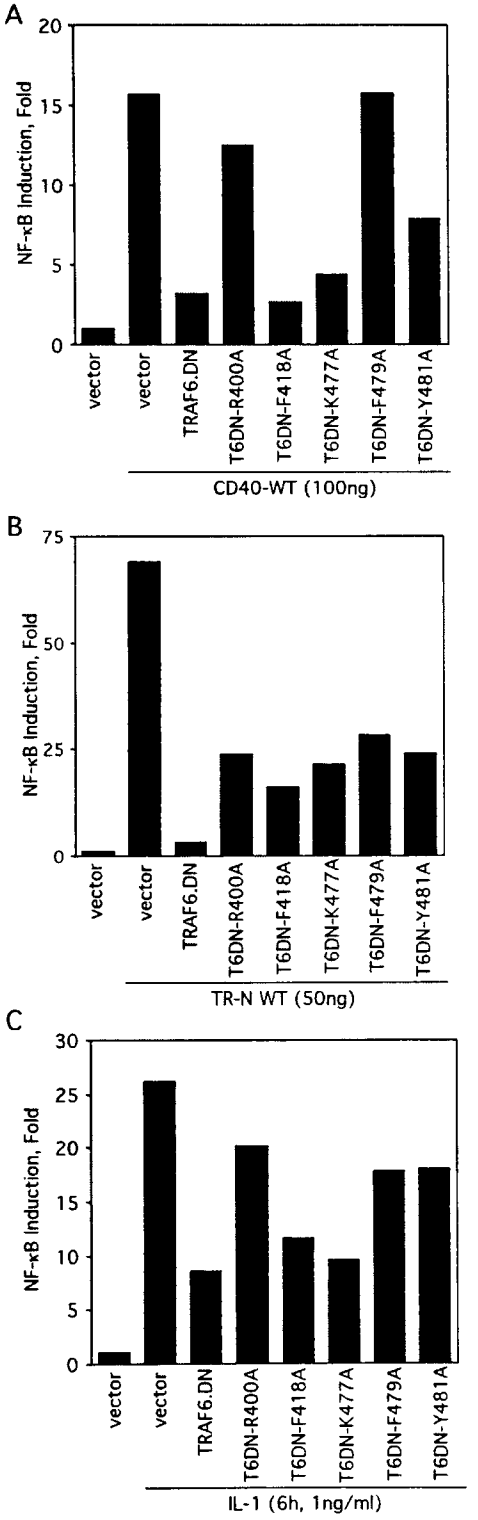

Figure 3.2.6. Functional importance of predicted receptor and IRAK-binding sites on TRAF6.

A. Residues 289-530 of mouse TRAF6 (T6DN, wild-type and mutants as indicated, 800 ng) was cotransfected with wild-type mouse CD40 (100 ng). NF-κB activity was determined as in A.

B. As in A, but with wild-type TR-N (50 ng) instead of CD40.

C. T6.DN (wild-type and mutants as indicated, 800 ng) was transfected as in A and B. Cells were treated with 1 ng/ml recombinant hIL-1α 6 hours prior to harvesting.

3.2.3.7 Relative contributions of each TRAF6-binding site in TRANCE-R and IRAK

Since there are multiple predicted TRAF6-binding sites in both TRANCE-R and IRAK, we assayed the relative contribution of each of these binding sites to signaling by full-length TRANCE-R and IRAK. As mutation of the P_0 glutamate residue was sufficient to substantially reduce TRAF6-mediated signaling by all of the consensus sites examined in Figure 3.2.5, we employed only P_0 E-A mutations to examine each predicted site in full-length TRANCE-R and IRAK individually and in combination. Single mutations of E342A, E375A, and E449A resulted in minimal effects on the ability of overexpressed TRANCE-R to activate NF-κB. Double mutations, particularly 342/375 and 342/449, and to a lesser extent 375/449, slightly reduced NF-κB activation by TRANCE-R, but not substantially. Triple mutation of E342A, E375A, and E449A (E3A) markedly reduced TRANCE-R-mediated NF-κB activation (Figure 3.2.7A). It is somewhat surprising that any single TRAF6-binding site was sufficient to mediate TRANCE-R-induced NF-κB activation almost to the level of the wild-type sequence. The functional NF-κB activating potential by these mutants was confirmed structurally by their ability to bind to and immunoprecipitate TRAF6. The relative amount of TRAF6 immunoprecipitated by TRANCE-R mutants correlated with the relative ability of these mutants to activate NF-κB. In particular, double mutants with the site 1 E342A mutation (342/375 and 342/449) co-precipitated less TRAF6 than did the 375/449 mutant, and the E3A mutant co-precipitated a negligible amount of TRAF6 (Figure 3.2.7B).

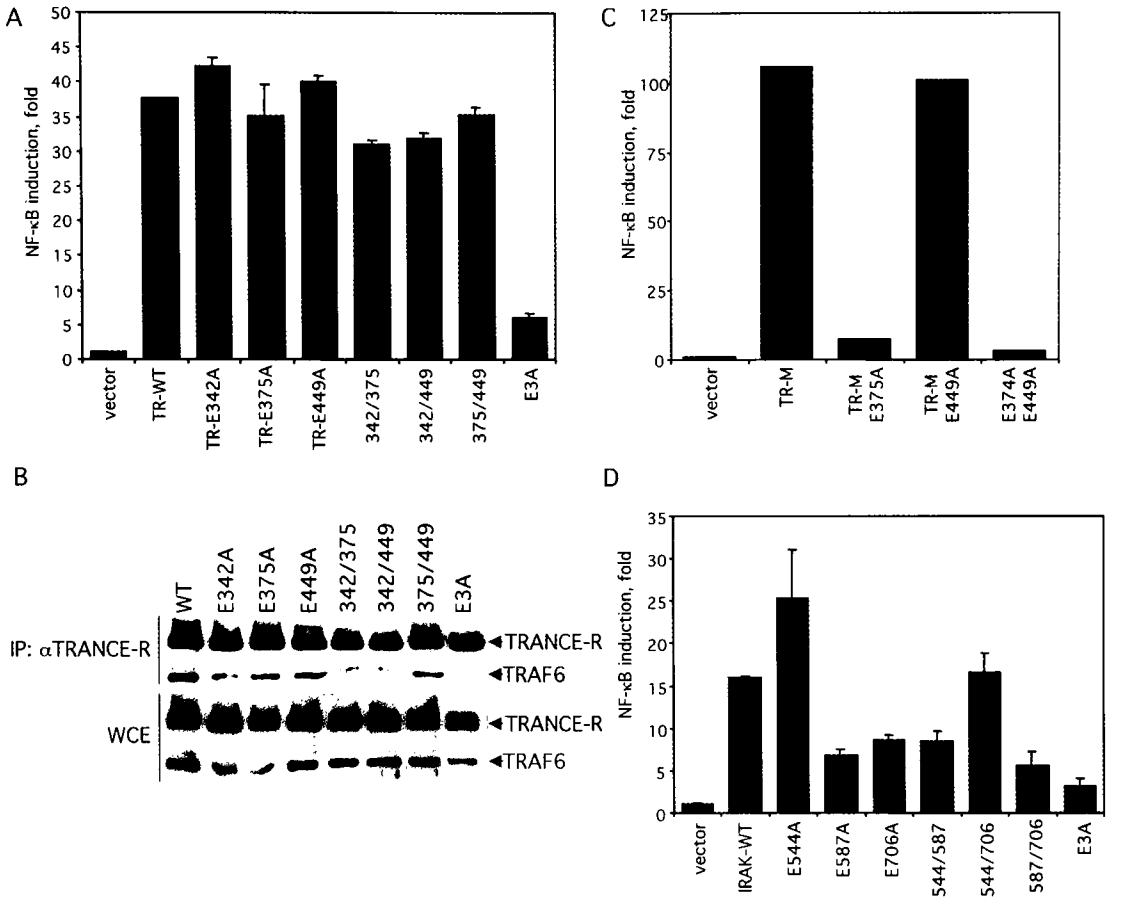

Figure 3.2.7. Relative contributions of each TRAF6-binding site to TRANCE-R and IRAK signaling.

A. Full-length TRANCE-R (wild-type and mutants as indicated, 50 ng) was transfected and NF-κB activity was determined as in Figure 3.2.5.

B. TRANCE-R (wild-type and mutants as indicated, 500 ng) was co-transfected with wild-type TRAF6 (500 ng). Cell lysates were immunoprecipitated with an antibody to TRANCE-R (1E6.66) and immunoprecipitates (IP) and whole cell lysates (WCE) were probed with anti-Flag M2 antibody.

C. TRANCE-R constructs consisting of the ectodomain/transmembrane region and residues 354-536 of the cytoplasmic tail (TR-M, see Figure 3.2.4B for diagram, wild-type and mutants as indicated, 50 ng) were transfected as in A.

D. Full-length IRAK (wild-type and mutants as indicated, 100 ng) was transfected as in A.

In the double mutants, mutation of the two "M" region glutamates (375/449) consistently produced less inhibition of NF-κB activation than did either double mutant involving the "N" region glutamate (342/375 and 342/449), so we examined the relative contribution of sites 2 and 3 in the context of the "M" region alone. Unexpectedly, the E375A mutation almost completely abolished TR-M-mediated NF-κB activation, while the E449A

mutation had minimal effect (Figure 3.2.7C). Similar results were obtained with 342/375 and 342/449 mutants of the "N+M" regions, omitting the "C" region (data not shown). This discrepancy between the "M" region or "N+M" region alone and the full cytoplasmic tail of TRANCE-R suggests a potential cooperativity between the C-terminal portion of TRANCE-R and the third TRAF6 binding site. Whether this is due to binding of other TRAFs to the "C" region or differential protein folding leading to masking of site 3 in the absence of the "C" region is unclear at this point.

Mutation of the three predicted TRAF6-binding sites in IRAK revealed, unexpectedly, a slight gain of NF-κB-activating potential by mutation of site 1 (E544A), as full-length IRAK with a single E544A mutation activated NF-κB slightly more strongly than wild-type full-length IRAK. Single mutation of E587A and E706A both reduced NF-κB activation by IRAK, while double mutation of E544A with either E587A or E706A slightly increased NF-κB activation over single mutants of either E587A or E706A. Triple mutation of E544A, E587A, and E706A almost completely reduced NF-κB activation by IRAK to background levels (Figure 3.2.7D). The increased activity observed in single and double mutants containing E544A suggests that site 1 in IRAK may somehow negatively regulate IRAK signaling by sequestering TRAF6 away from the other two sites while not allowing it to activate downstream signals or through some other, unknown mechanism. Given that the mode of TRAF6 interaction with TNFR family receptors is trimeric, it is likely that the mode of TRAF6 interaction with IRAK is similarly trimeric. There may be structural constraints of trimeric IRAK that prevent trimeric TRAF6 from interacting properly with site 1, thus sequestering monomeric TRAF6 at that site, preventing it from trimerizing and activating downstream signals.

3.2.3.8 The role of TRAF6 in ligand-dependent signaling by TR-N

In order to confirm the functional role of TRAF6 in TRANCE-mediated signaling, we generated stable HEK 293 cell lines expressing TR-N and TR-N-E342A. Stable clones were selected that expressed similar levels of surface TR-N as determined by FACS analysis (data not shown). In response to TRANCE stimulation, 293/TR-N cells demonstrated strong activation of JNK as measured by an in vitro kinase assay, while 293/TR-N-E342A cells did not activate JNK in response to TRANCE (Figure 3.2.8A, top). Similarly, TRANCE stimulation of 293/TR-N cells resulted in p38 activation as measured with phospho-p38 specific antibodies, while TRANCE did not activate p38 in 293/TR-N-E342A cells (Figure 3.2.8A, bottom). In order to measure TRANCE-dependent NF-κB activation, we transfected 293/TR-N and 293/TR-N-E342A cells with an NF-κB reporter plasmid, stimulated the cells for 24h with soluble TRANCE, and measured reporter activity. While 293/TR-N cells showed a robust activation of NF-κB in response to TRANCE, 293/TR-N-E342A cells had no measurable NF-κB induction in response to TRANCE (Figure 3.2.8B). Thus, the observed effects achieved by transient overexpression of TR-N constructs elsewhere in this report are similar to those found in a ligand-dependent situation with stable expression of TR-N. Furthermore, TR-N is functional for surface expression and ligand-dependent signaling through TRAF6 and will be a useful tool to isolate TRAF6-specific signals emanating from TRANCE-R.

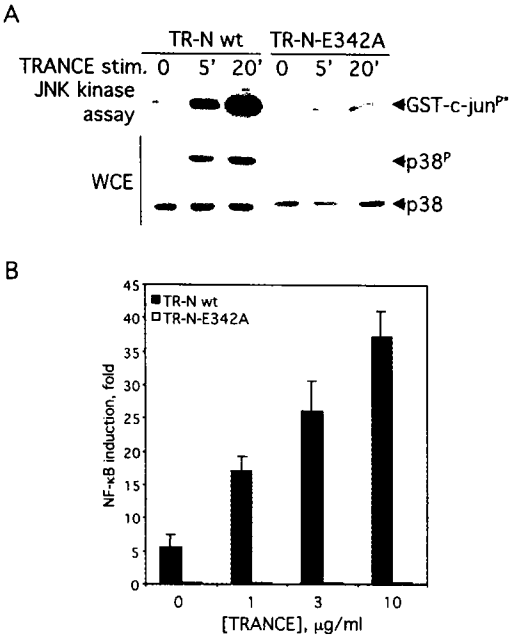

Figure 3.2.8. Ligand-dependent TRAF6 signaling by TR-N.

A. Stably transfected 293 cells expressing TR-N (wild-type and E342A as indicated) were stimulated with TRANCE (2 μg/ml) for the indicated time, lysed, and subjected to an *in vitro* JNK assay. Whole cell extracts (WCE) were probed with phospho-specific and total αp38 antibodies as indicated.

B. Stably transfected 293 cells expressing TR-N (wild-type and E342A as indicated) were transfected with an NF-κB luciferase reporter plasmid and stimulated for 24h with the indicated amount of soluble TRANCE. Luciferase activity was measured as in Figure 3.2.5.

3.2.3.9 Relative contributions of TRAF6-binding sites and TRAF1/2/3/5 binding sites to CD40 and TRANCE-R signaling

To account for residual, non-TRAF6-mediated NF-κB activation by TRANCE-R and CD40, we overexpressed an excess of TRAF2 or TRAF6 constructs lacking the N-terminal zinc-binding domains as dominant negatives (T2.DN and T6.DN). With wild-type full-length CD40, T6.DN inhibited NF-κB activation slightly more than did T2.DN. CD40-E239A activated NF-κB as strongly as CD40-WT + T6.DN, while CD40-E239A + T2.DN showed no NF-κB activation, suggesting that the defined TRAF6-binding site in CD40 is the only TRAF6-binding site in CD40 capable of signaling (Figure 3.2.9A). A similar set of transfections with TR-N and TR-N-E342A shows that TR-N activates NF-κB exclusively through TRAF6, as T2.DN had no effect on the ability of TR-N to activate NF-κB (Figure 3.2.9B). To determine if the C-terminal TRAF1/2/3/5 binding site(s) contribute to the residual non-TRAF6-dependent NF-κB activation in full-length TRANCE-R, we transfected the TR-FL-E3A construct with T6.DN and T2.DN. T6.DN had minimal effect on the residual activity of TR-FL-E3A to activate NF-κB, while T2.DN abolished the residual NF-κB activity (Figure 3.2.9C). Since TRANCE-R has

three TRAF6-binding sites, in order to more directly compare it to CD40, we deleted the "M" region, which contains two TRAF6-binding sites, leaving only site 1 (TR-ΔM, Figure 3.2.4B). The relative inhibition of TR-ΔM and TR-ΔM-E342A signaling by T6.DN and T2.DN (Figure 3.2.9D) was comparable to that observed in CD40-WT and CD40-E239A (Figure 3.2.9A). Thus, the presence of three TRAF6-binding sites in TRANCE-R as opposed to a single TRAF6-binding site in CD40 suggests that TRAF6 may play a more dominant role in TRANCE-R signaling. This may explain the observed absence of NF-κB, JNK, and p38 activation in TRAF6-/- cells upon TRANCE treatment despite the presence of other TRAFs in those cells (22).

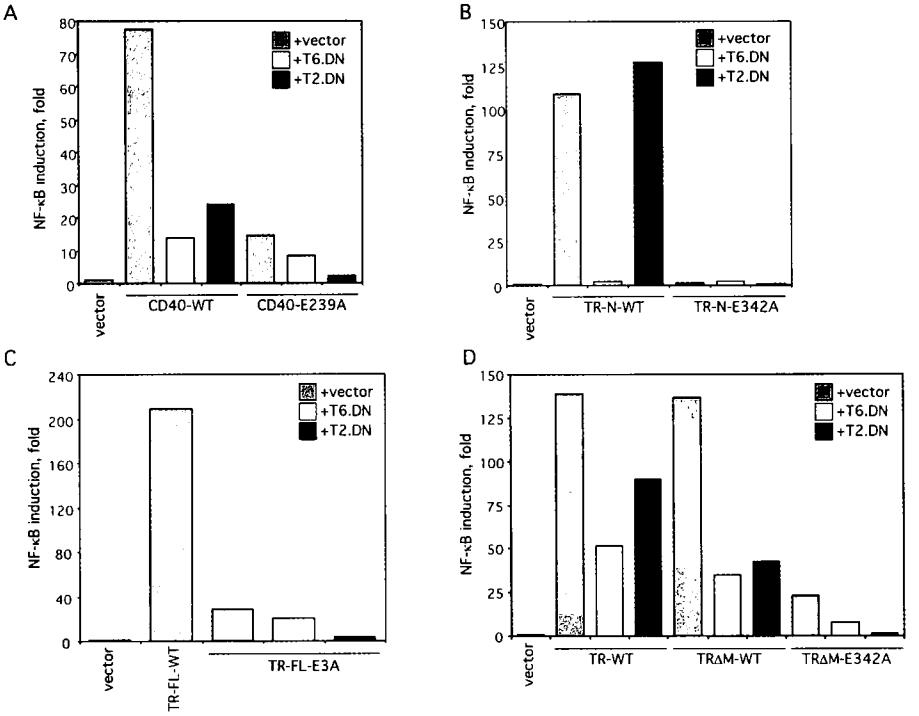

Figure 3.2.9. Relative contributions of TRAF6- and TRAF1/2/3/5-binding sites to CD40 and TRANCE-R signaling.

A. CD40 constructs (wild-type and E239A as indicated, 100 ng) were cotransfected with empty vector, TRAF6 residues 289-530 (T6.DN, 800 ng) or TRAF2 residues 241-501 (T2.DN, 800 ng) and subjected to an NF-κB reporter assay as in Figure 3.2.5.

B. As in A, but with TR-N (50 ng) instead of CD40.

C. As in A, but with full-length TRANCE-R (TR-FL, WT and triple E-A mutant, E3A, as indicated, 50 ng).

D. As in A, but with full-length TRANCE-R or TRANCE-R with a deletion of residues 254-536 (TR-ΔM, WT and E342A as indicated).

3.2.10 Formation of a ternary complex consisting of TRAF6, CD40, and c-Src

A specific role of TRAF6 in anti-apoptotic signaling (17,35) has recently been demonstrated by its activation of the Ser/Thr kinase Akt, which specifically phosphorylates and inactivates pro-apoptotic molecules such as BAD and pro-caspase-9 (36-38). For TRANCE-R, this Akt activation appears to be mediated by c-Src, a member of the Src tyrosine kinase family, with TRAF6 acting upstream of c-Src activation. Mice deficient in either TRAF6 (11,12) or c-Src (18) share similar phenotypes in TRANCE-R-mediated osteoclast function. A universal role of TRAF6 as an upstream event for Akt activation through the Src kinases has also been implicated for CD40 (39-41) and IL-1/Toll-like receptor signaling (17).

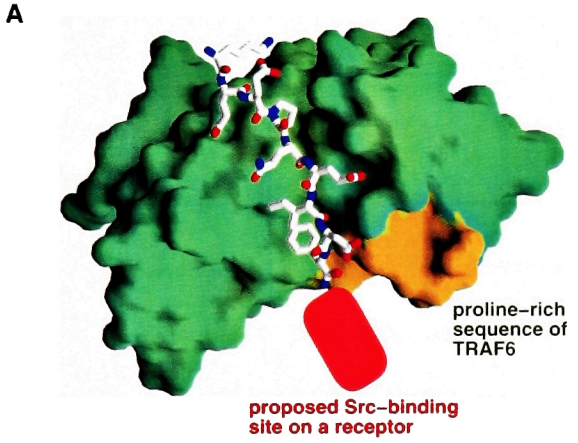

Figure 3.2.10. Ternary complex formation of TRAF6, CD40, and c-Src.
A. Mapping of the potential Src-binding site on the surface of the TRAF6/CD40 complex. CD40 peptide: ball and stick model; TRAF6: green; proline-rich sequence of TRAF6: orange.
B. Native gel electrophoresis, showing the ternary complex formation of TRAF6, GST-CD40cyt, and c-Src.

The structures of TRAF6 in complex with CD40 and TRANCE-R suggest a model for a ternary complex formation among TRAF6, a receptor and a Src kinase (Figure 3.2.10A). TRAF6 is the only TRAF family member that contains a putative SH3 domain binding sequence (462-PxxPxxP-468) as an insertion between the strands β6 and β7. Interestingly, residues 465-468 within this proline-rich sequence are disordered in the free TRAF6 structure and become ordered in the CD40 and TRANCE-R-bound structures. Previous binding studies using *in vitro* translated proteins suggested that c-Src interacts with TRAF6 via these proline-rich sequences and with TRANCE-R via a region immediately after its TRAF6-binding site. In the TRAF6 complexes, these proline-rich residues are situated adjacent to the bound CD40 and TRANCE-R receptor peptides, suggesting that a Src kinase may interact with an integrated surface formed by both TRAF6 and the receptors.

To determine whether such a ternary complex can be assembled *in vitro*, we assessed the pattern of native gel shift using purified TRAF6, GST-CD40 intracellular domain and c-Src (Figure 3.2.10B). The native gel clearly shows that a ternary complex is formed upon addition of the three proteins, as shown by the disappearance of protein bands corresponding to the individual components. Therefore, the biochemical evidence further suggests a cooperative (rather than a competitive) assembly of the ternary complex, providing an explanation for the interdependence of TRAF6 and Src kinases in the signal transduction of these receptors. The experiment also demonstrates a direct linkage of a TRAF protein to a downstream molecule among the many that have been implicated but not proven in TRAF signaling.

3.2.4 Conclusion

Our studies of the TRAF domain of TRAF6 reveal the structural underpinnings of its interaction with TNFR family proteins and components of the IL-1R/Toll-like receptor family signaling complex. Previous studies have demonstrated that TRAF6 is divergent from other TRAF proteins in that it binds to unique receptor sites and participates in signal transduction downstream of non-TNFR family proteins through its association with IRAK. While the overall structure of TRAF6 is similar to that of TRAF2, its mode of binding to receptor sequences is quite divergent, although it centers around a critical binding residue on the receptor (Glu) similar to TRAF2's binding to a central receptor residue (Gln). This suggests an evolutionary point of divergence between TRAF6 and TRAFs 1, 2, 3, and 5. Identification of a consensus binding sequence for TRAF6 revealed that there are numerous TRAF6 binding sites in TRANCE-R and IRAK, which we have confirmed functionally. Finally, we propose a potential mechanism by which TRAF6 links TNFR family proteins and the c-Src tyrosine kinase, in a cooperative ternary complex assembly. Given the importance of TRAF6 in bone remodeling and adaptive and innate immunity, structural information about its mode of signaling is an important starting point for the rational design of anti-osteoporotic and anti-inflammatory molecules.

3.2.5 Experimental Procedures

3.2.5.1 Isothermal titration calorimetry

Peptides containing putative TRAF6-binding sequences were chemically synthesized with amino-terminal acetylation and carboxy-terminal amidation. The molecular mass of each peptide was verified by MALDI-TOF mass spectrometry. A different TRAF6 construct (residues 333-508), which was expressed and purified similarly as described for

the TRAF6 construct used for crystallization, was used for ITC experiments due to its higher expression yield. Both protein and peptides were dialyzed extensively against 50mM sodium phosphate at pH 7.5 for at least two days at 4°C to ensure buffer equilibration. Accurate concentrations of the protein and peptide samples after dialysis were determined by quantitative amino acid analysis. ITC experiments were performed at 20°C for determination of binding enthalpy and affinity using a microcalorimetry system (MicroCal Inc.). Approximately 20 to 45 injections were titrated for each measurement. The data were analyzed by the ORIGIN data analysis software (MicroCal Inc.) (26). The heat of dilution obtained from injecting a ligand into the buffer was subtracted before the fitting process.

3.2.5.2 Protein expression, purification and crystallization

The TRAF proteins contain an N-terminal effector domain of RING and zinc-fingers and a C-terminal TRAF domain that can be further divided into a coiled-coil region and a homologous TRAF-C domain for receptor interaction (1,19). A combination of genetic, biochemical and crystallographic methods was used in identifying a crystallizable TRAF domain construct of TRAF6 and a detailed description will be presented elsewhere. In summary, the TRAF6 construct (residues 346-504) containing a carboxy-terminal His-tag was expressed in *E. coli* with overnight IPTG induction at 20°C. The protein was purified by Ni-affinity chromatography and gel filtration. The protein was concentrated to around 3 mg/ml and crystallized under 5-25% PEG8K and 100mM Tris at pH 7.5. For complex formation with TRAF6-binding peptides, a 10-fold molar excess of a mutant human CD40 peptide (230-KQEPQEIDF-238) or a human TRANCE-R peptide (342-QMPTEDEY-349) was included in the crystallization drops. Crystals grew as thin plates and chunky prisms respectively in the absence and presence of the peptides.

3.2.5.3 Data collection and structure determination

Preliminary crystal characterization and screening were conducted on an Raxis-IV imaging plate detector mounted on a Rigaku RU300 X-ray generator. Diffraction data were collected at the X4A beamline of NSLS and the A1 beamline of CHESS. The structures were determined by molecular replacement using the program Replace . The atomic model of the TRAF domain of TRAF2 was modified by removing the side chains of residues that are not conserved in TRAF6. Structural refinement was performed by the simulated annealing protocol in CNS (42). Ribbon and stick models were created using Setor (43) and the molecular surface representation was calculated and presented by Grasp (44).

3.2.5.4 Native gel shift and size exclusion chromatography

Native gel shift experiments were performed using the PhastGel system (Pharmacia) and 8-25% gradient polyacrylamide gel. Size exclusion chromatography was carried out using a Superdex 200 (10/30) column (Pharmacia).

3.2.5.5 Transfection and Reporter Assays

Mouse CD40 was cloned by RT-PCR from whole spleen mRNA and inserted into the pFLAG-CMV1 cloning vector (Sigma). Flag-tagged mouse TRANCE-R consisting of the extracellular domain and residues 235-368 or 354-536 of the cytoplasmic tail (TR-N and TR-M), mouse TRAF6 consisting of residues 289-530 (T6.DN), and TRAF2 consisting of residues 241-501 (T2.DN) have been described (20). Site-directed mutagenesis of CD40, TRANCE-R, IRAK, and TRAF6.DN was performed on the indicated residues by the Quick-change method (Stratagene). All constructs were confirmed by sequencing.

3. Results

293T HEK cells were transfected in 6-well plates with the indicated amounts of CD40, TR-N, and TRAF6.DN and mutants along with 75 ng of an NF–κB-luciferase reporter plasmid and 25 ng of a β-galactosidase plasmid to control for transfection efficiency. Transfection amounts were kept constant at 1 μg by addition of empty pFLAG-CMV1 vector. Cells were harvested 24-30 h after transfection and reporter activity was assayed as described (20). Where indicated, cells were treated with 1 ng/ml recombinant human IL-1 (R & D) 6 hour prior to harvesting.

For the *in vitro* JNK kinase assay, full length TRANCE-R, TR-N, TR-N mutants, and MEKK1 were transfected along with JNK1 (100 ng) as indicated. JNK1 was immunoprecipitated with an α–JNK1 antibody (E-17, Santa Cruz Biotechnology), and the immunoprecipitates were subjected to an *in vitro* kinase assay using recombinant GST-c-jun (1-79, Calbiochem) as a substrate as described previously (45). Transfection efficiency was confirmed by western blotting the immunoprecipitates with α-JNK1.

Stable cell lines expressing TR-N and TR-N-E342A were generated by co-transfection of HEK 293 cells with the indicated expression vectors with a vector carrying a neomycin resistance plasmid (pcDNA3.1). Cells were selected for resistance to G418 and cloned by limiting dilution. Clones with similar levels of surface expression of TR-N were identified by FACS analysis.

3.2.6 Acknowledgements

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3.2.7 References

1. Arch, R. H., Gedrich, R. W., and Thompson, C. B. (1998) Tumor necrosis factor receptor-associated factors (TRAFs)--a family of adapter proteins that regulates life and death. *Genes Dev* **12**(18), 2821-30.
2. Cao, Z., Xiong, J., Takeuchi, M., Kurama, T., and Goeddel, D. V. (1996) TRAF6 is a signal transducer for interleukin-1. *Nature* **383**(6599), 443-6.
3. Medzhitov, R., and Janeway, C. A., Jr. (1998) Innate immune recognition and control of adaptive immune responses. *Semin Immunol* **10**(5), 351-3.
4. van Kooten, C., and Banchereau, J. (2000) CD40-CD40 ligand. *J Leukoc Biol* **67**(1), 2-17.
5. Wong, B. R., Josien, R., and Choi, Y. (1999) TRANCE is a TNF family member that regulates dendritic cell and osteoclast function. *J Leukoc Biol* **65**(6), 715-24.
6. Fata, J. E., Kong, Y. Y., Li, J., Sasaki, T., Irie-Sasaki, J., Moorehead, R. A., Elliott, R., Scully, S., Voura, E. B., Lacey, D. L., Boyle, W. J., Khokha, R., and Penninger, J. M. (2000) The osteoclast differentiation factor osteoprotegerin-ligand is essential for mammary gland development. *Cell* **103**(1), 41-50.
7. Medzhitov, R., Preston-Hurlburt, P., Kopp, E., Stadlen, A., Chen, C., Ghosh, S., and Janeway, C. A., Jr. (1998) MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. *Mol Cell* **2**(2), 253-8.
8. Kawai, T., Adachi, O., Ogawa, T., Takeda, K., and Akira, S. (1999) Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* **11**(1), 115-22.
9. Adachi, O., Kawai, T., Takeda, K., Matsumoto, M., Tsutsui, H., Sakagami, M., Nakanishi, K., and Akira, S. (1998) Targeted disruption of the MyD88 gene results in loss of IL-1- and IL- 18-mediated function. *Immunity* **9**(1), 143-50.
10. Li, X., Commane, M., Jiang, Z., and Stark, G. R. (2001) IL-1-induced NFkappa B and c-Jun N-terminal kinase (JNK) activation diverge at IL-1 receptor-associated kinase (IRAK). *Proc Natl Acad Sci U S A* **98**(8), 4461-5.

11. Lomaga, M. A., Yeh, W. C., Sarosi, I., Duncan, G. S., Furlonger, C., Ho, A., Morony, S., Capparelli, C., Van, G., Kaufman, S., van der Heiden, A., Itie, A., Wakeham, A., Khoo, W., Sasaki, T., Cao, Z., Penninger, J. M., Paige, C. J., Lacey, D. L., Dunstan, C. R., Boyle, W. J., Goeddel, D. V., and Mak, T. W. (1999) TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. *Genes Dev* **13**(8), 1015-24.

12. Naito, A., Azuma, S., Tanaka, S., Miyazaki, T., Takaki, S., Takatsu, K., Nakao, K., Nakamura, K., Katsuki, M., Yamamoto, T., and Inoue, J. (1999) Severe osteopetrosis, defective interleukin-1 signalling and lymph node organogenesis in TRAF6-deficient mice. *Genes Cells* **4**(6), 353-62.

13. Kong, Y. Y., Yoshida, H., Sarosi, I., Tan, H. L., Timms, E., Capparelli, C., Morony, S., Oliveira-dos-Santos, A. J., Van, G., Itie, A., Khoo, W., Wakeham, A., Dunstan, C. R., Lacey, D. L., Mak, T. W., Boyle, W. J., and Penninger, J. M. (1999) OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* **397**(6717), 315-23.

14. Kim, D., Mebius, R. E., MacMicking, J. D., Jung, S., Cupedo, T., Castellanos, Y., Rho, J., Wong, B. R., Josien, R., Kim, N., Rennert, P. D., and Choi, Y. (2000) Regulation of peripheral lymph node genesis by the tumor necrosis factor family member TRANCE. *J Exp Med* **192**(10), 1467-78.

15. Dougall, W. C., Glaccum, M., Charrier, K., Rohrbach, K., Brasel, K., De Smedt, T., Daro, E., Smith, J., Tometsko, M. E., Maliszewski, C. R., Armstrong, A., Shen, V., Bain, S., Cosman, D., Anderson, D., Morrissey, P. J., Peschon, J. J., and Schuh, J. (1999) RANK is essential for osteoclast and lymph node development. *Genes Dev* **13**(18), 2412-24.

16. Soriano, P., Montgomery, C., Geske, R., and Bradley, A. (1991) Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. *Cell* **64**(4), 693-702.

17. Wong, B. R., Besser, D., Kim, N., Arron, J. R., Vologodskaia, M., Hanafusa, H., and Choi, Y. (1999) TRANCE, a TNF family member, activates Akt/PKB through a signaling complex involving TRAF6 and c-Src. *Mol Cell* **4**(6), 1041-9.

18. Xing, L., Venegas, A. M., Chen, A., Garrett-Beal, L., Boyce, B. F., Varmus, H. E., and Schwartzberg, P. L. (2001) Genetic evidence for a role for Src family kinases in TNF family receptor signaling and cell survival. *Genes Dev* **15**(2), 241-53.

19. Rothe, M., Wong, S. C., Henzel, W. J., and Goeddel, D. V. (1994) A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. *Cell* **78**(4), 681-92.

20. Wong, B. R., Josien, R., Lee, S. Y., Vologodskaia, M., Steinman, R. M., and Choi, Y. (1998) The TRAF family of signal transducers mediates NF-kappaB activation by the TRANCE receptor. *J Biol Chem* **273**(43), 28355-9.
21. Pullen, S. S., Dang, T. T., Crute, J. J., and Kehry, M. R. (1999) CD40 signaling through tumor necrosis factor receptor-associated factors (TRAFs). Binding site specificity and activation of downstream pathways by distinct TRAFs. *J Biol Chem* **274**(20), 14246-54.
22. Kobayashi, N., Kadono, Y., Naito, A., Matsumoto, K., Yamamoto, T., Tanaka, S., and Inoue, J. (2001) Segregation of TRAF6-mediated signaling pathways clarifies its role in osteoclastogenesis. *Embo J* **20**(6), 1271-80.
23. Pullen, S. S., Labadia, M. E., Ingraham, R. H., McWhirter, S. M., Everdeen, D. S., Alber, T., Crute, J. J., and Kehry, M. R. (1999) High-affinity interactions of tumor necrosis factor receptor-associated factors (TRAFs) and CD40 require TRAF trimerization and CD40 multimerization. *Biochemistry* **38**(31), 10168-77.
24. Darnay, B. G., Ni, J., Moore, P. A., and Aggarwal, B. B. (1999) Activation of NF-kappaB by RANK requires tumor necrosis factor receptor- associated factor (TRAF) 6 and NF-kappaB-inducing kinase. Identification of a novel TRAF6 interaction motif. *J Biol Chem* **274**(12), 7724-31.
25. Pullen, S. S., Miller, H. G., Everdeen, D. S., Dang, T. T., Crute, J. J., and Kehry, M. R. (1998) CD40-tumor necrosis factor receptor-associated factor (TRAF) interactions: regulation of CD40 signaling through multiple TRAF binding sites and TRAF hetero-oligomerization. *Biochemistry* **37**(34), 11836-45.
26. Ye, H., and Wu, H. (2000) Thermodynamic characterization of the interaction between TRAF2 and tumor necrosis factor receptor peptides by isothermal titration calorimetry. *Proc Natl Acad Sci U S A* **97**(16), 8961-6.
27. McWhirter, S. M., Pullen, S. S., Holton, J. M., Crute, J. J., Kehry, M. R., and Alber, T. (1999) Crystallographic analysis of CD40 recognition and signaling by human TRAF2. *Proc Natl Acad Sci U S A* **96**(15), 8408-13.
28. Park, Y. C., Burkitt, V., Villa, A. R., Tong, L., and Wu, H. (1999) Structural basis for self-association and receptor recognition of human TRAF2. *Nature* **398**(6727), 533-8.
29. Ye, H., Park, Y. C., Kreishman, M., Kieff, E., and Wu, H. (1999) The structural basis for the recognition of diverse receptor sequences by TRAF2. *Mol Cell* **4**(3), 321-30.
30. Banner, D. W., D'Arcy, A., Janes, W., Gentz, R., Schoenfeld, H. J., Broger, C., Loetscher, H., and Lesslauer, W. (1993) Crystal structure of the soluble human 55 kd TNF receptor-human TNF beta complex: implications for TNF receptor activation. *Cell* **73**(3), 431-45.

31. Khursigara, G., Orlinick, J. R., and Chao, M. V. (1999) Association of the p75 neurotrophin receptor with TRAF6. *J Biol Chem* **274**(5), 2597-600.
32. Xia, X. Z., Treanor, J., Senaldi, G., Khare, S. D., Boone, T., Kelley, M., Theill, L. E., Colombero, A., Solovyev, I., Lee, F., McCabe, S., Elliott, R., Miner, K., Hawkins, N., Guo, J., Stolina, M., Yu, G., Wang, J., Delaney, J., Meng, S. Y., Boyle, W. J., and Hsu, H. (2000) TACI is a TRAF-interacting receptor for TALL-1, a tumor necrosis factor family member involved in B cell regulation. *J Exp Med* **192**(1), 137-43.
33. Takeuchi, M., Rothe, M., and Goeddel, D. V. (1996) Anatomy of TRAF2. Distinct domains for nuclear factor-kappaB activation and association with tumor necrosis factor signaling proteins. *J Biol Chem* **271**(33), 19935-42.
34. Song, H. Y., Regnier, C. H., Kirschning, C. J., Goeddel, D. V., and Rothe, M. (1997) Tumor necrosis factor (TNF)-mediated kinase cascades: bifurcation of nuclear factor-kappaB and c-jun N-terminal kinase (JNK/SAPK) pathways at TNF receptor-associated factor 2. *Proc Natl Acad Sci U S A* **94**(18), 9792-6.
35. Arron, J. R., Vologodskaia, M., Wong, B. R., Naramura, M., Kim, N., Gu, H., and Choi, Y. (2001) A positive regulatory role for Cbl family proteins in tumor necrosis factor-related activation-induced cytokine (trance) and CD40L-mediated Akt activation. *J Biol Chem* **276**(32), 30011-7.
36. Yano, S., Tokumitsu, H., and Soderling, T. R. (1998) Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway. *Nature* **396**(6711), 584-7.
37. Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. (1998) Regulation of cell death protease caspase-9 by phosphorylation. *Science* **282**(5392), 1318-21.
38. Coffer, P. J., Jin, J., and Woodgett, J. R. (1998) Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. *Biochem J* **335**(Pt 1), 1-13.
39. Ren, C. L., Morio, T., Fu, S. M., and Geha, R. S. (1994) Signal transduction via CD40 involves activation of lyn kinase and phosphatidylinositol-3-kinase, and phosphorylation of phospholipase C gamma 2. *J Exp Med* **179**(2), 673-80.
40. Vidalain, P. O., Azocar, O., Servet-Delprat, C., Rabourdin-Combe, C., Gerlier, D., and Manie, S. (2000) CD40 signaling in human dendritic cells is initiated within membrane rafts. *Embo J* **19**(13), 3304-13.
41. Hostager, B. S., Catlett, I. M., and Bishop, G. A. (2000) Recruitment of CD40 and tumor necrosis factor receptor-associated factors 2 and 3 to membrane microdomains during CD40 signaling. *J Biol Chem* **275**(20), 15392-8.
42. Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R.

J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Crystallogr D Biol Crystallogr* **54**(Pt 5), 905-21.

43. Evans, S. V. (1993) SETOR: hardware-lighted three-dimensional solid model representations of macromolecules. *J Mol Graph* **11**(2), 134-8, 127-8.
44. Nicholls, A., Sharp, K. A., and Honig, B. (1991) Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbons. *Proteins* **11**(4), 281-96
45. Wong, B. R., Rho, J., Arron, J., Robinson, E., Orlinick, J., Chao, M., Kalachikov, S., Cayani, E., Bartlett, F. S., 3rd, Frankel, W. N., Lee, S. Y., and Choi, Y. (1997) TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. *J Biol Chem* **272**(40), 25190-4.

3.3 REGULATION OF THE SUBCELLULAR LOCALIZATION OF TRAF2 BY TRAF1 REVEALS MECHANISMS OF TRAF2 SIGNALING

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3.3.1 Summary

TRAF2 is a critical adaptor molecule for TNF receptors in inflammatory and immune signaling. In response to ligand engagement, TRAF2 is recruited to CD40 and translocates to lipid rafts in a RING finger-dependent process, which is required for its ability to activate downstream kinases. TRAF1 can displace TRAF2 and CD40 from raft fractions, and it promotes the ability of TRAF2 to sustain activation of signal cascades. Replacement of the RING finger of TRAF2 with a raft-targeting dual acylation signal restores JNK activation and association with the cytoskeletal protein Filamin, but not NF-κB activation. TRAF1-/- dendritic cells show attenuated responses to secondary stimulation by TRAF2-dependent factors. These findings offer insights into the mechanism of TRAF2 signaling and identify a physiological role for TRAF1 as a positive regulator of TRAF2 signaling.

3.3.2 Introduction

Tumor necrosis factor (TNF) family proteins are essential regulators of the life and death of hematopoietic cells, bone and mammary gland homeostasis, and embryonic development (1). Signaling through TNF receptor (TNFR) proteins is mediated in part by TNFR associated factor (TRAF) adaptor proteins, which have been shown to activate the transcription factor NF-κB, MAP kinases, and Src-family kinases (2). Of the six mammalian TRAFs, sequence conservation analysis has shown that TRAFs 1, 2, 3, and 5 are closely related, while TRAFs 4 and 6 are more evolutionarily divergent. Of these evolutionary relations, TRAF1 and TRAF2 appear to have arisen after duplication of a common precursor, while TRAF3 and TRAF5 have arisen from a different common precursor (3).

The general domain organization of TRAF proteins, of which TRAF2 is the archetype, comprises an N-terminal zinc-binding domain, specifically a RING finger followed by several Zn fingers, and a C-terminal TRAF domain, consisting of a coiled-coil which permits TRAF oligomerization (TRAF-N) and a receptor binding domain (TRAF-C) (4,5). There are some exceptions to this scheme, most notably in TRAF1, which has a C-terminal TRAF domain that is highly homologous to TRAF2, but lacks the RING and all but one of the Zn fingers. The specificity conferred by a particular TRAF-binding receptor is conferred by its relative affinity for the different TRAF proteins, the cell type-specific expression of TRAFs, and the stoichiometry of TRAFs in a given receptor complex. Trimeric TNF family ligands bind to trimerized TNFR family proteins, dictating a trimeric mode of binding of TRAFs in which the affinity and avidity of TRAF proteins for receptor complexes is greatly enhanced (6-9)

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Although TRAFs are essential adaptors for signaling through TNFR family proteins, they do not appear to possess intrinsic enzymatic activity and the precise mechanism of their action is as yet unknown. It is clear that the TRAF domain of TRAF2 is necessary for its direct interactions with TNFR proteins such as TNFR2, CD40, TRANCE-R, CD30 and others, as well as interactions with cytoplasmic factors including TRADD, RIP, NIK, ASK1, GCK, c-IAPs, I-TRAF, TRIP, A20 and others (2,10,11). However, expression of the TRAF domain alone inhibits signaling by TNF family ligands, and indeed, mutants of TRAF2 lacking the RING finger act as dominant negative factors for NF-κB and MAPK activation (12,13). The N-terminal RING and Zn fingers are therefore required for the activation of these cascades. The RING finger has been proposed to interact with MEKK1, a MAP3K that is potentially involved in TNF-α induced JNK activation (14,15), and the actin-binding protein Filamin (16), but physical association of the RING finger of TRAF2 and these proteins has not been rigorously demonstrated. TRAF2A, a splice variant of TRAF2 identified only in the murine *traf2* locus which carries a 7-amino acid insert in the RING finger, cannot activate NF-κB, but is capable of JNK activation (3,17). It has been recently shown in vitro that the RING fingers of TRAF2 and TRAF6 can serve as E3 ubiquitin ligases, mediating non-degradative K63 polyubiquitination of some interacting partners of TRAFs as well as TRAFs themselves. This polyubiquitination is linked to the ability to activate the IKK complex upstream of NF-κB (18,19). Ubiquitination of TRAFs may also lead to signal-dependent degradation, thus serving as a means of downregulating TRAF-dependent signals (20).

While most TRAFs are constitutively present in the cell types in which they are expressed, TRAF1 is absent in most resting cells (21). Expression of TRAF1 is rapidly upregulated in response to NF-κB and AP-1 activation by a variety of inflammatory mediators, including TNF-α, CD40L, LPS, and lymphocyte receptors (22-24). By itself,

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TRAF1 does not appear to activate signaling cascades activated by TRAF2, although it can hetero-oligomerize with TRAF2 (4) and interact with many of the same receptors and cytoplasmic proteins as TRAF2 (11). While the exact physiological role of TRAF1 is unknown, it appears to positively regulate survival signals mediated by TRAF2 (22,25,26). Recently, TRAF1 was found to be a target of caspases, and the resulting cleavage product negatively regulated the anti-apoptotic signals of TRAF2 during TNF-induced cell death (27,28).

An emerging theme in cell surface receptor signaling is detergent-resistant liquid-ordered lipid membrane microdomains, or lipid rafts (29-32). Contrary to the long-held fluid-mosaic model of membranes as proteins floating freely in a sea of phospholipids, it appears that the membrane lipids may have regions of higher-order organization enriched in cholesterol and sphingolipids that coalesce around activated transmembrane receptor protein complexes. These complexes are resistant to solubilization at low temperatures in non-ionic detergents such as Triton X-100 and thus may serve to assemble or exclude various signaling complex components, which may enhance signaling specificity. Recently, several reports have demonstrated that CD40 engagement or Epstein-Barr virus LMP1 expression results in recruitment of CD40 or LMP1, TRAF2, TRAF3, and several other proteins to lipid rafts, which are thought to be intrinsic to some of the signaling functions of CD40 and LMP1 (33-37).

In this report, we examine the role of the translocation of TRAF2 into detergent-insoluble complexes in the TRAF2-dependent activation of NF-κB and JNK. In response to CD40 stimulation, TRAF2 translocates into lipid rafts in a RING finger-dependent process, which is required for kinase activation. TRAF1, which is upregulated in response to TRAF2-mediated signals, regulates the removal of CD40 and TRAF2 from insoluble

complexes, but increases the ability of TRAF2 to mediate sustained activation of NF-κB and JNK. Substitution of the RING finger of TRAF2 with a lipid raft-targeting dual acylation signal rescues JNK activation, but not NF-κB activation by TRAF2, suggesting that raft translocation is necessary and sufficient for JNK activation, but insufficient for NF-κB activation. Finally, in TRAF1-/- dendritic cells, we show that maturation by CD40L leads to a loss of soluble TRAF2 and a concomitant reduction in TNF, TRANCE, and CD40L-mediated survival, revealing a positive physiological role for TRAF1 in the regulation of TRAF2-dependent signaling.

3.3.3 Results

3.3.3.1 TRAF1 increases the detergent solubility of TRAF2

Recently, it has been shown by several groups that CD40 engagement results in translocation of TRAF2 to detergent resistant lipid-ordered membrane microdomains, or lipid rafts (33-37). Since TRAF1 can hetero-oligomerize with TRAF2 and interact with the TRAF2 binding site of CD40, we investigated the effect of TRAF1 on the solubility of TRAF2 in non-ionic detergent (0.75% Triton X-100). Transient overexpression of TNFR or TRAF proteins leads to self-aggregation and signaling, mimicking ligand engagement, so we co-transfected HEK 293T cells with constant amounts of plasmids driving the expression CD40 and TRAF2, while titrating the amount of TRAF1. In the absence of TRAF1, a majority of TRAF2 was found in the insoluble fraction, while the addition of TRAF1 resulted in a dose-dependent redistribution of TRAF2 to the soluble fraction (Fig. 3.3.1A). The N-terminal zinc-binding RING finger of TRAF2 has been shown to be essential for NF-κB (12) and JNK (13) activation. It has also been suggested that the zinc binding capacity of TRAF2 is required for its translocation into lipid rafts (33). To determine if the RING finger of TRAF2 mediates translocation into the insoluble

fraction, we cotransfected CD40 and wild-type TRAF2 or TRAF2 with a deletion of the first 87 amino acids (T2Δ87), which comprise the RING finger, with or without TRAF1. T2Δ87 was found completely in the soluble fraction, regardless of TRAF1 expression (Fig. 3.3.1B). Like TRAF1, TRAF5 has been shown to hetero-oligomerize with TRAF2 (38). In order to determine if TRAF5 can mediate solubilization of TRAF2, we cotransfected CD40, TRAF2, and TRAF1 or TRAF5. While TRAF1 can mediate solubilization of TRAF2, TRAF5 cannot (Fig. 3.3.1C), suggesting a unique role for TRAF1.

To determine if TRAF1 could indeed mediate translocation of TRAF2 out of lipid rafts under sustained signaling conditions, we performed sucrose density gradient centrifugation on extracts of cells transfected with CD40, TRAF2 or T2Δ87, with or without TRAF1, treated for the last 6 h prior to lysis with soluble CD40L. In cells transfected with CD40 and TRAF2, both CD40 and TRAF2 could be found in the low-density raft fractions, co-migrating with the known raft-associated tyrosine kinase Lyn (Fig. 3.3.1D, upper left). However, addition of TRAF1 resulted in redistribution of both CD40 and TRAF2 out of the raft fractions (Fig. 3.3.1D, upper right). T2Δ87 was not found in significant amounts in the raft fractions with or without TRAF1, and even in the absence of overexpressed TRAF1, T2Δ87 coexpression resulted in a steady-state reduction of CD40 in the raft fractions as compared to coexpression with wild-type TRAF2 (Fig. 3.3.1D, bottom). Although TRAF1 resulted in a complete loss of TRAF2 from the raft fractions, there was still a considerable amount of insoluble TRAF2 in the crude cell extract, which may represent cytoskeleton-associated TRAF2 ((35) and further discussion below). To visually examine the distribution of TRAF2 in the cell, we co-transfected TRAF2 fused at its C terminus to EGFP (TRAF2-GFP) with or without CD40 and TRAF1. Fluorescence microscopy 24 hours after transfection revealed that TRAF2-

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GFP by itself was distributed throughout the cytoplasm with several aggregates in the cell, which is consistent with the ability of overexpressed TRAF2 to self-associate. Coexpression of CD40 resulted in the complete redistribution of TRAF2-GFP to punctate structures distributed throughout the cell. Addition of TRAF1 to TRAF2-GFP alone or with CD40 resulted in the continuous distribution of TRAF2 throughout the cytoplasm (Fig. 3.3.1E).

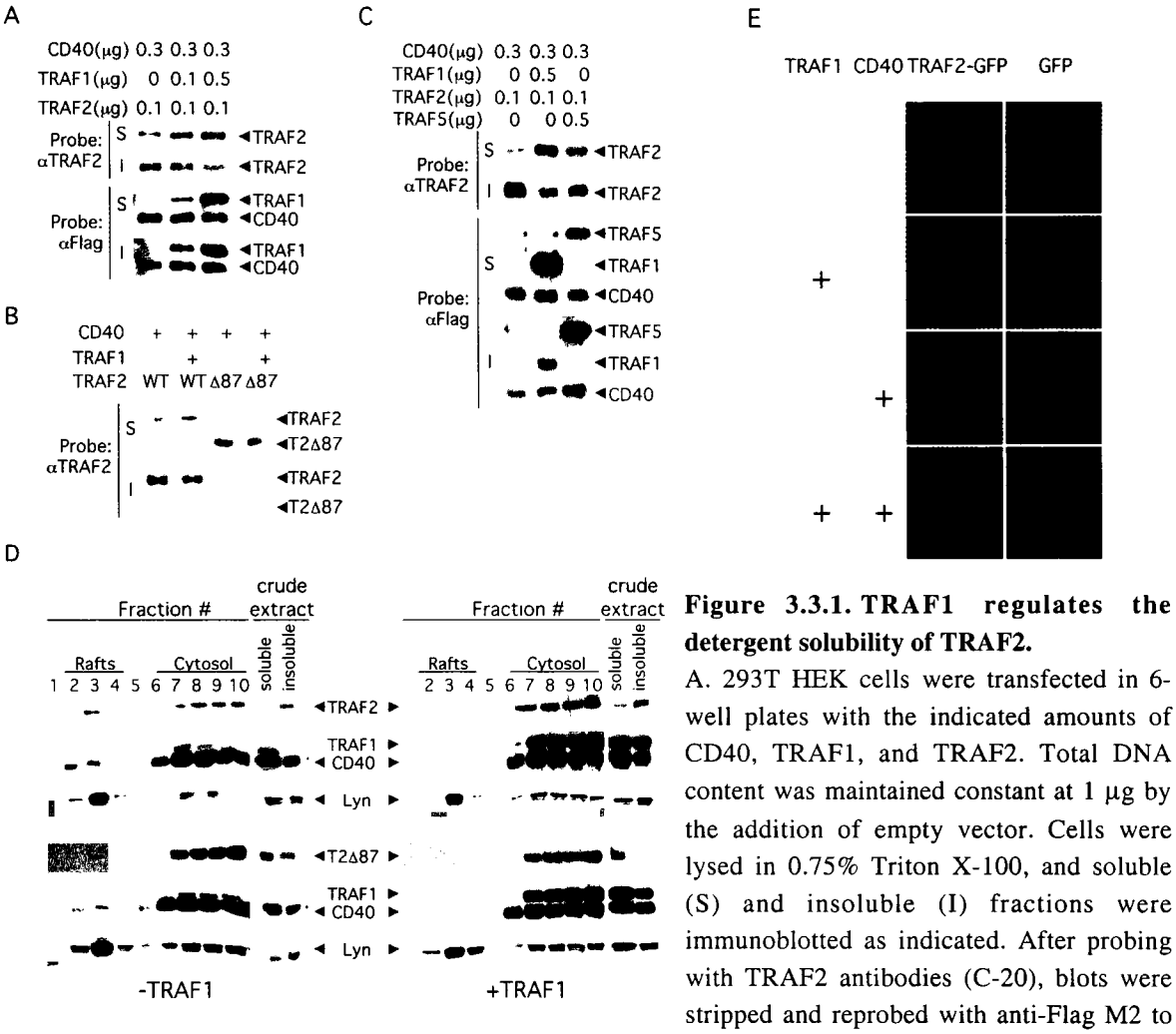

Figure 3.3.1. TRAF1 regulates the detergent solubility of TRAF2.

A. 293T HEK cells were transfected in 6-well plates with the indicated amounts of CD40, TRAF1, and TRAF2. Total DNA content was maintained constant at 1 μg by the addition of empty vector. Cells were lysed in 0.75% Triton X-100, and soluble (S) and insoluble (I) fractions were immunoblotted as indicated. After probing with TRAF2 antibodies (C-20), blots were stripped and reprobed with anti-Flag M2 to detect TRAF1 and CD40.

B. As in (A), but with 0.1 μg of TRAF2 or an N-terminal truncation mutant removing the first 87 residues (comprising the RING finger) of TRAF2 (T2Δ87). 0.5 μg of TRAF1 was transfected where indicated (+).

C. As in (A), with TRAF5 where indicated.

D. 293T cells were transfected with 1.5 μg of TRAF2 or T2Δ87, 2.5 μg of TRAF1, and 1.0 μg of CD40 where indicated. Cells were treated with CD40L 6 h prior to harvesting then subjected to sucrose gradient density centrifugation as described in Experimental Procedures and immunoblotted as indicated.

E. 293T cells were transfected as in (A) with TRAF2-GFP or GFP alone (0.2 μg), CD40 (0.1 μg), and TRAF1 (0.3 μg) as indicated. Cells were visualized by fluorescent microscopy 24 h after transfection.

3.3.3.2 Differential effects of TRAF2 and TRAF6 on CD40 localization

While oligomerization at the receptor appears to be necessary for the translocation of TRAF2 and CD40 into lipid rafts (33), it is unclear whether receptor engagement per se is sufficient for stable translocation of the receptor. The cytoplasmic tail of CD40 has two defined TRAF binding sites, one that is proximal to the membrane to which TRAF6 binds, and a more distal site which binds to TRAFs 1, 2, 3, and 5 (7). While TRAF2 and TRAF3 have been shown to be recruited to membrane rafts by CD40 engagement in primary cells (34), TRAF6 does not appear to play a prominent role in CD40-associated lipid rafts (33). In order to compare the contributions of TRAF2 and TRAF6 to CD40 translocation, we generated point mutants of CD40 that are deficient in binding to TRAF2, TRAF6, or both. Based on structural studies, we (section 3.2) and others (7) have identified E239 of mouse CD40 (corresponding to E235 of human CD40) as a critical binding residue for TRAF6 and Q253 (corresponding to Q252 of human CD40) as a critical binding residue for TRAF2. Cotransfection of CD40 and alanine mutations of E239, Q253, or both with TRAF2 followed by immunoprecipitation of CD40 demonstrated that TRAF2 binding to CD40-Q253A is greatly attenuated (Fig. 3.3.2A, top). A similar cotransfection of CD40 constructs with TRAF6 and immunoprecipitation of TRAF6 demonstrated that TRAF6 binding to CD40-E239A is greatly attenuated (Fig. 3.3.2A, bottom). Sucrose density gradient fractionation revealed that wild-type CD40 and CD40-E239A translocated to raft fractions, but to a greater extent in the presence of overexpressed TRAF2 than in the presence of overexpressed TRAF6. However, CD40-Q253A and the double E239A/Q253A mutant remained in the soluble fraction in the presence of overexpressed TRAF2 or TRAF6 (Fig. 3.3.2B). Since CD40-WT is found in

the raft fraction in the presence of overexpressed TRAF6 at similar levels to those of CD40-E239A, raft translocation of CD40 appears to be dependent on its ability to bind to TRAF2, but not to TRAF6. Furthermore, a higher level of raft-associated CD40-E239A than CD40-WT was observed in the presence of overexpressed TRAF2, which suggests that TRAF6 binding may actually decrease the steady-state affinity of CD40 for the raft fraction.

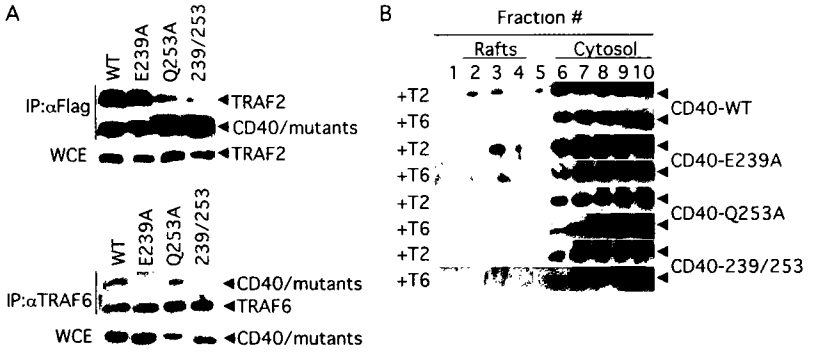

Figure 3.3.2. TRAF2 regulates the steady-state detergent solubility of CD40.

A. 293T cells were transfected with Flag-tagged CD40 constructs (0.5 μg of WT or mutants as indicated) and TRAF2 (0.5 μg, top) or Flag-tagged TRAF6 (0.5 μg, bottom). CD40 was immunoprecipitated with anti-Flag M2 antibodies and immunoprecipitates were probed for TRAF2 and CD40 as indicated. TRAF6 was immunoprecipitated with anti-TRAF6 antibodies and immunoprecipitates were probed for CD40 and TRAF6 as indicated.

B. As in Fig. 1D, but cells were transfected with CD40-WT or the indicated mutants (1.0 μg) and TRAF2 or TRAF6 (1.5 μg) and subjected to sucrose density gradient centrifugation and immunoblotting.

3.3.3.3 TRAF1 promotes sustained TRAF2-mediated signaling

It has previously been shown that stable expression of TRAF1 promotes sustained JNK activation by TNF-α (22). We found that, with the transfection of limiting amounts of TRAF2 (100 ng) and treatment with TNF-α (5 ng/ml) over the final 6 hours prior to cell lysis, TRAF1 co-expression increased the steady-state level of JNK activation, which correlates with the redistribution of TRAF2 from the insoluble fraction to the soluble fraction (Fig. 3.3.3A). Short-term treatment by TNF-α (<30 minutes) induced high levels of JNK activation in untransfected cells or cells transfected with TRAF2 and varying amounts of TRAF1 ((22) and data not shown). In an NF-κB-luciferase reporter assay,

levels of luciferase activity should reflect the integrated total of NF-κB activation over the time between transfection and cell lysis. We found that, although increasing levels of TRAF1 expression had little effect on the ability of limiting amounts of CD40 (50 ng) alone to activate NF-κB, in the presence of limiting amounts of TRAF2 (100 ng), TRAF1 overexpression could increase sustained NF-κB reporter activity (Fig. 3.3.3B). Thus, the ability of TRAF2 to mediate the sustained activation of downstream signal cascades appears to correlate with its solubility.

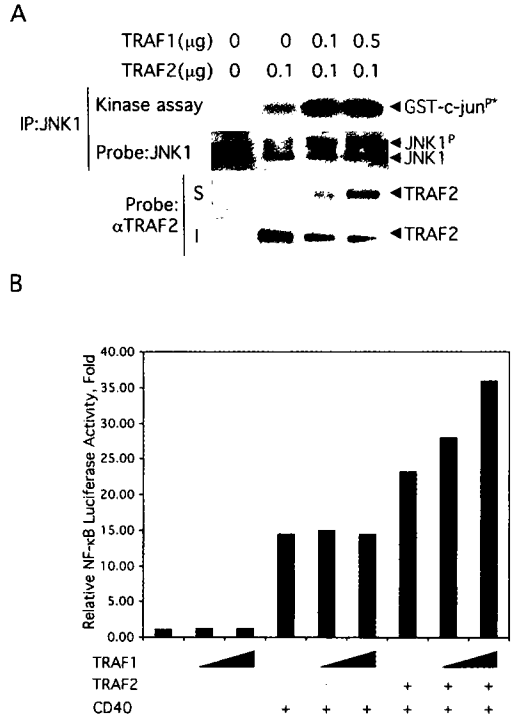

Figure 3.3.3. TRAF1 promotes sustained TRAF2-mediated JNK and NF-κB activation.
A. 293T cells were transfected with TRAF1 and TRAF2 as indicated. 6 h prior to harvesting, 10 ng/ml TNF-α was added to the culture medium. Cells were lysed in 0.75% Triton X-100 and subjected to an in vitro JNK kinase assay or fractionation as in Fig. 1.
B. 293T cells were transfected with varying amounts of TRAF1 (0, 0.1, or 0.5 μg, indicated by broadening line), CD40 (50 ng), and TRAF2 (0.1 μg) as indicated and subjected to an NF-κB reporter assay. Values are indicated as fold increase over background, and are normalized against an internal standard (β–galactosidase).

3.3.3.4 Raft translocation of TRAF2 is necessary and sufficient for JNK, but not NF-κB activation

Since the RING finger of TRAF2 is necessary for NF-κB (12) and JNK (13) activation, as well as for raft translocation (Fig. 3.3.2), we investigated whether raft translocation is sufficient for the ability of TRAF2 to activate these signals. Many Src-family kinases are acylated, leading to their accumulation in membrane rafts (29). Previously, fusion of the

3. Results

N-terminal myristoylation/palmitoylation signal sequence of Lck to Akt has been used to force membrane localization of Akt, leading to constitutive Akt activity (39). We therefore generated cDNA encoding the myristoylation-palmitoylation signal from Lck linked to the N-terminus of T2Δ87 (M/P-T2Δ87), thus directing redistribution of T2Δ87 to the membrane raft fraction in a RING finger-independent manner. When overexpressed with or without CD40 and/or TRAF1, M/P-T2Δ87 was localized primarily in the insoluble fraction, although co-expression of TRAF1 could redistribute a small amount of M/P-T2Δ87 to the soluble fraction (Fig. 3.3.4A). M/P-T2Δ87 could not substantially activate NF-κB (Fig. 3.3.4B). However, in a JNK assay, overexpressed M/P-T2Δ87 was able to rescue the deficiency in JNK activation by T2Δ87, although only about half as efficiently as wild-type TRAF2 (Fig. 3.3.4C). This bifurcation of the ability to activate JNK and NF-κB indicates that the RING finger's ability to mediate raft translocation is necessary and sufficient for JNK activation. However, raft translocation is not sufficient to activate NF-κB, suggesting that the RING finger has an essential function in addition to raft translocation that is required for NF-κB activation.

Since it has been suggested that the RING finger of TRAF2 is necessary to interact with the MAP3K MEKK1 (14), which may be, in turn, necessary for JNK activation (15), we tested the ability of M/P-T2Δ87 to induce the translocation of MEKK1 to detergent-resistant membranes. Co-expression of wild-type TRAF2, T2Δ87, and M/P-T2Δ87 with MEKK1 and lysis in 0.75% Triton X-100 showed that the ability of TRAF2 to mediate translocation of MEKK1 into the insoluble fraction correlated with its ability to activate JNK. Wild-type TRAF2 and M/P-T2Δ87 induced the translocation of MEKK1, but T2Δ87 did not (Fig. 3.3.4D, top). ASK1, another MAP3K known to interact with TRAF2 and activate JNK, interacts with the TRAF domain of TRAF2. Unlike MEKK1, the association of ASK1 and TRAF2 is not dependent on the RING finger of TRAF2 (40,41).

3. Results

Accordingly, ASK1 did not translocate to detergent-resistant membranes upon co-expression with TRAF2 (Fig. 3.3.4D, bottom). The apparent necessity of the RING finger for interaction with MEKK1 actually reflects a requirement for lipid raft translocation but not the physical presence of the RING finger. Thus, since MEKK1 was previously shown to interact with a TRAF2 fusion construct with the C-terminal TRAF domain replaced by FKBP (14), it appears that MEKK1 interacts with the Zn fingers of TRAF2, and this interaction is dependent upon the ability of TRAF2 to translocate to lipid rafts.

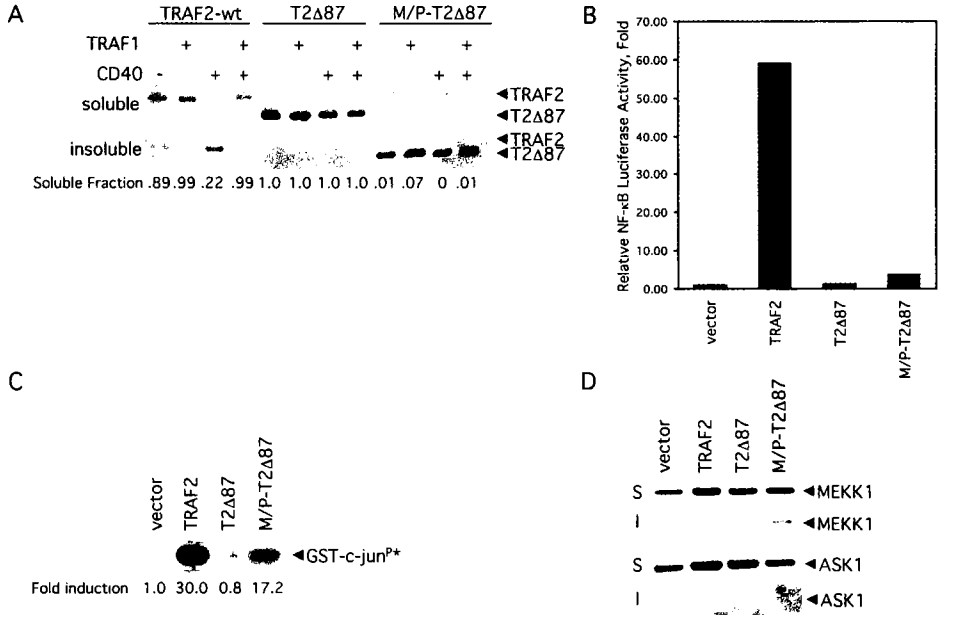

Figure 3.3.4. Forced raft localization of TRAF2 is sufficient to activate JNK, but not NF-κB.

A. 0.1 μg of wild-type TRAF2 (wt), T2Δ87, or T2Δ87 with an N-terminal myristoylation-palmitoylation signal peptide (M/P-T2Δ87) was cotransfected with or without TRAF1 (+, 0.5 μg), with or without CD40 (+, 0.2 μg) as indicated. Soluble and insoluble fractions were prepared as in Fig. 3.3.1. The relative proportion of soluble TRAF2 or its mutants was determined by densitometry and is indicated below the immunoblots (Note: this is a reflection of values relative to one another, but does not provide an absolute measure of solubility).

B. Cells were transfected with the indicated TRAF2 constructs (0.4 μg) and subjected to an NF-κB reporter assay as in Fig. 3.3.3B.

C. Cells were transfected as in (B) and subjected to an in vitro JNK assay as in Fig. 3.3.3A.

D. Cells were transfected with the indicated TRAF2 constructs (0.3 μg) and MEKK1 or ASK1 (0.3 μg) as indicated and soluble and insoluble fractions were prepared and immunoblotted.

3.3.3.5 Raft translocation is necessary for the interaction of TRAF2 with the actin-binding protein Filamin

Some TRAF proteins have been shown, upon activation, to localize to the actin cytoskeleton (35), potentially mediated through binding to Filamin (16). Furthermore, although nearly all of the known interactions TRAF2 has with other proteins appear to be mediated through the TRAF-C domain, it was proposed that TRAF2 interacts with Filamin through its RING finger domain (16). The presence of non-raft-associated, insoluble TRAF2 but not T2Δ87 in crude extracts (Fig. 3.3.2A) led us to investigate whether raft translocation was necessary for binding to cytoskeletal components. We cotransfected Filamin with TRAF2, T2Δ87, or M/P-T2Δ87 in the presence or absence of TRAF1. We then lysed the cells in 0.5% NP-40 to more efficiently dissociate M/P-T2Δ87 from membrane rafts, and separately lysed a small aliquot of cells in 0.75% Triton X-100 to show raft association. Immunoprecipitation of Filamin and western blotting revealed that, surprisingly, M/P-T2Δ87 could interact with Filamin as efficiently as wild-type TRAF2, while non-acylated T2Δ87 could not (Fig. 3.3.5, left side). Thus, it appears that, as in the case of MEKK1 (Fig. 3.3.4D), the RING finger is not essential for physical interaction of TRAF2 and Filamin, but raft translocation, ordinarily mediated by the RING finger, is necessary for Filamin binding. Furthermore, TRAF1 was able to compete for binding to Filamin with TRAF2, thus in the presence of overexpressed TRAF1, TRAF2 did not bind to Filamin (Fig. 3.3.5, right side). This suggests that sequestration into the cytoskeleton by Filamin may serve to inactivate TRAF2 after it has translocated into membrane rafts, thus downregulating sustained or repeated TRAF2 signaling by internalization and possible degradation (36). By preventing TRAF2 from interacting with Filamin, TRAF1 may therefore prolong and enhance TRAF2 mediated signaling.

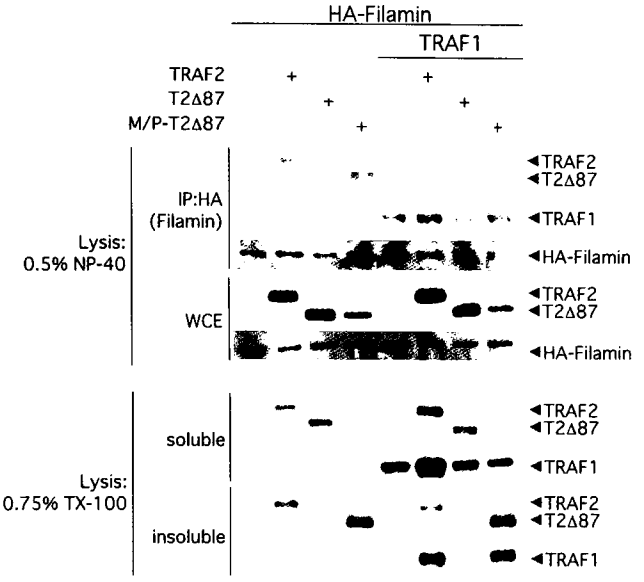

Figure 3.3.5. Interactions with TRAF2 and the actin-binding protein Filamin are dependent on raft translocation of TRAF2.
Cells were transfected as indicated with HA-tagged Filamin (a.a. 1644-2118; 0.3 μg), TRAF2 constructs (0.3 μg), and TRAF1 (0.3 μg) as indicated. Upon harvesting, 80% of the cells were lysed in 0.5% NP-40, subjected to immunoprecipitation of Filamin with antibodies against HA, and immunoblotted as indicated. The remaining cells were lysed in 0.75% Triton X-100 and soluble and insoluble fractions were immunoblotted as indicated.

3.3.3.6 TRAF1 recycles TRAF2 for signaling via serial receptor engagement in dendritic cells

TRAF1 is not ordinarily expressed at high levels in non-activated cells, but it is rapidly upregulated by NF-κB activation downstream of TNF family ligand stimulation (22). Therefore, we hypothesized that TRAF1 may play a physiological role in situations where multiple TNF family ligands that signal through TRAF2 engage cells over time. Dendritic cells (DCs) are known to respond to numerous TNF family members throughout their life cycle, including TNF-α, CD40L, and TRANCE. Furthermore, histological studies have shown that TRAF1 expression is consistently elevated in DCs, perhaps more so than in any other cell type examined (21). Using bone marrow-derived DCs from wild-type mice and mice with a targeted deletion of the TRAF domain of TRAF1 (TRAF1-/-; Y. Pewzner-Jung et al, manuscript in preparation), we found that CD40L or LPS maturation induces comparable levels of CD86 expression on CD11c+ cells (Fig. 3.3.6A), suggesting that TRAF1 is not required for DC differentiation or maturation.

In wild-type DCs, TRAF1 expression is relatively low in the immature stage, but is greatly upregulated by maturation in CD40L or LPS (Fig. 3.3.6B). Treatment of immature or LPS-matured DCs from wild-type or TRAF1-/- mice with CD40L induced similar levels of NF-κB activation as measured by IκB degradation (Fig. 3.3.6B, lanes 1-2, 5-6, 7-8, and 11-12). In DCs matured in CD40L, there was a marked deficiency in NF-κB activation by CD40L restimulation in TRAF1-/- DCs as compared to wild-type DCs (Fig. 3.3.6B, lanes 3-4 and 9-10). Consistent with this deficiency in NF-κB activation, there was substantially less pre-existing soluble TRAF2 in CD40L-matured TRAF1-/- DCs than in CD40L-matured wild-type DCs (Fig. 3.3.6B, lanes 1 and 3 vs. lanes 7 and 9). Similar levels of pre-existing soluble TRAF2 were observed in immature and LPS-matured TRAF1-/- DCs and wild-type DCs prior to CD40L restimulation (Fig. 3.3.6B, lanes 5 and 11). CD40L restimulation in all cases resulted in similar short-term (20 minutes) reductions of soluble TRAF2, consistent with previous observations (34,36).

Once mature, DCs quickly undergo apoptosis in the absence of survival stimuli provided by activated T cells including TNF family ligands such as TNF-α, CD40L, and TRANCE (42). We found that TRAF1-/- DCs matured in CD40L display severely impaired survival in response to TNF-α stimulation and partially impaired survival in response to CD40L, with a negligible defect in TRANCE-mediated survival. TRAF1-/- DCs matured in LPS, which does not signal through TRAF2, had comparable survival responses to wild-type DCs under stimulation by TNF-α, CD40L, and TRANCE (Fig. 3.3.6C). Thus, TRAF1 appears to be dispensable for the first TRAF2-dependent signal (CD40L-induced maturation), but its upregulation by the maturation signal maintains high levels of soluble TRAF2, enabling subsequent TRAF2-dependent signals (TNF-α or CD40L-induced survival) to occur. While TRANCE can activate NF-κB through TRAF2 in vitro, (43), it

predominantly signals through TRAF6 (43,44), which may explain the negligible difference in survival observed in CD40L-matured wild-type and TRAF1-/- DCs.

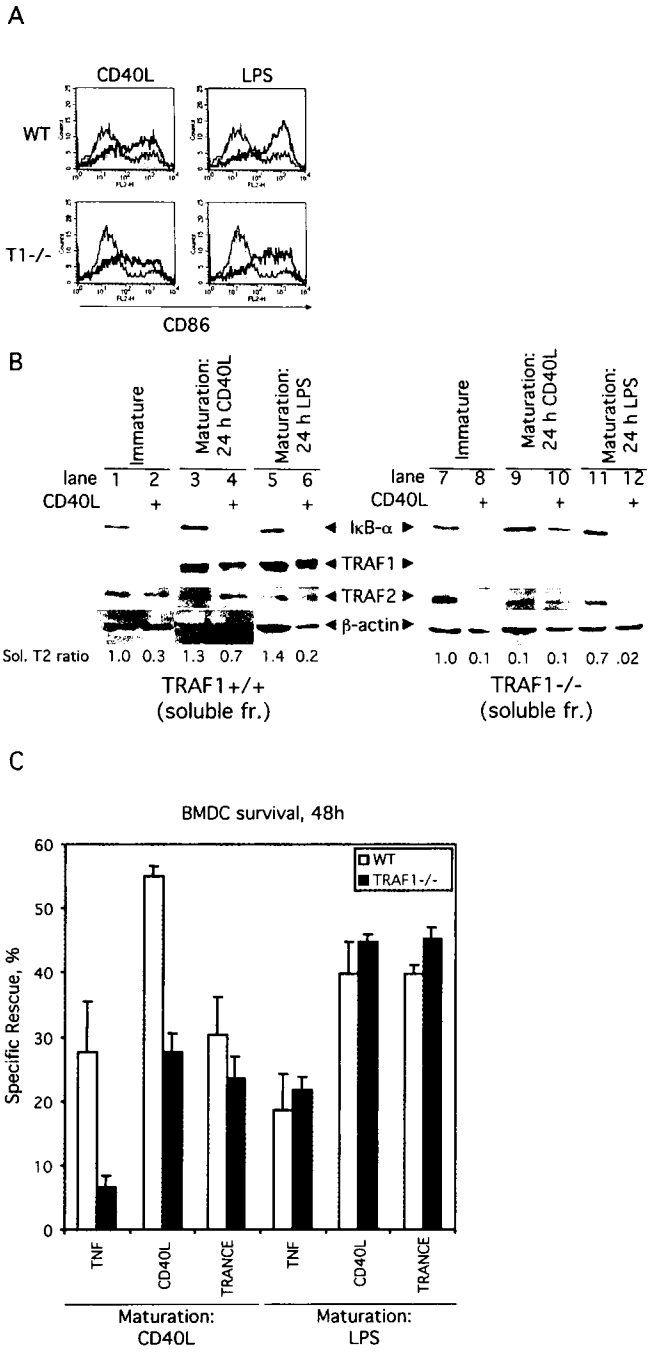

Figure 3.3.6. TRAF1-/- dendritic cells have deficient secondary responses to TRAF2-dependent signals.

A. Wild-type and TRAF1-/- (T1-/-) DCs were matured by overnight culture in CD40L (1:200) or LPS (100 ng/ml) and CD86 expression was monitored by FACS analysis. Immature cells are shown as shaded areas on the histogram and matured cells are shown as broad dark lines.

B. DCs were matured as in (A) (immature, lanes 1-2 and 7-8; CD40L matured, lanes 3-4 and 9-10; LPS matured, lanes 5-6 and 11-12), starved in medium containing 0.5% serum for 2 h, and restimulated with CD40L (1:200) as indicated for 20 minutes (even numbered lanes; odd numbered lanes were not restimulated). Cells were lysed and the soluble fractions were immunoblotted as indicated. Normalized ratios of soluble TRAF2 relative to the level of soluble TRAF2 in unstimulated immature cells were determined by densitometry and are indicated below the β–actin blots.

C. DCs prepared as in (A) were incubated in normal medium, or medium containing TNF-α (10 ng/ml), CD40L (1:200), or TRANCE (1 μg/ml) for 48 h as indicated. Survival was determined by PI exclusion FACS. Specific rescue is represented as [% of surviving cells (stimulated)-% of surviving cells (unstimulated)]/[(100 % of surviving cells (unstimulated)].

3.3.4 Discussion

The data presented in this report reconcile widely varying observations about TRAF2 signaling to provide a potential mechanism of TRAF2's action. First, the mechanism of TRAF2 signaling centers on its ability to translocate to lipid rafts. The N-terminal RING finger domain of TRAF2 is necessary both for TRAF2's ability to activate signaling cascades and to translocate to lipid rafts. Enforced raft translocation of TRAF2 lacking its RING finger (T2Δ87) rescues T2Δ87's ability to activate JNK, but not NF-κB. Second, under conditions of sustained stimulation through TRAF2-dependent receptors, TRAF2 becomes mostly insoluble and total cellular TRAF2 decreases. Third, TRAF1 is known to be upregulated by activation of NF-κB and AP-1, often in TRAF2-dependent signaling. TRAF1 can displace TRAF2 from rafts and promote sustained TRAF2-mediated signaling in response to a single stimulus or multiple stimuli over time. Thus, a dynamic model emerges of translocation of the receptor complex mediated by TRAF2, which is likely to be the trigger for signal activation in response to a stimulus. The physiological role for TRAF1 is to "reset" the system by dissociating TRAF2 from insoluble complexes and enabling subsequent stimuli to transduce signals through TRAF2.

Prior reports have suggested that a receptor, such as CD40, translocates to rafts upon ligand engagement and this leads to binding of TRAF proteins (33,34). In agreement with the potential requirement for zinc binding ability for TRAF2 to translocate to rafts (33), we have found that TRAF2 requires its N-terminal RING finger domain to translocate to the insoluble fraction (Fig. 3.3.1). Nevertheless, a mutant of TRAF2 lacking the RING finger, T2Δ87, is fully capable of binding to CD40 and acting as a dominant-negative for TRAF signaling (12,13). Furthermore, we have found that T2Δ87 overexpression actually reduces the amount of raft-associated CD40 (Fig. 3.3.1D), so the TRAF domain of

TRAF2 can clearly interact with CD40 in the soluble fraction. Although short-term stimulation of a CD40 mutant that cannot bind to TRAF2 resulted in its apparent translocation to lipid rafts (33), our data show that steady-state residence in lipid rafts by CD40 is dependent on its ability to bind to TRAF2 (Fig. 3.3.2). This suggests that the ability to activate downstream signals upon binding to a receptor is tied to the ability of TRAF2 to translocate the receptor complex to lipid rafts. It is thought that the N-terminal domain of TRAF2 somehow activates kinases that lead to NF-kB and MAPK signaling. However, with the exception of MEKK1 (14), all of the kinases known to interact with TRAF2 interact with the C-terminal receptor-binding domain (11). Our data suggest that MEKK1 does not physically associate with the RING finger of TRAF2 since enforced raft translocation of M/P-T2Δ87 induces JNK activation and translocation of MEKK1 (Fig. 3.3.4). This apparent discrepancy may be explained by the observation that wild-type TRAF2 did not interact with MEKK1 until TNF-α stimulation (14). TNF-α stimulation likely induced the translocation of TRAF2 to lipid rafts, where it may have had better access to MEKK1 or to intermediary proteins linking TRAF2 to MEKK1. Taken together with observations by the same authors that a construct with replacement of the TRAF-C domain of TRAF2 with an inducible multimerization signal could interact with and activate MEKK1, our data suggest that TRAF2 interacts with MEKK1 via its Zn fingers, but can only do so upon raft translocation. Regardless, the importance of MEKK1 in TNF-α mediated JNK activation is disputable, as genetic deletion targeting different regions of MEKK1 has shown opposite results with regard to its role in TNF-mediated JNK activation (15,45). Thus, all of the known protein-protein interactions mediated by TRAF2 appear to be via the C-terminal receptor-binding TRAF domain or Zn fingers, but not the RING finger. This favors a model wherein receptor engagement by a ligand recruits TRAF2 and cytoplasmic factors including MAP3Ks, first in the soluble fraction. Subsequently, the RING finger mediates translocation of the receptor

complex to lipid rafts, which may simultaneously activate and release the kinases in a concerted mechanism, the details of which are still unclear.

While the ability of TRAF2 to translocate to rafts appears to be tied to its ability to activate signal cascades, we have found that in primary cells under circumstances of sustained receptor engagement (~16h) in the absence of TRAF1, the steady-state level of TRAF2 in the soluble fraction decreases and TRAF2-dependent receptors become refractory to further stimulation (Fig. 3.3.6). Others have demonstrated that, under short-term periods of signaling (<1 h), TRAF2 becomes insoluble and degrades in response to CD40 (36) and CD30 engagement (46). Stimulation of TNFR2 has resulted in a depletion of soluble TRAF2, potentiating TNFR1-mediated cell death (47). Thus, while translocation into lipid rafts is essential for TRAF2 to activate signaling processes, once translocated, it appears that a given complex of TRAF2 is inactivated, thus supporting the idea that translocation and kinase activation are concerted, instantaneous processes. TRAF1, however, is able to displace TRAF2 away from the insoluble fraction back into the cytosolic fraction. The mechanism of how this happens is unclear, but it is possible that TRAF1 hetero-oligomerizes with TRAF2, displacing it from rafts, and/or it competes with TRAF2 for binding sites on the receptor. In support of this, in transient transfection assays with prolonged TNF-α stimulation or overexpression of CD40 and TRAF2, which simulates prolonged stimulation, TRAF1 not only increases the solubility of TRAF2, but it also increases JNK and NF-κB activation (Fig. 3.3.3).

The presence of considerable amounts of TRAF2 in insoluble complexes that are not raft-associated (Fig. 3.3.1D) indicates that there may be another subcellular location of insoluble TRAF2 complexes. TRAF2 has been shown to interact with Caveolin-1 (48) and the actin-binding protein Filamin (Fig. 3.3.5 and (16)) and has been suggested to

accumulate in perinuclear (49) or cytoskeletal compartments after signaling (35). This may ultimately lead to degradation of TRAF2 (36,46). Given TRAF2's association with lipid rafts and caveolae, a reasonable model suggests that, upon receptor enagement, TRAF2 first translocates to membrane rafts, where it can activate kinase cascades. It then may be internalized via caveolae, whereupon it is trafficked to cytoskeletal compartments and/or degraded. Since we found that only TRAF2 that is capable of raft translocation can bind to Filamin and that TRAF1 can disrupt the interaction of TRAF2 and Filamin (Fig. 3.3.5), it appears that cytoskeletal trafficking of TRAF2 is a consequence of raft translocation. Furthermore, it is likely that TRAF1 influences the intracellular trafficking of activated TRAF2 by solubilizing it not only from lipid rafts, but also from cytoskeletal structures. This may, in turn, prevent or reduce the degradation of activated TRAF2 complexes, thereby increasing the available levels of soluble TRAF2 for subsequent signaling by engagement of multiple TNF receptor family proteins over time (Fig. 3.3.6).

The implications of TRAF1's ability to regulate the solubility of TRAF2 leading to positive signaling outcomes are supported by several studies. Previously, we have found that transgenic overexpression of TRAF1 in T cells leads to prolonged survival of activated CD8+ T cells that may otherwise be subject to TNF-α induced apoptosis (26). Others have shown that TRAF1, in concert with TRAF2, c-IAP1, and c-IAP2, contributes to the suppression of TNF-α induced caspase-8 activation and subsequent cytoprotection (25). In stable transfectants overexpressing full-length TRAF1, but not in transfectants expressing an N-terminal truncation of TRAF1, NF-κB and JNK activation was sustained (22). It has recently been observed that TRAF1 is a target of caspases during apoptosis, which results in a decrease in its cytoprotective properties and a concomitant decrease in sustained antiapoptotic signaling by TRAF2 (27,28). During the preparation of this manuscript, it was reported that TRAF1 deficiency results in "positive" modulation of

TNF-α signaling in T lymphocytes (50). The increased TNF-induced skin necrosis observed in those TRAF1-/- mice is reminiscent of the "feed-forward" TNF-induced TNF overproduction in TRAF2-/- cells (51), and thus may point to a "negative" role of TRAF1 in one aspect of TNF signaling but that "negative" role is in terms of a "positive" role in TRAF2 regulation.

In this study, in TRAF1-/- DCs, CD40 signaling in the short term (up to 20 minutes) on immature cells is intact and comparable to that of wild-type cells, enabling NF-κB activation and functional maturation, as well as depletion of TRAF2 from the soluble fraction (Fig. 3.3.6). Since the signaling and functional outcomes in immature wild-type and TRAF1-/- DCs are indistinguishable, and since immature wild-type DCs express low levels of TRAF1, it is likely that the initial stimulation is TRAF1-independent. However, after 24 hours of stimulation by CD40L and a brief starvation period followed by restimulation, there is a marked difference between wild-type and TRAF1-/- cells. Despite similar CD40L-dependent induction of CD40 expression in wild-type and TRAF1-/- DCs (data not shown), CD40L can re-activate NF-κB in wild-type cells, but NF-κB activation in TRAF1-/- cells is greatly attenuated. This correlates directly with the level of pre-existing TRAF2 in the soluble fraction. These signaling events correlate strongly with substantially reduced DC survival mediated by the TRAF2-dependent factors TNF-α and CD40L in TRAF1-/- DCs matured in CD40L as compared to those matured in LPS, a TRAF2-independent factor. In TRAF1-/- DCs matured in CD40L, TNF-α provided virtually no survival effect, while CD40L was able to promote survival, albeit to a lesser extent than in wild-type DCs, and TRANCE promoted similar survival levels in wild-type and TRAF1-/- DCs. This is consistent with the fact that TRAF2 is the predominant TRAF protein that mediates survival signaling downstream of TNFR1,

while CD40 and TRANCE-R can signal through TRAF6 as well as through TRAF2 (7,43).

The role of TNF family ligand signaling in dendritic cell homeostasis is well documented. Dendritic cells residing in peripheral tissues become activated by the presence of inflammatory mediators, including IL-1, LPS, and TNF-α, or the presence of activated T cells, which may express TNF-α, CD40L, or TRANCE (52). Recently, it has been shown that transgenic overexpression of CD40L in epidermis leads to chronic skin inflammation and autoimmunity, mediated in large part by the excessive activation and maturation of Langerhans cells, which are epidermal DCs (53). TNF-α deficient mice have attenuated antiviral responses, in part due to deficiencies in maturation and upregulation of costimulatory factors due to a lack of auctocrine TNF-α production (54). There are two receptors for TNF-α: TNFR1 and TNFR2. TNFR1 can activate both the apoptotic caspase cascade and, via the interaction of TRADD with TRAF2, it can activate pro-survival signaling, while TNFR2 lacks a death domain and cannot activate caspases (1). The spontaneous apoptosis of mature DCs appears to be at least partially due to autocrine activation of TNFR1, as TNFR1-/- DCs in culture are highly resistant to spontaneous apoptosis (55). Given that TRAF1 is highly expressed in DCs (21) and it regulates the availability of TRAF2 for anti-apoptotic signaling, it is likely that the balance between caspase activation and pro-survival signals is regulated to some extent in DCs by TRAF1. This hypothesis is consistent with our finding in TRAF1-/- DCs that maturation of DCs by CD40L tilts the balance of TNF-α signaling from survival to apoptosis due to a depletion of soluble TRAF2.

What remains unclear is the mechanism of action of the RING finger that enables translocation of TRAF2 and the associated receptor complex. One potential mechanism

has emerged recently in studies examining a non-classical polyubiquitination mechanism mediated by the RING finger of TRAF6 (18,19). In these elegant *in vitro* studies, a protein complex that can activate IKK, leading to NF-κB activation, was biochemically purified. It was found that TRAF6 associates with the ubiquitin-conjugating enzymes Ubc13 and Uev1A, which results in polyubiquitination on K63 of ubiqutin (as opposed to proteasome-targeting K48 polyubiquitination), which is attached to TRAF6 itself, in a process dependent on the RING finger and oligomerization of TRAF6. This ubiquitination step, in turn, activates a complex consisting of the TAK1 (a MAP3K), TAB1, and TAB2, which can subsequently activate the IKK complex upstream of NF-κB as well as MKK6, which is upstream of JNK. Interestingly, TAB2 has been shown to translocate from membrane-associated fractions to the cytoplasm upon activation of the TAK1/TAB1/TAB2 complex (56). While it is unclear whether TRAF2 undergoes the same process as TRAF6, it is tempting to speculate that the formation of K63-linked polyubiquitinated complexes is tied to the translocation of TRAF2 to lipid rafts. This is a particularly intriguing hypothesis in light of the fact that various forms of ubiquitination have been tied to trafficking between cellular compartments (57). It is possible, then, that ubiquitinated TRAF2 can then interact with raft-associated proteins, possibly including the TAK1/TAB1/TAB2 complex or an analog of this complex with similar function, displacing them into the cytoplasm where they can phosphorylate substrates leading to NF-κB and JNK activation. Clearly, the role of ubiquitination in RING-dependent raft translocation and kinase activation merits further investigation.

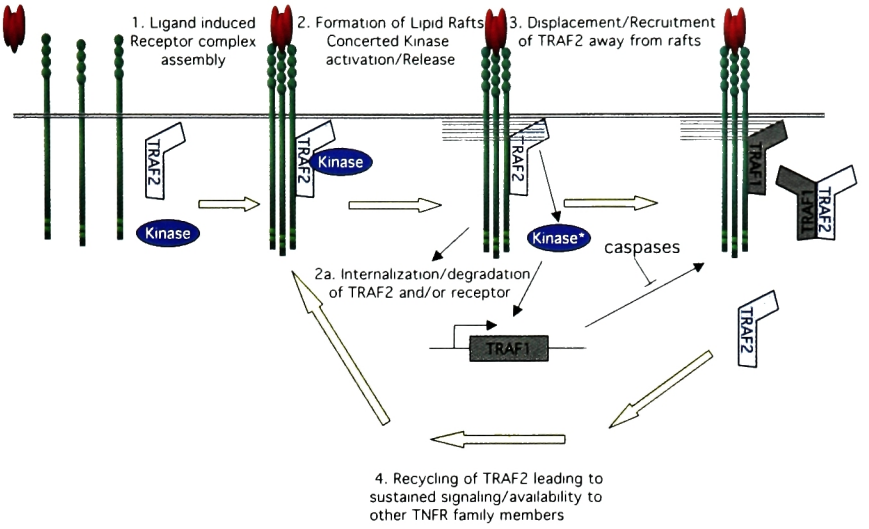

Figure 3.3.7. Proposed model of the mechanism of TRAF2 signaling and its regulation by TRAF1. Upon ligand engagment, a TNFR family protein recruits TRAF2 and various kinases via the C-terminal TRAF domain of TRAF2. The receptor complex assembles in the soluble fraction. Upon complex assembly, the N-terminal RING finger of TRAF2 mediates translocation of the receptor complex into detergent-resistant lipid rafts. This translocation event simultaneously activates and releases the kinases, while isolating TRAF2 in an insoluble complex that may be internalized and/or degraded. The activated kinases ultimately activate transcription factors such as NF-κB and AP-1, which upregulate the expression of TRAF1. TRAF1 then releases TRAF2 from insoluble complexes by hetero-oligomerization with TRAF2 or competing for receptor binding sites. This results in an increase of soluble TRAF2 that is available for subsequent signaling events mediated by other TRAF2-dependent TNFR family proteins.

Conclusion

While previous reports have concluded that TRAF signaling takes place within the rafts, our results suggest a slightly altered model (Fig. 3.3.7). Since the receptor, TRAF2, and downstream signaling components can interact in soluble lysates, and especially since T2Δ87 cannot translocate to rafts but can still interact with both the receptor and downstream components, it appears that the act of translocation is coupled to the activating event. Thus, upon receptor engagement, the receptor, TRAF2, and downstream signaling molecules assemble in the soluble fraction. Subsequently, TRAF2 translocates with the receptor to lipid rafts, simultaneously releasing and activating the downstream kinase. It is unclear what the exact mechanism of activation of the downstream kinase is, but it is possible that K63-linked polyubiquitination and/or raft-associated kinases such as

c-Src either directly or indirectly activate these kinases. TRAF2 is now sequestered in the rafts, unable to stimulate additional molecules of downstream kinases. As TRAF2 has been shown to interact with caveolin-1 (48) and the actin-binding protein Filamin (16), which is a raft translocation-dependent process (Fig. 3.3.6), it is possible that TRAF2 and/or other components of the receptor complexes are internalized and degraded. As a result of this initial signaling, NF-κB and AP-1 are activated and TRAF1 gene expression is turned on. TRAF1 protein levels rise, and now TRAF1 can relocate TRAF2 to the soluble cytoplasmic fraction and potentially protect it from degradation, where it can re-assemble receptor signaling complexes and continue the cycle. This allows for what appears to be "sustained" signaling or restimulation through the same receptor, or stimulation through multiple TNFR family proteins that bind to TRAF2 over time.

3.3.5 Experimental Procedures

3.3.5.1 Reagents

Recombinant mouse TNF-α, IL-4, and GM-CSF were from R & D Systems, LPS (*E. Coli* 055:B5) was from Sigma, soluble hCD8-TRANCE (TRANCE) was purified from insect cells as described (58), and soluble mCD8-CD40L (CD40L) was generated in insect cells and supernatant was used at a 1:200 dilution as described (59).

Antibodies (Abs) specific for IκB-α were from New England Biolabs; TRAF2 (N-19 and C-20), TRAF1 (N-19), Lyn (44), JNK1 (N-19), MEKK1 (C-22), and ASK1 (H-300) were from Santa Cruz Biotechnology; β-actin (Ab-1) was from Calbiochem; HA (12CA5) from Boehringer Mannheim; the Flag epitope (M2) was from Sigma; and TRAF6 was generously provided by Dr. Sankar Ghosh (Yale University).

3.3.5.2 Constructs

Mouse CD40 was cloned by RT-PCR from whole spleen mRNA and inserted into the pFLAG-CMV1 cloning vector (Sigma). Mouse TRAF2 and T2Δ87 in pcDNA3.1 have been previously described (60). To make M/P-T2Δ87, complementary oligonucleotides encoding the N-terminal 12 residues of Lck (MGCVCSSNPEDD) with appropriate flanking restriction sites were annealed, digested, and ligated into the expression vector encoding T2Δ87 at the 5' end of T2Δ87. To make TRAF2-GFP, cDNA encoding EGFP from the EGFP-N3 vector (Clontech) was ligated into pcDNA3.1. TRAF2 was amplified by PCR using a C-terminal primer that fused sequence encoding a flexible linker $(GGGS)_2$ to the C-terminus and eliminated the stop codon, and this product was ligated in frame upstream of EGFP. ASK1 in pcDNA3 was generously provided by Dr. James Woodgett (Ontario Cancer Institute, University of Toronto) and HA-tagged Filamin (a.a. 1644-2118) was generously provided by Dr. Ulrich Siebenlist (NIAID, NIH). MEKK1 in pCFL and Flag-tagged mouse TRAF1, TRAF5, and HA- and Flag-tagged TRAF6 have been previously described (43). Site-directed mutagenesis of CD40 was performed on the indicated residues by the Ex-Site method (Stratagene). All constructs were confirmed by sequencing.

3.3.5.3 Dendritic cells

Dendritic cells were generated from bone marrow precursors via a modification of existing protocols (61,62). Bone marrow precursors were plated in 24-well tissue culture plates at a density of 10^6/ml, 1 ml/well, in RPMI-1640 medium containing 5% heat-inactivated FBS, 10 mM HEPES (pH 7.0), β-mercaptoethanol, penicillin, streptomycin, and rmGM-CSF (25 ng/ml) and rmIL-4 (5 ng/ml) for 7 days in a 37°, 5% CO_2 incubator, with replacement of 800 µl of medium on days 2 and 4 and the addition of 500 µl of

medium on day 6. On day 7, cells were left alone or stimulated overnight with CD40L (1:200) or LPS (100 ng/ml). On day 8, cells were removed for FACS analysis, transferred to a new 24 well plate for restimulation (see below), or transferred into 96-well plates (10^5 cells/well in 200 µl of medium without GM-CSF or IL-4, containing 5% serum and the indicated stimuli [TNF-α, 10 ng/ml; CD40L, 1:200; or TRANCE, 1 µg/ml] in triplicate) for survival assays. Maturation was assayed by FACS analysis of CD86, CD80, and I-A^b expression, gated on CD11c+ cells on a FACSCalibur (Becton-Dickinson). Survival was assayed by FACS analysis of propidium iodide exclusion after 48 h as described previously (58).

3.3.5.4 Cell Stimulation, Transfection, and Analysis

In vitro differentiated dendritic cells were extensively washed to remove exogenous growth factors, cultured in medium with low serum (0.5% FBS, 2-4 h), then stimulated by adding TNF-α, TRANCE, or CD40L as indicated. After stimulation, cells were washed with ice-cold PBS, lysed, and subject to SDS-PAGE and western blotting. In order to control for equal loading of each timepoint, the protein concentration of each sample was determined and samples were normalized for total protein content prior to further processing.

293T cells were transfected in 6-well plates by calcium phosphate precipitation as described (43). The amount of transfected DNA was held constant to 1 µg by addition of empty vector DNA where necessary. Cells were processed for analysis 24-30 h after transfection. For NF-κB reporter assays, cells were transfected with the indicated amounts of expression constructs and mutants along with 75 ng of an NF-κB-luciferase reporter plasmid and 25 ng of a β-galactosidase plasmid to control for transfection efficiency. Transfection amounts were kept constant at 1 µg by addition of empty

pFLAG-CMV1 vector. Luciferase and β-galactosidase activity was measured as described (43). In vitro JNK assays were performed on cell lysates as described (59). All transfection experiments were repeated at least three times and representative results are shown.

Where indicated, cells were harvested in 1 ml ice-cold PBS, then lysed in a solution of 150 mM NaCl, 20 mM HEPES (pH 7.0), 10% glycerol, and 0.75% Triton X-100 with protease and phosphatase inhibitors (1 mM PMSF, 1 μg/ml leupeptin, 0.1 U/ml aprotinin, 10 mM NaF, and 5 mM Na_3VO_4). For lysis in NP-40, cells were lysed in HNE buffer (20 mM HEPES, pH 7.0; 150 mM NaCl, 5 mM EDTA) with 0.5% NP-40 and protease inhibitors. Cell lysates were incubated on ice for 20-30 minutes, vortexed extensively, and centrifuged in a microfuge at maximum speed at 4° for 10 minutes. Soluble fractions were removed and subjected to SDS-PAGE or immunoprecipitation. Insoluble fractions were washed extensively in lysis buffer and solubilized via the addition of SDS gel-loading buffer, vortexing, and boiling for 10 minutes. Immunoprecipitation was carried out by the addition of an antibody as indicated to the soluble fraction, rotation at 4° for 2-3 h, followed by the addition of 15 μl protein G-sepharose equilibrated in lysis buffer and rotation at 4° for 1 h. The beads were washed 3 x in lysis buffer containing detergent and once in lysis buffer without detergent. SDS gel-loading buffer was added and samples were boiled and subjected to SDS-PAGE and western blotting.

3.3.5.5 Sucrose density gradient centrifugation

293T cells were transfected as indicated in 10 cm tissue culture plates with 4 μg total DNA. Six hours prior to harvesting, CD40L (1:200) was added to the culture medium. Cells were harvested in ice-cold PBS and lysed in 1 ml of HNE containing 0.25% Triton X-100, incubated on ice for 30 minutes, and vortexed extensively. One ml of an 80%

sucrose solution in HNE was mixed with the lysate, and this was overlaid with 2 ml of a 30% sucrose solution in HNE, followed by 1 ml of a 5% sucrose solution in HNE. The samples were centrifuged in a Beckman SW55Ti rotor at 200,000 x g overnight at 4° as described (35). 0.5 ml fractions were taken from the top of the gradient to which 250 µl of 2x SDS gel-loading buffer was added. 30 µl of each fraction was subjected to SDS-PAGE and western blotting.

3.3.6 References

1. Locksley, R. M., Killeen, N., and Lenardo, M. J. (2001) The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* **104**(4), 487-501.
2. Wajant, H., Henkler, F., and Scheurich, P. (2001) The TNF-receptor-associated factor family: scaffold molecules for cytokine receptors, kinases and their regulators. *Cell Signal* **13**(6), 389-400.
3. Grech, A., Quinn, R., Srinivasan, D., Badoux, X., and Brink, R. (2000) Complete structural characterisation of the mammalian and Drosophila TRAF genes: implications for TRAF evolution and the role of RING finger splice variants. *Mol Immunol* **37**(12-13), 721-34.
4. Rothe, M., Wong, S. C., Henzel, W. J., and Goeddel, D. V. (1994) A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. *Cell* **78**(4), 681-92.
5. Park, Y. C., Burkitt, V., Villa, A. R., Tong, L., and Wu, H. (1999) Structural basis for self-association and receptor recognition of human TRAF2. *Nature* **398**(6727), 533-8.
6. Ye, H., Park, Y. C., Kreishman, M., Kieff, E., and Wu, H. (1999) The structural basis for the recognition of diverse receptor sequences by TRAF2. *Mol Cell* **4**(3), 321-30.
7. Pullen, S. S., Dang, T. T., Crute, J. J., and Kehry, M. R. (1999) CD40 signaling through tumor necrosis factor receptor-associated factors (TRAFs). Binding site specificity and activation of downstream pathways by distinct TRAFs. *J Biol Chem* **274**(20), 14246-54.
8. Chan, F. K., Chun, H. J., Zheng, L., Siegel, R. M., Bui, K. L., and Lenardo, M. J. (2000) A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. *Science* **288**(5475), 2351-4.

9. McWhirter, S. M., Pullen, S. S., Holton, J. M., Crute, J. J., Kehry, M. R., and Alber, T. (1999) Crystallographic analysis of CD40 recognition and signaling by human TRAF2. *Proc Natl Acad Sci U S A* **96**(15), 8408-13.
10. Arch, R. H., Gedrich, R. W., and Thompson, C. B. (1998) Tumor necrosis factor receptor-associated factors (TRAFs)--a family of adapter proteins that regulates life and death. *Genes Dev* **12**(18), 2821-30.
11. Wajant, H., and Scheurich, P. (2001) Tumor necrosis factor receptor-associated factor (TRAF) 2 and its role in TNF signaling. *Int J Biochem Cell Biol* **33**(1), 19-32.
12. Takeuchi, M., Rothe, M., and Goeddel, D. V. (1996) Anatomy of TRAF2. Distinct domains for nuclear factor-kappaB activation and association with tumor necrosis factor signaling proteins. *J Biol Chem* **271**(33), 19935-42.
13. Song, H. Y., Regnier, C. H., Kirschning, C. J., Goeddel, D. V., and Rothe, M. (1997) Tumor necrosis factor (TNF)-mediated kinase cascades: bifurcation of nuclear factor-kappaB and c-jun N-terminal kinase (JNK/SAPK) pathways at TNF receptor-associated factor 2. *Proc Natl Acad Sci U S A* **94**(18), 9792-6.
14. Baud, V., Liu, Z. G., Bennett, B., Suzuki, N., Xia, Y., and Karin, M. (1999) Signaling by proinflammatory cytokines: oligomerization of TRAF2 and TRAF6 is sufficient for JNK and IKK activation and target gene induction via an amino-terminal effector domain. *Genes Dev* **13**(10), 1297-308.
15. Xia, Y., Makris, C., Su, B., Li, E., Yang, J., Nemerow, G. R., and Karin, M. (2000) MEK kinase 1 is critically required for c-Jun N-terminal kinase activation by proinflammatory stimuli and growth factor-induced cell migration. *Proc Natl Acad Sci U S A* **97**(10), 5243-8.
16. Leonardi, A., Ellinger-Ziegelbauer, H., Franzoso, G., Brown, K., and Siebenlist, U. (2000) Physical and functional interaction of filamin (actin-binding protein-280) and tumor necrosis factor receptor-associated factor 2. *J Biol Chem* **275**(1), 271-8.
17. Brink, R., and Lodish, H. F. (1998) Tumor necrosis factor receptor (TNFR)-associated factor 2A (TRAF2A), a TRAF2 splice variant with an extended RING finger domain that inhibits TNFR2-mediated NF-kappaB activation. *J Biol Chem* **273**(7), 4129-34.
18. Deng, L., Wang, C., Spencer, E., Yang, L., Braun, A., You, J., Slaughter, C., Pickart, C., and Chen, Z. J. (2000) Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell* **103**(2), 351-61.
19. Wang, C., Deng, L., Hong, M., Akkaraju, G. R., Inoue, J., and Chen, Z. J. (2001) TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature* **412**(6844), 346-51.

20. Takayanagi, H., Ogasawara, K., Hida, S., Chiba, T., Murata, S., Sato, K., Takaoka, A., Yokochi, T., Oda, H., Tanaka, K., Nakamura, K., and Taniguchi, T. (2000) T-cell-mediated regulation of osteoclastogenesis by signalling cross- talk between RANKL and IFN-gamma. *Nature* **408**(6812), 600-5.
21. Zapata, J. M., Krajewska, M., Krajewski, S., Kitada, S., Welsh, K., Monks, A., McCloskey, N., Gordon, J., Kipps, T. J., Gascoyne, R. D., Shabaik, A., and Reed, J. C. (2000) TNFR-associated factor family protein expression in normal tissues and lymphoid malignancies. *J Immunol* **165**(9), 5084-96.
22. Schwenzer, R., Siemienski, K., Liptay, S., Schubert, G., Peters, N., Scheurich, P., Schmid, R. M., and Wajant, H. (1999) The human tumor necrosis factor (TNF) receptor-associated factor 1 gene (TRAF1) is up-regulated by cytokines of the TNF ligand family and modulates TNF-induced activation of NF-kappaB and c-Jun N-terminal kinase. *J Biol Chem* **274**(27), 19368-74.
23. Dunn, I. F., Sannikova, T. Y., Geha, R. S., and Tsitsikov, E. N. (2000) Identification and characterization of two CD40-inducible enhancers in the mouse TRAF1 gene locus. *Mol Immunol* **37**(16), 961-73.
24. Carpentier, I., and Beyaert, R. (1999) TRAF1 is a TNF inducible regulator of NF-kappaB activation. *FEBS Lett* **460**(2), 246-50.
25. Wang, C. Y., Mayo, M. W., Korneluk, R. G., Goeddel, D. V., and Baldwin, A. S., Jr. (1998) NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c- IAP2 to suppress caspase-8 activation. *Science* **281**(5383), 1680-3.
26. Speiser, D. E., Lee, S. Y., Wong, B., Arron, J., Santana, A., Kong, Y. Y., Ohashi, P. S., and Choi, Y. (1997) A regulatory role for TRAF1 in antigen-induced apoptosis of T cells. *J Exp Med* **185**(10), 1777-83.
27. Leo, E., Deveraux, Q. L., Buchholtz, C., Welsh, K., Matsuzawa, S., Stennicke, H. R., Salvesen, G. S., and Reed, J. C. (2001) Traf1 is a substrate of caspases activated during tumor necrosis factor receptor-alpha -induced apoptosis. *J Biol Chem* **276**(11), 8087-93.
28. Jang, H. D., Chung, Y. M., Baik, J. H., Choi, Y. G., Park, I. S., Jung, Y. K., and Lee, S. Y. (2001) Caspase-cleaved TRAF1 negatively regulates the antiapoptotic signals of TRAF2 during TNF-induced cell death. *Biochem Biophys Res Commun* **281**(2), 499-505.
29. Brown, D. A., and London, E. (1998) Functions of lipid rafts in biological membranes. *Annu Rev Cell Dev Biol* **14**, 111-36
30. Simons, K., and Ikonen, E. (1997) Functional rafts in cell membranes. *Nature* **387**(6633), 569-72.
31. Cherukuri, A., Dykstra, M., and Pierce, S. K. (2001) Floating the raft hypothesis: lipid rafts play a role in immune cell activation. *Immunity* **14**(6), 657-60.

32. Galbiati, F., Razani, B., and Lisanti, M. P. (2001) Emerging themes in lipid rafts and caveolae. *Cell* **106**(4), 403-11.
33. Hostager, B. S., Catlett, I. M., and Bishop, G. A. (2000) Recruitment of CD40 and tumor necrosis factor receptor-associated factors 2 and 3 to membrane microdomains during CD40 signaling. *J Biol Chem* **275**(20), 15392-8.
34. Vidalain, P. O., Azocar, O., Servet-Delprat, C., Rabourdin-Combe, C., Gerlier, D., and Manie, S. (2000) CD40 signaling in human dendritic cells is initiated within membrane rafts. *Embo J* **19**(13), 3304-13.
35. Higuchi, M., Izumi, K. M., and Kieff, E. (2001) Epstein-Barr virus latent-infection membrane proteins are palmitoylated and raft-associated: protein 1 binds to the cytoskeleton through TNF receptor cytoplasmic factors. *Proc Natl Acad Sci U S A* **98**(8), 4675-80.
36. Brown, K. D., Hostager, B. S., and Bishop, G. A. (2001) Differential signaling and tumor necrosis factor receptor-associated factor (TRAF) degradation mediated by CD40 and the Epstein-Barr virus oncoprotein latent membrane protein 1 (LMP1). *J Exp Med* **193**(8), 943-54.
37. Kaykas, A., Worringer, K., and Sugden, B. (2001) CD40 and LMP-1 both signal from lipid rafts but LMP-1 assembles a distinct, more efficient signaling complex. *Embo J* **20**(11), 2641-54.
38. Aizawa, S., Nakano, H., Ishida, T., Horie, R., Nagai, M., Ito, K., Yagita, H., Okumura, K., Inoue, J., and Watanabe, T. (1997) Tumor necrosis factor receptor-associated factor (TRAF) 5 and TRAF2 are involved in CD30-mediated NFkappaB activation. *J Biol Chem* **272**(4), 2042-5.
39. Andjelkovic, M., Alessi, D. R., Meier, R., Fernandez, A., Lamb, N. J., Frech, M., Cron, P., Cohen, P., Lucocq, J. M., and Hemmings, B. A. (1997) Role of translocation in the activation and function of protein kinase B. *J Biol Chem* **272**(50), 31515-24.
40. Hoeflich, K. P., Yeh, W. C., Yao, Z., Mak, T. W., and Woodgett, J. R. (1999) Mediation of TNF receptor-associated factor effector functions by apoptosis signal-regulating kinase-1 (ASK1). *Oncogene* **18**(42), 5814-20.
41. Nishitoh, H., Saitoh, M., Mochida, Y., Takeda, K., Nakano, H., Rothe, M., Miyazono, K., and Ichijo, H. (1998) ASK1 is essential for JNK/SAPK activation by TRAF2. *Mol Cell* **2**(3), 389-95.
42. Wong, B. R., Josien, R., and Choi, Y. (1999) TRANCE is a TNF family member that regulates dendritic cell and osteoclast function. *J Leukoc Biol* **65**(6), 715-24.
43. Wong, B. R., Josien, R., Lee, S. Y., Vologodskaia, M., Steinman, R. M., and Choi, Y. (1998) The TRAF family of signal transducers mediates NF-kappaB activation by the TRANCE receptor. *J Biol Chem* **273**(43), 28355-9.

44. Kobayashi, N., Kadono, Y., Naito, A., Matsumoto, K., Yamamoto, T., Tanaka, S., and Inoue, J. (2001) Segregation of TRAF6-mediated signaling pathways clarifies its role in osteoclastogenesis. *Embo J* **20**(6), 1271-80.

45. Yujiri, T., Ware, M., Widmann, C., Oyer, R., Russell, D., Chan, E., Zaitsu, Y., Clarke, P., Tyler, K., Oka, Y., Fanger, G. R., Henson, P., and Johnson, G. L. (2000) MEK kinase 1 gene disruption alters cell migration and c-Jun NH2-terminal kinase regulation but does not cause a measurable defect in NF- kappa B activation. *Proc Natl Acad Sci U S A* **97**(13), 7272-7.

46. Duckett, C. S., and Thompson, C. B. (1997) CD30-dependent degradation of TRAF2: implications for negative regulation of TRAF signaling and the control of cell survival. *Genes Dev* **11**(21), 2810-21.

47. Weiss, T., Grell, M., Siemienski, K., Muhlenbeck, F., Durkop, H., Pfizenmaier, K., Scheurich, P., and Wajant, H. (1998) TNFR80-dependent enhancement of TNFR60-induced cell death is mediated by TNFR-associated factor 2 and is specific for TNFR60. *J Immunol* **161**(6), 3136-42.

48. Feng, X., Gaeta, M. L., Madge, L. A., Yang, J. H., Bradley, J. R., and Pober, J. S. (2001) Caveolin-1 associates with TRAF2 to form a complex that is recruited to tumor necrosis factor receptors. *J Biol Chem* **276**(11), 8341-9.

49. Arch, R. H., Gedrich, R. W., and Thompson, C. B. (2000) Translocation of TRAF proteins regulates apoptotic threshold of cells. *Biochem Biophys Res Commun* **272**(3), 936-45.

50. Tsitsikov, E. N., Laouini, D., Dunn, I. F., Sannikova, T. Y., Davidson, L., Alt, F. W., and Geha, R. S. (2001) Traf1 is a negative regulator of tnf signaling. enhanced tnf signaling in traf1-deficient mice. *Immunity* **15**(4), 647-57.

51. Nguyen, L. T., Duncan, G. S., Mirtsos, C., Ng, M., Speiser, D. E., Shahinian, A., Marino, M. W., Mak, T. W., Ohashi, P. S., and Yeh, W. C. (1999) TRAF2 deficiency results in hyperactivity of certain TNFR1 signals and impairment of CD40-mediated responses. *Immunity* **11**(3), 379-89.

52. Banchereau, J., and Steinman, R. M. (1998) Dendritic cells and the control of immunity. *Nature* **392**(6673), 245-52.

53. Mehling, A., Loser, K., Varga, G., Metze, D., Luger, T. A., Schwarz, T., Grabbe, S., and Beissert, S. (2001) Overexpression of cd40 ligand in murine epidermis results in chronic skin inflammation and systemic autoimmunity. *J Exp Med* **194**(5), 615-28.

54. Trevejo, J. M., Marino, M. W., Philpott, N., Josien, R., Richards, E. C., Elkon, K. B., and Falck-Pedersen, E. (2001) TNF-alpha -dependent maturation of local dendritic cells is critical for activating the adaptive immune response to virus infection. *Proc Natl Acad Sci U S A* **98**(21), 12162-12167.

55. Funk, J. O., Walczak, H., Voigtlander, C., Berchtold, S., Baumeister, T., Rauch, P., Rossner, S., Steinkasserer, A., Schuler, G., and Lutz, M. B. (2000) Cutting edge: resistance to apoptosis and continuous proliferation of dendritic cells deficient for TNF receptor-1. *J Immunol* **165**(9), 4792-6.

56. Takaesu, G., Kishida, S., Hiyama, A., Yamaguchi, K., Shibuya, H., Irie, K., Ninomiya-Tsuji, J., and Matsumoto, K. (2000) TAB2, a novel adaptor protein, mediates activation of TAK1 MAPKKK by linking TAK1 to TRAF6 in the IL-1 signal transduction pathway. *Mol Cell* **5**(4), 649-58.

57. Pickart, C. M. (2001) Ubiquitin enters the new millennium. *Mol Cell* **8**(3), 499-504.

58. Wong, B. R., Josien, R., Lee, S. Y., Sauter, B., Li, H. L., Steinman, R. M., and Choi, Y. (1997) TRANCE (tumor necrosis factor [TNF]-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor. *J Exp Med* **186**(12), 2075-80.

59. Lee, S. Y., Reichlin, A., Santana, A., Sokol, K. A., Nussenzweig, M. C., and Choi, Y. (1997) TRAF2 is essential for JNK but not NF-kappaB activation and regulates lymphocyte proliferation and survival. *Immunity* **7**(5), 703-13.

60. Lee, S. Y., Kandala, G., Liou, M. L., Liou, H. C., and Choi, Y. (1996) CD30/TNF receptor-associated factor interaction: NF-kappa B activation and binding specificity. *Proc Natl Acad Sci U S A* **93**(18), 9699-703.

61. Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., and Steinman, R. M. (1992) Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* **176**(6), 1693-702.

62. Labeur, M. S., Roters, B., Pers, B., Mehling, A., Luger, T. A., Schwarz, T., and Grabbe, S. (1999) Generation of tumor immunity by bone marrow-derived dendritic cells correlates with dendritic cell maturation stage. *J Immunol* **162**(1), 168-75.

3.4 SUPPLEMENTARY RESULTS

The results presented in this section do not clearly fit anywhere into the three major results sections, but comprise some important observations about TRAF signaling that contribute to the continuity of this thesis. Although the results presented here are far from comprehensive, they are presented in support of ideas that will be addressed in the general discussion in section 4. These results pertain to TRAF-mediated activation of Src-family kinases, which represents a new TRAF-activated signaling pathway previously identified by our laboratory (1).

3.4.1 Phosphorylation of TRANCE-R by c-Src downregulates the surface expression of TRANCE-R

Joseph R. Arron, Masha Vologodskaia, and Yongwon Choi

TRANCE and c-Src are essential components of the signaling processes that underlie osteoclast biology. Gene targeting experiments have demonstrated that TRANCE is required for the differentiation of osteoclasts from hematopoietic precursors (2). Although mice deficient in c-Src have osteoclasts, osteoclast function in c-Src-/- mice is severely impaired, leading to osteopetrosis (3). Osteoclasts from these mice fail to undergo cytoskeletal rearrangements such as the formation of ruffled borders and actin rings necessary for bone resorption (4). Since TRANCE is necessary to induce these morphological and functional changes in mature osteoclasts (5), we reasoned that TRANCE and c-Src acted in a shared biochemical pathway. We recently demonstrated that TRANCE treatment results in activation of Akt in osteoclasts and dendritic cells, and that this process is dependent upon c-Src activity. Exposure to TRANCE leads to the

association of c-Src with TRANCE-R and an increase in the kinase activity of Src-family kinases (1). Furthermore, as we have shown in Section 3.1, c-Src mediates tyrosine phosphorylation of TRANCE-R on a specific residue (Y468 in mouse TRANCE-R) (6). We therefore investigated the role of tyrosine phosphorylation of TRANCE-R in its expression and signaling.

Although a Y468F mutation in mouse TRANCE-R abolished phosphorylation of TRANCE-R by c-Src, it did not affect the ability of TRANCE-R to interact with c-Cbl or Cbl-b (6). In order to further investigate the role of TRANCE-R tyrosine phosphorylation in TRANCE-mediated signaling, we attempted to generate cell lines that stably expressed wild-type TRANCE-R or TRANCE-R with a Y468F mutation (TR-wt or TR-Y468F). Using the pMI retroviral vector, we generated retroviruses containing a construct driving the expression of TR-wt or TR-Y468F followed by an internal ribosomal entry site (IRES) and cDNA encoding human CD2. Using these retroviruses, we infected KMls8.3.5.1 (K8) T cell hybridomas, which do not ordinarily express TRANCE-R on their surface as determined by FACS analysis (data not shown). Two days after infection, FACS analysis of the bulk cell population revealed the presence of $hCD2^+$, $TRANCE\text{-}R^+$ cells infected with wild-type TR-wt and TR-Y468F viruses (Figure 3.4.1, left panels). Ten days after infection and selection in 500 μg/ml G418, the bulk population of cells contained $hCD2^+$, $TRANCE\text{-}R^+$ cells only in the sample infected with TR-Y468F. In samples infected with TR-wt, there were no $TRANCE\text{-}R^+$ cells despite the presence of $hCD2^+$ cells (Figure 3.4.1, middle panels), suggesting that, despite the presence of TR-wt mRNA, cell surface expression of TR-wt was impaired. Infected cells were cloned by limiting dilution, and representative clones are shown in the right panels of Figure 3.4.1.

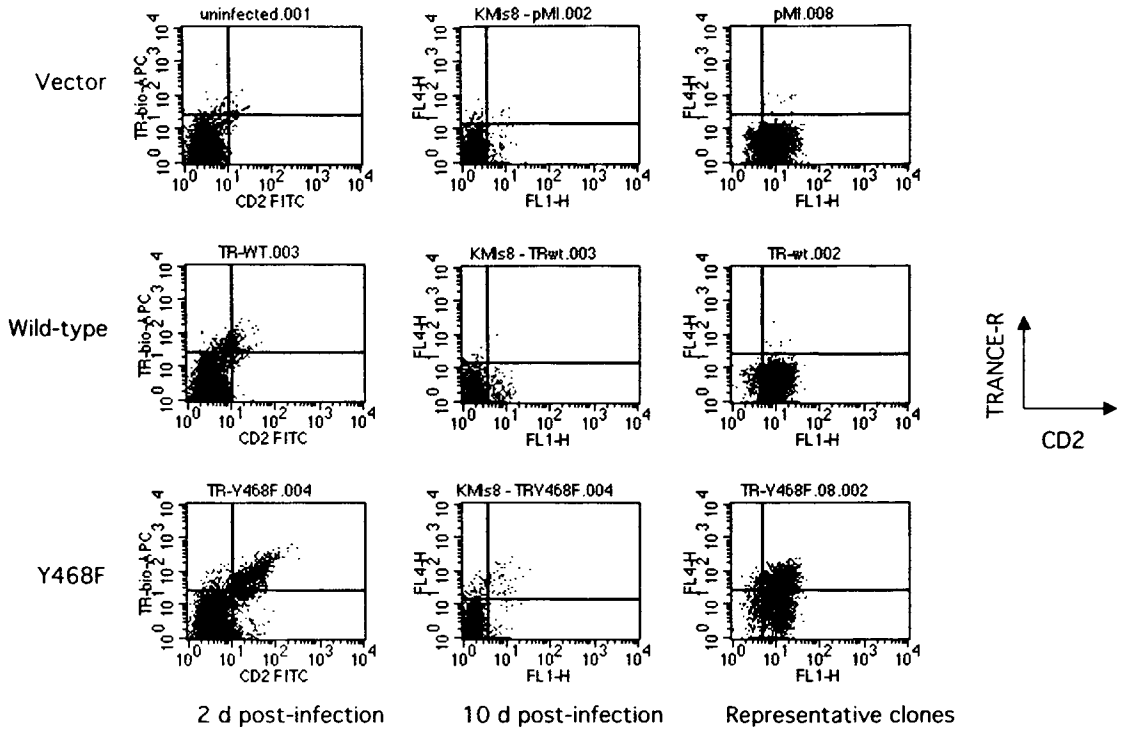

Figure 3.4.1. Phosphorylation of TRANCE-R on Y468 prevents stable cell surface expression of TRANCE-R.

KMls8.3.5.1 T cell hybridoma cells (K8) were infected with retroviruses driving expression of wild-type TRANCE-R (TR-wt) or TR-Y468F upstream of an IRES-hCD2 insert. FACS analysis of infected K8 cells is shown 2 d after infection (left panels), 10 d after infection and G418 selection (middle panels), and representative clones obtained by limiting dilution (right panels).

We next determined whether TRANCE-mediated signaling leading to the activation of Src-family kinase dependent pathways is affected by the Y468F mutation in TRANCE-R. We have previously found that the activation of Akt by TRANCE is dependent on TRAF6 and Src-family kinases (1), so we investigated TRANCE-mediated Akt activation in K8/TR-Y468F stable cells. While K8 cells alone did not show activation of Akt or JNK in response to TRANCE stimulation, K8/TR-Y468 cells showed Akt and JNK activation in response to TRANCE (Figure 3.4.2). K8/TR-wt cells did not show activation of any signaling pathways in response to the addition of soluble TRANCE, consistent with the observation that they do not express TRANCE-R on their surface (data not shown).

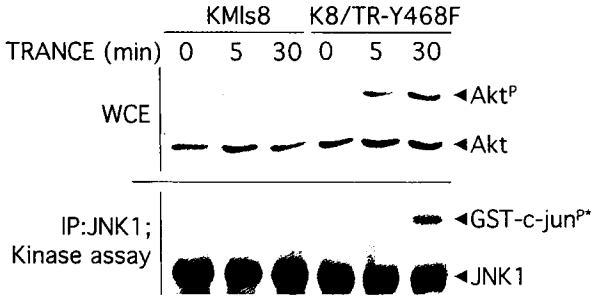

Figure 3.4.2. Y468 of TRANCE-R is not required for TRANCE-activated signaling. Parental K8 cell line (left) or a stable clone expressing TR-Y468F (right) were starved in 0.5% FBS for 2-3 h and stimulated for the indicated number of minutes with soluble TRANCE (2 μg/ml). Cell lysates were immunoblotted with antibodies to phospho-Akt and total Akt (WCE, top), or subjected to an *in vitro* JNK kinase assay (bottom).

Previously, we have attempted to generate stable cell lines expressing wild-type full-length TRANCE-R on the cell surface, and these attempts have failed in a wide variety of common cell lines, including HEK 293, Cos, CHO, HeLa, and others (data not shown). While TRANCE-R mRNA expression has been detected in numerous cell types and tissues, including skeletal muscle, thymus, liver, colon, small intestine, and adrenal gland (7), high levels of cell surface expression of TRANCE-R have only been observed on mature DCs. Notably, high surface expression of TRANCE-R has not been observed on immature DCs, macrophages, or naïve lymphocytes (8). Osteoclasts and activated lymphocytes respond to TRANCE stimulation, but cell surface expression of TRANCE-R on these cell types is very low relative to the expression of TRANCE-R on mature DCs. Taken together with the finding that TR-Y468 can be stably expressed on the cell surface, these observations suggest that tyrosine phosphorylation of TRANCE-R on Y468 in most cell types may activate a strong internalization or degradation signal.

Why, then, is TRANCE-R expressed at such high levels on mature DCs? As DCs mature, they downregulate endocytosis so as to express the highest possible levels of peptide-MHC complexes and costimulatory molecules on their surface for efficient T cell priming (9). The downregulation of endocytosis by mature DCs appears to be tied to the regulation of Rho-family GTPases, particularly Cdc42 (10). Thus, the high levels of

surface TRANCE-R expression in mature DCs may be a by-product of low endocytic activity in mature DCs. The normal or elevated endocytic activity in the cell lines mentioned above or immature DCs may be sufficient to prevent high stable levels of surface TRANCE-R expression in those cells. Further investigation of the role of phosphorylation of Y468 in TRANCE-R and cytoplasmic factors that bind to phospho-Y468 may reveal important regulatory processes that underlie the efficient endocytosis displayed by immature DCs.

3.4.2 The N-terminus of TRAF6 has a unique role in c-Src activation

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TRAF6 and c-Src have overlapping roles in TRANCE signaling in osteoclasts, as determined by genetic and biochemical experiments (1,3,4,11-13). We have found that TRAF6 can interact with c-Src (1) and c-Cbl (6). Furthermore, while the interaction with c-Src and c-Cbl appears to be dependent only on the TRAF domain of TRAF6, expression of full-length TRAF6 leads to the activation of c-Src, resulting in the tyrosine phosphorylation of c-Cbl. Thus, similar to the activation of IKK and JNK signal cascades (14,15), there is a requirement for the N-terminal RING and Zn fingers of TRAF6 to activate c-Src. We have shown in that the RING finger of TRAF2 is necessary for its translocation to lipid rafts and the activation of IKK and JNK cascades (Section 3.3). It has been well established that acylated Src-family kinases localize to lipid rafts (16). In order to determine if the N-terminal RING and Zn fingers of TRAF2 and TRAF6 have unique roles, we generated chimeric TRAF proteins, comprising the N-terminus of one TRAF and the TRAF domain of another TRAF. This approach has been previously used to dissect the differential signals transduced by TRAF3 and TRAF5 (17).

3. Results

We generated two chimeric TRAF2-TRAF6 constructs: 1) Residues 1-240 of mTRAF2 fused to residues 289-530 of mTRAF6 (N2-C6), and 2) residues 1-288 of TRAF6 fused to residues 241-501 of TRAF2 (N6-C2). Overexpression of TRAF2, TRAF6, N2-C6, and N6-C2 induced NF-κB and JNK activation (Figure 3.4.3A and B), although the N2-C6 construct activated NF-κB at 3-4-fold levels lower than did similar amounts of TRAF2, TRAF6, or N6-C2. When we transfected the various TRAF constructs with c-Cbl and limiting amounts of c-Src and examined tyrosine phosphorylation of c-Cbl, we found that TRAF6 and the N6-C2 construct could promote efficient phosphorylation of c-Cbl, but TRAF2 and N2-C6 could not, although there was a slight increase over background levels in phospho-c-Cbl in the presence of overexpressed TRAF2 (Figure 3.4.3C). Thus, while the TRAF domain is sufficient for TRAF6 to interact with c-Src and c-Cbl, the N-terminal RING and Zn fingers have a unique role in the activation of c-Src, and this function cannot be substituted by the N-terminal domain of TRAF2. Conversely, the N-terminal RING and Zn fingers of TRAF6 are able to confer c-Src activating ability to the TRAF domain of TRAF2.

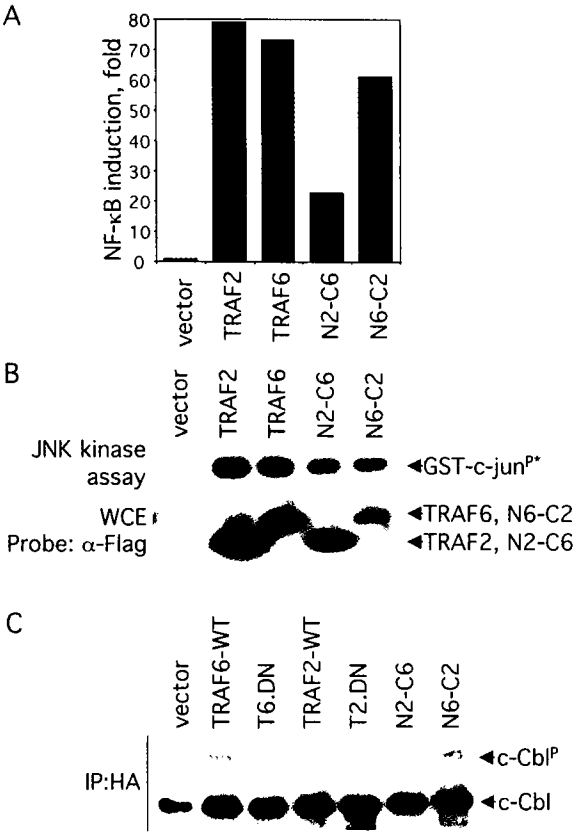

Figure 3.4.3. Differential abilities of the N-terminal RING and Zn fingers of TRAF2 and TRAF6 to activate c-Src.

A. TRAF2, TRAF6, and chimeras consisting of the N-terminal half of TRAF2 fused to the C-terminal half of TRAF6 (N2-C6) and the N-terminal half of TRAF6 fused to the C-terminal half of TRAF2 (N6-C2) (0.5 μg) were transfected into 293T cells with an NF-κB-responsive reporter plasmid and luciferase activity was measured 24h after transfection.

B. As in (A), but an *in vitro* JNK assay was performed on the cell lysates.

C. TRAF6, T6.DN, TRAF2, T2.DN, N2-C6, and N6-C2 (0.5 μg) were co-transfected as indicated with HA-tagged c-Cbl (0.3 μg) and c-Src (10 ng). c-Cbl was immunoprecipitated with antibodies to HA and immunoprecipitates were immunoblotted with antibodies to phosphotyrosine (4G10).

Despite numerous observations that TRAF2 and TRAF6 activate similar signaling pathways through the action of their RING and Zn fingers (18), our findings suggest that the functions of these domains may be divergent, and this may point to divergent mechanisms of action between TRAF2 and TRAF6. Thus, there are two potential mechanisms whereby TRAF2 and TRAF6 can achieve signaling specificity: 1) binding to different receptor sites (Section 3.2) and 2) differential ability to activate signaling cascades through the action of the N-terminal RING and Zn fingers.

3.4.3 References

1. Wong, B. R., Besser, D., Kim, N., Arron, J. R., Vologodskaia, M., Hanafusa, H., and Choi, Y. (1999) TRANCE, a TNF family member, activates Akt/PKB through a signaling complex involving TRAF6 and c-Src. *Mol Cell* **4**(6), 1041-9.

2. Kong, Y. Y., Yoshida, H., Sarosi, I., Tan, H. L., Timms, E., Capparelli, C., Morony, S., Oliveira-dos-Santos, A. J., Van, G., Itie, A., Khoo, W., Wakeham, A., Dunstan, C. R., Lacey, D. L., Mak, T. W., Boyle, W. J., and Penninger, J. M. (1999) OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* **397**(6717), 315-23.
3. Soriano, P., Montgomery, C., Geske, R., and Bradley, A. (1991) Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. *Cell* **64**(4), 693-702.
4. Boyce, B. F., Yoneda, T., Lowe, C., Soriano, P., and Mundy, G. R. (1992) Requirement of pp60c-src expression for osteoclasts to form ruffled borders and resorb bone in mice. *J Clin Invest* **90**(4), 1622-7.
5. Fuller, K., Wong, B., Fox, S., Choi, Y., and Chambers, T. J. (1998) TRANCE is necessary and sufficient for osteoblast-mediated activation of bone resorption in osteoclasts. *J Exp Med* **188**(5), 997-1001.
6. Arron, J. R., Vologodskaia, M., Wong, B. R., Naramura, M., Kim, N., Gu, H., and Choi, Y. (2001) A positive regulatory role for Cbl family proteins in tumor necrosis factor-related activation-induced cytokine (trance) and CD40L-mediated Akt activation. *J Biol Chem* **276**(32), 30011-7.
7. Anderson, D. M., Maraskovsky, E., Billingsley, W. L., Dougall, W. C., Tometsko, M. E., Roux, E. R., Teepe, M. C., DuBose, R. F., Cosman, D., and Galibert, L. (1997) A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature* **390**(6656), 175-9.
8. Wong, B. R., Josien, R., Lee, S. Y., Sauter, B., Li, H. L., Steinman, R. M., and Choi, Y. (1997) TRANCE (tumor necrosis factor [TNF]-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor. *J Exp Med* **186**(12), 2075-80.
9. Mellman, I., and Steinman, R. M. (2001) Dendritic cells: specialized and regulated antigen processing machines. *Cell* **106**(3), 255-8.
10. Garrett, W. S., Chen, L. M., Kroschewski, R., Ebersold, M., Turley, S., Trombetta, S., Galan, J. E., and Mellman, I. (2000) Developmental control of endocytosis in dendritic cells by Cdc42. *Cell* **102**(3), 325-34.
11. Lomaga, M. A., Yeh, W. C., Sarosi, I., Duncan, G. S., Furlonger, C., Ho, A., Morony, S., Capparelli, C., Van, G., Kaufman, S., van der Heiden, A., Itie, A., Wakeham, A., Khoo, W., Sasaki, T., Cao, Z., Penninger, J. M., Paige, C. J., Lacey, D. L., Dunstan, C. R., Boyle, W. J., Goeddel, D. V., and Mak, T. W. (1999) TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. *Genes Dev* **13**(8), 1015-24.
12. Naito, A., Azuma, S., Tanaka, S., Miyazaki, T., Takaki, S., Takatsu, K., Nakao, K., Nakamura, K., Katsuki, M., Yamamoto, T., and Inoue, J. (1999) Severe

osteopetrosis, defective interleukin-1 signalling and lymph node organogenesis in TRAF6-deficient mice. *Genes Cells* **4**(6), 353-62.

13. Xing, L., Venegas, A. M., Chen, A., Garrett-Beal, L., Boyce, B. F., Varmus, H. E., and Schwartzberg, P. L. (2001) Genetic evidence for a role for Src family kinases in TNF family receptor signaling and cell survival. *Genes Dev* **15**(2), 241-53.
14. Takeuchi, M., Rothe, M., and Goeddel, D. V. (1996) Anatomy of TRAF2. Distinct domains for nuclear factor-kappaB activation and association with tumor necrosis factor signaling proteins. *J Biol Chem* **271**(33), 19935-42.
15. Song, H. Y., Regnier, C. H., Kirschning, C. J., Goeddel, D. V., and Rothe, M. (1997) Tumor necrosis factor (TNF)-mediated kinase cascades: bifurcation of nuclear factor-kappaB and c-jun N-terminal kinase (JNK/SAPK) pathways at TNF receptor-associated factor 2. *Proc Natl Acad Sci U S A* **94**(18), 9792-6.
16. Simons, K., and Ikonen, E. (1997) Functional rafts in cell membranes. *Nature* **387**(6633), 569-72.
17. Dadgostar, H., and Cheng, G. (1998) An intact zinc ring finger is required for tumor necrosis factor receptor-associated factor-mediated nuclear factor-kappaB activation but is dispensable for c-Jun N-terminal kinase signaling. *J Biol Chem* **273**(38), 24775-80.
18. Wajant, H., Henkler, F., and Scheurich, P. (2001) The TNF-receptor-associated factor family: scaffold molecules for cytokine receptors, kinases and their regulators. *Cell Signal* **13**(6), 389-400.

4. GENERAL DISCUSSION

This thesis investigates the proximal components of TNFR family protein signal transduction, specifically pertaining to the assembly and regulation of signaling complexes mediated by TRAF proteins. Evolutionary, structural, and functional studies have all established a distinction between the TRAF proteins that activate signal cascades downstream of TNFR family proteins. This distinction is exemplified in examination of TRAF2 and TRAF6. While TRAF2 and TRAF6 appear to interact with an overlapping subset of intracellular molecules and TNFR family proteins, there are key distinctions to be made. Not only do TRAF2 and TRAF6 interact with different receptor sequences via their TRAF domains, but the functions of their N-terminal domains are also divergent. While it has long been understood that TRAFs were crucial for assembling signaling complexes upon TNF family ligand engagement of TNFRs, the mechanism of action of TRAFs has not been well characterized. The work presented in this thesis, along with other recent reports, makes significant progress toward delineating a mechanism of action for TRAF proteins. Two emerging themes in the mechanisms of TRAF signaling are ubiquitination and lipid raft translocation. Another important consideration in TRAF signaling lies in the other, often-ignored side of signal transduction – the negative regulation of signaling. Mechanisms of negative regulation of TRAF signaling appear to be tied to the very things that are required for signal activation, and it will be interesting to examine these phenomena in the context of signaling in general.

4.1 Distinctions between TRAF6 and other TRAFs

Examples of the two major types of signal-activating TRAFs are TRAF6 and TRAF2. TRAF1, 3, and 5 have more in common with TRAF2 than with TRAF6, although there are some key differences between them. Of TRAF1, 3, and 5, only TRAF5 has been

shown to activate NF-κB and AP-1 similar to TRAF2 (1,2). However, while TRAF2 interacts directly with many receptors, TRAF5 may interact only indirectly with some receptors via hetero-oligomerization with TRAF3 (3). TRAF3 does not activate NF-κB or AP-1, but it is apparently essential for normal development and immune regulation (4). This essential role of TRAF3 may lie in its ability to recruit TRAF5 to receptors, or there may be additional regulatory roles for it that have not yet been identified. TRAF1 is highly homologous to TRAF2 in its TRAF domain, but it lacks the N-terminal structures (RING and multiple Zn fingers) that enable TRAF2 to activate signals (5). Nonetheless, as we have seen in section 3.3, TRAF1 is a key regulator of TRAF2-mediated signaling, most likely due to its ability to hetero-oligomerize with TRAF2 and regulate its subcellular distribution. TRAF1, 2, 3, and 5 all interact with similar receptor sites, albeit with slightly different affinity/avidity profiles (6).

TRAF6 has the most evolutionarily divergent TRAF domain, although its oligomerization appears to activate NF-κB and AP-1 very similarly to TRAF2. TRAF6 binds to a distinct receptor sequence from TRAF2, as we have defined in section 3.2. Thus, one obvious level of specificity that may be conferred by TRAF6 is that it can potentially interact with a completely different array of receptors. Furthermore, TRAF6 can interact with IRAK proteins, which, unlike TNFR proteins, are intracellular adaptors for non-TNFR family receptors (7,8). The IL-1/TLR family of receptors is very important in general inflammation and innate immunity, thus TRAF6 is a bridge between innate and adaptive immunity. Not only does TRAF6 have key roles in immunity, but the same inflammatory factors and repetitive sequences that mediate innate immunity can also affect bone homeostasis (section 2.3.2). TRAF6 has been shown to be vital for osteoclast physiology, and we have recently found that dendritic cells, which arise from the same hematopoietic precursors as osteoclasts, are also dependent on TRAF6 for their

maturation (T. Kobayashi et al., unpublished data). Thus, by virtue of the fact that DCs and OCs come from the same precursors, and the same factors are responsible for the final stage of their maturation and activation, and these factors are all dependent on TRAF6 for signal transduction, it is apparent that TRAF6 plays a central role in osteoimmunology. Figure 4.1 details the roles of TRAF6 in the parallel development of DCs and OCs.

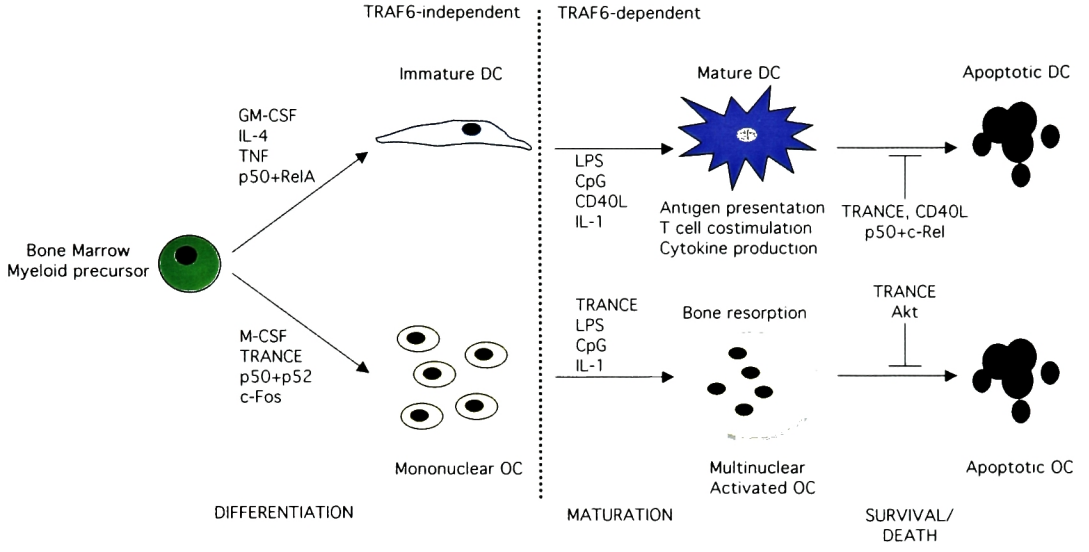

Figure 4.1. Parallel lifecycles of dendritic cells and osteoclasts and the role of TRAF6 in osteoimmunology.
DCs and OCs differentiate from common myeloid hematopoietic precursors. Factors mediating DC differentiation include GM-CSF, IL-4, and TNF. DC differentiation is dependent on the combination of the NF-κB subunits p50 and RelA (F. Ouaaz, J. Arron, Y. Zhang, Y. Choi, and A. Beg, manuscript submitted). OC differentiation is dependent on M-CSF, TRANCE, and the transcription factors c-Fos and the combination of NF-κB subunits p50 and p52 (9). The maturation of DCs and OCs are both mediated by TRAF6-dependent factors, including LPS, CpG, and IL-1. CD40L also induces DC maturation, while TRANCE induces OC maturation. Mature, activated DCs and OCs rapidly undergo apoptosis in the absence of survival signals provided by TRANCE and CD40L. TRANCE-mediated DC survival is dependent on the combination of the NF-κB subunits p50 and cRel, while TRANCE-mediated OC survival is dependent on Akt.

Since it appears likely that the TRAF domain of TRAF2 and TRAF6 is responsible for most protein-protein interactions (Sections 2.2 and 3.3), it might be reasonable to conclude that the specificity of signaling mediated by TRAF2 and TRAF6 is encoded in the TRAF domain, because it determines which upstream molecules are coupled to which

downstream molecules. For example, IRAK has three TRAF6 binding sites and no TRAF2 binding sites while TNFR2 has one TRAF2 binding site and no TRAF6 binding sites. Since TRAF2 and TRAF6 interact with overlapping but distinct subsets of intracellular signaling molecules, one would expect there to be a qualitative difference between the effects of IL-1 and TNF on a given cell. Nonetheless, the specificity of TRAF signaling does not appear to be localized exclusively to the TRAF domain. As we have shown in section 3.3, the RING finger of TRAF2 mediates its translocation to lipid rafts, and without TRAF1, TRAF2 remains in insoluble complexes after translocation. TRAF6, on the other hand, does not appear to accumulate in insoluble complexes ((10) and data not shown). While translocation appears to be necessary for the ability of TRAF2 to activate signaling cascades, it is unclear whether the same can be said for TRAF6.

4.2 c-Src activation by TRAF6

While the N-terminal RING and Zn fingers of both TRAF2 and TRAF6 mediate the activation of NF-κB and AP-1, the N-terminal half of TRAF6 can also strongly activate c-Src, while the N-terminal half of TRAF2 can only weakly activate c-Src, if at all (section 3.4). This result is particularly interesting in light of the fact that we previously observed that c-Src and c-Cbl interacted with TRAF1, TRAF3, and TRAF6, but not with TRAF2 or TRAF5 ((11) and data not shown). In fact, the pattern of co-immunoprecipitation of c-Src and c-Cbl with various TRAFs correlates with the tendency of the TRAFs to localize in the soluble fraction of cells lysed in Triton X-100 (data not shown). Thus, it is possible that the reason we did not observe co-immunoprecipitation of c-Src and c-Cbl with TRAF2 is because that interaction may have taken place in the insoluble fraction, which, at the time, we ignored. In support of the notion that the TRAF domain of TRAF2 can in fact interact with c-Src and c-Cbl, the N6-C2 chimeric TRAF

construct could activate c-Src-mediated c-Cbl phosphorylation (section 3.4). Alternatively, while there is no doubt that the TRAF domain of TRAF6 can interact with c-Src, it may be possible that elements in the N-terminal half of TRAF6 can also interact with c-Src when oligomerized (as is the case in the N6-C2 construct, where the TRAF domain of TRAF2 mediates oligomerization). Previously, we did not observe interaction between c-Src with the N-terminal half of TRAF6 alone (11), but it is apparent that the N-terminal half of TRAF6 can activate signaling by itself if it is oligomerized artificially by some means other than the TRAF domain (12,13). However, our biochemical and strucutural studies (section 3.2) suggest that c-Src can interact with a specific part of the TRAF domain distal to the N-terminal half of TRAF6. Thus, if c-Src can interact with only the TRAF domain, then there is a unique ability of the RING and Zn fingers of TRAF6 to mediate c-Src activation. If c-Src can also interact with the oligomerized RING and Zn fingers of TRAF6 but not TRAF2, then the specific ability of the N-terminal half of TRAF6 to interact with c-Src may mediate c-Src activation. While the currently available data do not completely rule out either possibility, the fact that c-Src can interact with the TRAF domain alone of TRAF6 favors the first possibility.

4.3 Ubiquitination as a mechanism of TRAF signaling

If the RING finger of TRAF proteins is not required for the interaction of TRAFs and any other proteins known to interact with TRAFs (section 3.3), how does it activate signaling cascades? One established function of RING finger domains is the ability to mediate ubiquitination of other proteins by acting as E3 ubiquitin ligases (14). For example, we (section 3.1) and others (15-17) have shown that Cbl family proteins mediate the degradation of activated signaling molecules in a RING finger-dependent manner, which is accomplished by the ubiquitination of target proteins. Recently, the RING fingers of TRAF2 and TRAF6 have been shown *in vitro* to mediate a novel form of

polyubiquitination (13,18). The well-characterized degradative pathway of ubiquitination involves polyubiquitination wherein ubiquitin chains are formed with covalent linkage of one ubiquitin molecule to the target protein and further molecules of ubiquitin are covalently linked to K48 of the previous ubiquitin. K48-linked polyubiquitination leads to proteasomal degradation of the targeted protein. The novel form of ubiquitination mediated by TRAF proteins involves the formation of polyubiquitin chains linked through K63 of ubiquitin. This does not appear to mediate degradation of the target proteins. However, TRAF6-mediated K63-linked polyubiquitination appears to be required for the activation of the IKK complex leading to NF-κB activation. In addition to ubiquitinating downstream signaling molecules, TRAF6 itself is polyubiquitinated through a K63 linkage. It is thought that TRAF2 undergoes a similar process.

While K63-linked polyubiquitination appears to be required for NF-κB activation, our findings in section 3.3 demonstrate that the RING finger is dispensable for the activation of JNK by TRAF2 if raft translocation of TRAF2 is artificially induced. Taken together with the findings that K63-linked polyubiquitination leads to the activation of NF-κB as well as JNK (13), our observations that M/P-T2Δ87 can activate JNK but not NF-κB (Fig. 3.3.4) suggest that RING finger-mediated polyubiquitination of TRAF2 may mediate its translocation into lipid rafts. Although translocation is sufficient for JNK activation, the ubiquitination process is likely to be required in addition to translocation for NF-κB activation. A caveat to this hypothesis is that the RING fingers on various TRAF proteins may function differently. TRAF3 does not translocate to insoluble complexes or activate JNK upon overexpression, but replacement of the RING finger of TRAF3 with the RING finger of TRAF5 has been shown to induce its translocation and its ability to activate JNK (19). However, endogenous TRAF3 has been shown to be strongly recruited to lipid rafts upon CD40 engagement or LMP1 expression (20-22),

which may be mediated by its hetero-oligomerization with TRAF5. The murine splice variant of TRAF2, TRAF2A, can activate JNK but not NF-κB (23), so it is possible that TRAF2A possesses an intrinsic property of the RING finger that enables raft translocation, but is not capable of mediating K63-linked polyubiquitination leading to NF-κB activation.

While the possibility remains that the RING finger mediates ubiqutination and raft translocation as completely separate processes, it is simpler and more likely that the RING finger mediates one process, which results in the other process. Thus, RING-mediated K63-linked polyubiquitination of TRAF proteins may increase the affinity of TRAFs for lipid rafts either intrinsically or by mediating the interaction of polyubiquitinated TRAFs with raft-specific proteins. Alternatively, the RING finger of oligomerized TRAF proteins may mediate translocation into rafts, where proteins mediating polyubiquitination reside, thus the co-localization of TRAFs and ubiquitinating enzymes enables their activation. Given that enforced raft localization of M/P-T2Δ87 can activate JNK signaling but not NF-κB activation in the absence of the RING finger, it seems likely that ubiquitination may precede raft translocation, since there is no evidence that raft translocation is absolutely necessary for NF-κB activation. However, ubiquitination is required for NF-κB activation. Therefore, an orderly progression of TRAF signaling may proceed as follows: 1) ligand-mediated receptor trimerization, 2) TRAF recruitment and trimerization, 3) recruitment of TRAF-interacting signaling molecules in the soluble fraction, 4) RING finger-mediated polyubiquitination of the IKK complex and TRAFs (leading to NF-κB activation), and 5) polyubiquitin-mediated TRAF translocation to lipid rafts leading to JNK activation (Figure 4.2). Clearly, the role of ubiquitination in RING-dependent raft translocation and kinase activation merits further investigation.

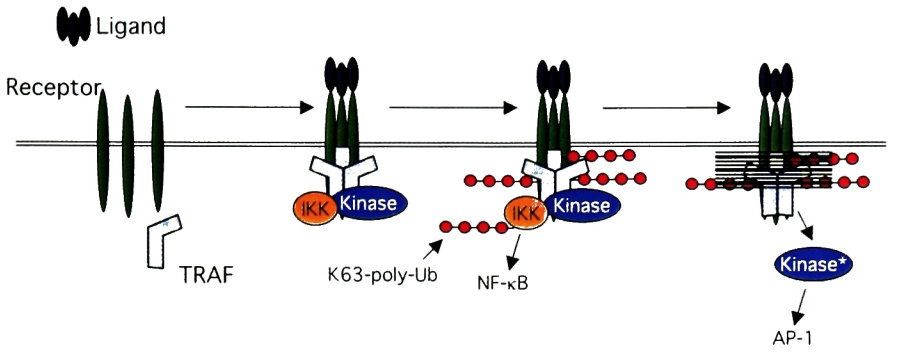

Figure 4.2. Proposed model of ubiqutin-mediated signaling by TRAF proteins. See text for details.

4.4 Raft translocation as a mechanism of TRAF signaling

The role of lipid rafts has been an emerging theme in signal transduction in recent years (24-26). Because of the differential affinities of various proteins for lipid-ordered microdomains, these domains may serve to concentrate certain proteins in close proximity to one another while excluding other proteins. Recently, rafts have also been suggested to be involved in cell polarity, by redistributing relative amounts of transmembrane and raft-associated proteins on different sides of a cell (27). We have observed a crucial role of raft translocation in TRAF2-mediated signaling (section 3.3). Of particular interest is that, while raft translocation of TRAF2 is necessary for its ability to activate certain signals, once it has translocated to lipid rafts, it does not relocalize to the soluble cytoplasmic fraction in the absence of TRAF1. Furthermore, when TRAF2 is trapped in insoluble complexes as a result of an initial receptor engagement, it cannot activate signals in response to subsequent receptor engagements. Thus, the translocation of TRAF2 to rafts appears to simultaneously activate downstream signals and inactivate TRAF2. While TRAF2 does accumulate in lipid rafts to some extent during steady-state signaling, it appears that a majority of insoluble TRAF2 may not be raft-associated. TRAF1, which is not expressed in naïve cells, is upregulated by TRAF2-dependent signals, and its function appears to be to regulate steady-state levels of soluble TRAF2. It also prevents the steady-state association of TRAF2 with the cytoskeletal protein Filamin. TRAF2 must be able to translocate to rafts in order to associate with Filamin. It appears

that TRAF2 undergoes a cycle of raft translocation, internalization (possibly mediated by interactions with caveolin), and sequestration in insoluble cytoskeletal complexes and/or degradation. While raft translocation is necessary for the positive effects of TRAF2 (activation of signaling cascades), it appears that raft translocation downregulates the ability of activated TRAF2 to activate further signaling cascades in the absence of TRAF1. Therefore, raft translocation serves a positive and negative role in TRAF2 signaling.

The temporally regulated positive and negative roles of raft translocation in TRAF2 signaling is reminiscent of the temporally regulated positive and negative roles of Cbl proteins in TRAF6-mediated Akt activation (section 3.1). In each case, the association of components of a TNFR protein signaling complex with a regulatory factor (Cbl in the case of TRAF6 and c-Src and lipid rafts in the case of TRAF2) is necessary for both the activation of a signaling pathway and the ultimate quenching of that signal. This type of complex regulation is seen in other examples of Cbl signaling, such as from the EGF receptor (16), and in Src-family kinase signaling (28), where only activated kinases are susceptible to degradation by ubiquitination or inactivation by phosphatases. It is striking that in all of these cases, downregulation of a given component takes place as a direct consequence of the component's activation.

Since TRAF2's solubility is regulated by TRAF1, how is TRAF6 regulated? While we have found that TRAF2 becomes insoluble in the steady state in the absence of TRAF1, we have not found the same to be true for TRAF6. Furthermore, while TRAF2 can induce steady-state raft translocation of CD40, TRAF6 appears to inhibit steady-state raft translocation of CD40 (Figure 3.3.2). TRAF1 or TRAF3 alone do not localize to insoluble complexes upon overexpression, while TRAF2 and TRAF5 do localize to

insoluble complexes (data not shown). This appears to directly correlate to the ability of overexpressed TRAF2 and TRAF5 to activate NF-κB and JNK, while overexpressed TRAF1 and TRAF3 cannot. Although it can activate NF-κB and JNK, TRAF6 does not accumulate in insoluble complexes upon overexpression, and it been suggested that TRAF6 does not translocate to lipid rafts upon CD40 stimulation (10). However, the methods used to identify raft-associated TRAFs are based on relative detergent solubility. It is possible that TRAF6 is in fact raft associated upon ligand stimulation, but not as strongly as TRAF2 and it was therefore not detected in defined "raft fractions."

In the case of LMP1 expression, it was found that TRAF2 and TRAF3 could be either detected or not detected in defined "raft fractions," depending on the lysis conditions (22). TRAF6 can interact with and is a potent activator of c-Src, a dually acylated raft-associated kinase. Given these observations, it appears likely that TRAF6 may be able to translocate to lipid rafts in order to activate kinase cascades in the same manner as TRAF2, but it is able to easily dissociate from rafts, whereupon it can activate subsequent signals. TRAF2, on the other hand, becomes stuck in rafts and/or other insoluble fractions, and its solubility is regulated by an additional factor, TRAF1. In order to thoroughly examine these possibilities, real-time fluorescence imaging of TRAF2 and TRAF6 in live cells may be necessary. Additionally, the regulation of TRAF6 after its activation should be closely examined. TRAF2 can interact with caveolin (29) and Filamin (30), thus it is possible that TRAF2 is internalized and sequestered in the cytoskeleton after raft translocation. It has been suggested that Filamin plays a role in TRAF6 signaling (30), but a direct interaction has not been demonstrated. A mode of activation-induced TRAF6 downregulation through degradative ubiquitination has been shown, and this pathway may be unique to TRAF6, while sequestration in insoluble

complexes (and possible subsequent degradation) may be unique to the downregulation of TRAF2.

4.5 Physiological regulation of TRAF signaling

Ultimately, the answers to many remaining questions about TRAF signaling lie in the observation of different cell types in the context of the whole organism. While TRANCE can activate a variety of signaling pathways in DCs and OCs, the functional roles of these signaling pathways are divergent. We have previously shown that the TRANCE-R/TRAF6/c-Src/PI3-K pathway leading to the activation of Akt is an important survival signal for OCs (11). Others have implicated this pathway in TRANCE-mediated survival of ductal epithelial cells in breast tissue (31). However, this pathway appears to be less crucial in TRANCE-mediated DC survival, as Cbl-b-/- DCs are deficient in TRANCE-mediated Akt activation, but have no survival defects relative to wild-type DCs (section 3.1). Recently, we have found that TRANCE-mediated survival in mature DCs is specifically dependent upon the NF-κB subunits p50 and cRel in combination (F. Ouaaz, J. Arron, Y. Zhang, Y. Choi, and A. Beg, manuscript submitted). While recent evidence has suggested that PI3-K activation may regulate NF-κB transcriptional activity (32), the cross-talk between these pathways appears to be highly cell type-specific, and in fact may be specific to the RelA NF-κB subunit. Another level of cell type specificity in TRAF signaling comes from the transcriptional regulation of TNFR proteins. It has recently been shown that there are numerous alternatively spliced forms of CD40 in different cell types that may have differential abilities to interact with TRAFs (33). Given the observations that tyrosine phosphorylation on a particular residue of TRANCE-R regulates its cell surface expression (section 3.4) and that TRAF6 deficiency completely abrogates TRANCE-mediated signal activation despite the ability of full-length TRANCE-R to signal through TRAF2 (34), it is possible that TRANCE-R is similarly

alternatively spliced. In addition to the specific effects of proximal components of the signaling complex, it is likely that other, TRAF-independent signals that are activated within the cell may affect signals downstream of TRAFs. For example, IFN-γ stimulation of OC precursors has been shown to induce the degradation of TRAF6 (35). Taken together, these and many other observations demonstrate that an understanding of TRAF signaling must be at a level deeper than simple protein-protein interactions.

4.6 Conclusions

It is clear is that the fine regulation of TRAF signaling in primary cells is dependent upon multiple factors, including: 1) the relative levels of various TRAFs within a given cell, 2) the availability of soluble TRAFs for ligand-dependent signaling, 3) the availability of downstream signaling components that interact with TRAFs, and 4) the recent history of TRAF-dependent signals in a given cell. These all contribute to the differential activation of various signaling pathways, ultimately leading to different gene expression profiles and cellular effects. While the work presented here has made considerable progress toward elucidating structural and functional features of the mechanisms of TRAF signaling, it has raised a number of further questions. What are the individual roles of the RING and each Zn finger in TRAF2 and TRAF6? While it is structurally similar to TRAF2, TRAF3 does not appear to regulate signaling in the same way as TRAF2 – what does TRAF3 do? What does TRAF4 do? We have identified a biochemical role for TRAF1, but there do not appear to be a strong phenotype in TRAF1-/- mice. What are some physiological situations in which TRAF1-mediated TRAF2 regulation might be important? TRANCE-R, a TNFR family protein, has three TRAF6 binding sites and two TRAF1/2/3/5 binding sites. These account for about 10% of its cytoplasmic tail. CD40, which binds to the same TRAFs, has a considerably shorter cytoplasmic tail. What does the rest of TRANCE-R do? What non-TRAF proteins does it interact with, in TRAF

dependent and independent fashions? Ultimately the observations about the molecular phenomena that underlie TNF family signaling must be extrapolated into a comprehensive understanding of and ability to manipulate the wide range of physiological processes linked to inflammation and immunity.

4.7 References

1. Ishida, T. K., Tojo, T., Aoki, T., Kobayashi, N., Ohishi, T., Watanabe, T., Yamamoto, T., and Inoue, J. (1996) TRAF5, a novel tumor necrosis factor receptor-associated factor family protein, mediates CD40 signaling. *Proc Natl Acad Sci U S A* **93**(18), 9437-42.
2. Nakano, H., Oshima, H., Chung, W., Williams-Abbott, L., Ware, C. F., Yagita, H., and Okumura, K. (1996) TRAF5, an activator of NF-kappaB and putative signal transducer for the lymphotoxin-beta receptor. *J Biol Chem* **271**(25), 14661-4.
3. Pullen, S. S., Miller, H. G., Everdeen, D. S., Dang, T. T., Crute, J. J., and Kehry, M. R. (1998) CD40-tumor necrosis factor receptor-associated factor (TRAF) interactions: regulation of CD40 signaling through multiple TRAF binding sites and TRAF hetero-oligomerization. *Biochemistry* **37**(34), 11836-45.
4. Xu, Y., Cheng, G., and Baltimore, D. (1996) Targeted disruption of TRAF3 leads to postnatal lethality and defective T-dependent immune responses. *Immunity* **5**(5), 407-15.
5. Rothe, M., Wong, S. C., Henzel, W. J., and Goeddel, D. V. (1994) A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. *Cell* **78**(4), 681-92.
6. Pullen, S. S., Labadia, M. E., Ingraham, R. H., McWhirter, S. M., Everdeen, D. S., Alber, T., Crute, J. J., and Kehry, M. R. (1999) High-affinity interactions of tumor necrosis factor receptor-associated factors (TRAFs) and CD40 require TRAF trimerization and CD40 multimerization. *Biochemistry* **38**(31), 10168-77.
7. Cao, Z., Xiong, J., Takeuchi, M., Kurama, T., and Goeddel, D. V. (1996) TRAF6 is a signal transducer for interleukin-1. *Nature* **383**(6599), 443-6.
8. Cao, Z., Henzel, W. J., and Gao, X. (1996) IRAK: a kinase associated with the interleukin-1 receptor. *Science* **271**(5252), 1128-31.
9. Franzoso, G., Carlson, L., Xing, L., Poljak, L., Shores, E. W., Brown, K. D., Leonardi, A., Tran, T., Boyce, B. F., and Siebenlist, U. (1997) Requirement for NF-kappaB in osteoclast and B-cell development. *Genes Dev* **11**(24), 3482-96.

10. Hostager, B. S., Catlett, I. M., and Bishop, G. A. (2000) Recruitment of CD40 and tumor necrosis factor receptor-associated factors 2 and 3 to membrane microdomains during CD40 signaling. *J Biol Chem* **275**(20), 15392-8.
11. Wong, B. R., Besser, D., Kim, N., Arron, J. R., Vologodskaia, M., Hanafusa, H., and Choi, Y. (1999) TRANCE, a TNF family member, activates Akt/PKB through a signaling complex involving TRAF6 and c-Src. *Mol Cell* **4**(6), 1041-9.
12. Baud, V., Liu, Z. G., Bennett, B., Suzuki, N., Xia, Y., and Karin, M. (1999) Signaling by proinflammatory cytokines: oligomerization of TRAF2 and TRAF6 is sufficient for JNK and IKK activation and target gene induction via an amino-terminal effector domain. *Genes Dev* **13**(10), 1297-308.
13. Wang, C., Deng, L., Hong, M., Akkaraju, G. R., Inoue, J., and Chen, Z. J. (2001) TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature* **412**(6844), 346-51.
14. Joazeiro, C. A., and Weissman, A. M. (2000) RING finger proteins: mediators of ubiquitin ligase activity. *Cell* **102**(5), 549-52.
15. Levkowitz, G., Waterman, H., Zamir, E., Kam, Z., Oved, S., Langdon, W. Y., Beguinot, L., Geiger, B., and Yarden, Y. (1998) c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. *Genes Dev* **12**(23), 3663-74.
16. Levkowitz, G., Waterman, H., Ettenberg, S. A., Katz, M., Tsygankov, A. Y., Alroy, I., Lavi, S., Iwai, K., Reiss, Y., Ciechanover, A., Lipkowitz, S., and Yarden, Y. (1999) Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. *Mol Cell* **4**(6), 1029-40.
17. Yokouchi, M., Kondo, T., Houghton, A., Bartkiewicz, M., Horne, W. C., Zhang, H., Yoshimura, A., and Baron, R. (1999) Ligand-induced ubiquitination of the epidermal growth factor receptor involves the interaction of the c-Cbl RING finger and UbcH7. *J Biol Chem* **274**(44), 31707-12.
18. Deng, L., Wang, C., Spencer, E., Yang, L., Braun, A., You, J., Slaughter, C., Pickart, C., and Chen, Z. J. (2000) Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell* **103**(2), 351-61.
19. Dadgostar, H., and Cheng, G. (2000) Membrane localization of TRAF 3 enables JNK activation. *J Biol Chem* **275**(4), 2539-44.
20. Brown, K. D., Hostager, B. S., and Bishop, G. A. (2001) Differential signaling and tumor necrosis factor receptor-associated factor (TRAF) degradation mediated by CD40 and the Epstein-Barr virus oncoprotein latent membrane protein 1 (LMP1). *J Exp Med* **193**(8), 943-54.

33. Tone, M., Tone, Y., Fairchild, P. J., Wykes, M., and Waldmann, H. (2001) Regulation of CD40 function by its isoforms generated through alternative splicing. *Proc Natl Acad Sci U S A* **98**(4), 1751-6.

34. Kobayashi, N., Kadono, Y., Naito, A., Matsumoto, K., Yamamoto, T., Tanaka, S., and Inoue, J. (2001) Segregation of TRAF6-mediated signaling pathways clarifies its role in osteoclastogenesis. *Embo J* **20**(6), 1271-80.

35. Takayanagi, H., Ogasawara, K., Hida, S., Chiba, T., Murata, S., Sato, K., Takaoka, A., Yokochi, T., Oda, H., Tanaka, K., Nakamura, K., and Taniguchi, T. (2000) T-cell-mediated regulation of osteoclastogenesis by signalling cross- talk between RANKL and IFN-gamma. *Nature* **408**(6812), 600-5.