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CD40-Induced TRAF degradation in immune regulation

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CD40-INDUCED TRAF DEGRADATION IN IMMUNE REGULATION

by

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An Abstract

Of a thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree
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Thesis Supervisor: Professor Gail A. Bishop

ABSTRACT

CD40 is a TNF receptor superfamily (TNFRSF) member central to the development of many aspects of the adaptive immune response. CD40 signaling promotes adaptive immunity in part by inducing the expression of cytokines, chemokines, and various adhesion and co-stimulatory molecules. The family of cytoplasmic adapter proteins, the TNFR-associated factors (TRAFs), serve as major mediators of TNFRSF pathways. CD40 regulates itself in part via the signaling induced degradation of TRAF2 and TRAF3. However, the effect of CD40-induced TRAF degradation on other TRAF dependent pathways is unknown. Here I provide evidence that CD40-mediated degradation of TRAFs 2 and 3 also influences the responsiveness of immune cells to CD40-independent, TRAF2- and 3-dependent pathways.

LMP1 is a functional mimic of CD40, but signals to B lymphocytes in an amplified and sustained manner. LMP1 contributes to the development of B cell lymphoma in immunosuppressed patients, and may exacerbate flares of certain autoimmune diseases. The cytoplasmic (CY) domain of LMP1 binds TRAF2 with lower avidity than the CY domain of CD40, and TRAF2 is needed for CD40-mediated degradation of TRAFs 2 and 3. LMP1 doesn't induce TRAF degradation, and employs TRAF3 as a positive mediator of cell signaling, whereas CD40 signals are inhibited by TRAF3. Here, I tested the hypothesis that relative affinity for TRAF2, and/or distinct sequence differences in the TRAF2/3 binding sites of CD40 vs. LMP1, controls the disparate ways in which CD40 and LMP1 use TRAFs 2 and 3. The results revealed that TRAF binding

affinity and TRAF binding site sequence dictate a distinct subset of CD40 vs. LMP1 signaling properties.

The E3 ubiquitin ligases, cIAP1 and cIAP2, have been reported to play a crucial role in CD40 signaling. Because LMP1 is a mimic of CD40 signals, I hypothesized that LMP1 requires the cIAPs for signaling. To elucidate the role of the cIAPs in CD40 and LMP1 signaling, I specifically depleted the cIAPs and found that the cIAPs are differentially utilized in CD40 and LMP1 signaling. I also sought to further the understanding of the molecular underpinnings of how CD40, but not LMP1 signaling induces TRAF2 and TRAF3 degradation upon signaling. To do this, I investigated the ability of various CD40 and LMP1 mutants to induce TRAF degradation in distinct TRAF or cIAP deficient models. I found that neither a high TRAF2 binding potential nor the presence of the cIAP molecules are required for this process. Thus, this work reveals important insights into the molecular mechanisms of and role of CD40-mediated TRAF degradation in the immune system.

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Graduate College
The University of Iowa
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CERTIFICATE OF APPROVAL

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PH.D. THESIS

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This is to certify that the Ph.D. thesis of

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Twenty years from now you will be more disappointed by the things that you didn't do than by the ones you did do. So throw off the bowlines. Sail away from the safe harbor. Catch the trade winds in your sails. Explore. Dream. Discover.

Mark Twain

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INTRODUCTION

CD40 and CD154

CD40, a type I transmembrane glycoprotein of the TNFR superfamily (1-3), was originally identified as both a B cell costimulatory receptor and a tumor antigen expressed in bladder carcinoma (4). CD40 is expressed constitutively on antigen presenting cells (APC) such as B cells, dendritic cells (DCs), and macrophages, and under specific conditions can also be expressed on epithelial cells, monocytes, basophils, eosinophils (5), T cells, endothelium, neuronal cells (6), and smooth muscle cells (2, 3, 7). Additional studies have demonstrated that while fibroblasts and keratinocytes constitutively express low levels of CD40, expression is upregulated in response to interferon γ (8, 9).

The natural ligand for CD40, CD154, was isolated from membranes of activated T cells (10-13). CD154 is a type II transmembrane glycoprotein of the TNF family which is transiently expressed on activated $CD4^+$ T cells, but can also be induced upon activation on $CD8^+$ T cells, mast cells, basophils, eosinophils (14), epithelial cells, NK cells (15) and platelets (2, 7, 16). Additional studies show that endothelial cells, smooth muscle cells, and macrophages constitutively express low levels of CD154, which is upregulated upon cell activation (7, 17). It has also been suggested that DCs can express CD154 mRNA, although their CD154 protein levels are barely detectable (18).

CD40 in Immunity

CD40 is central to many facets of the immune response, including promotion of humoral immunity mediated by B cells, as well as stimulation of APC function. The ligation of CD40 on APCs represents a point at which activated helper T cells and activated APCs cooperate to induce a highly regulated, specific, and powerful adaptive immune response (19). An APC can promote immune responses only if it has internalized antigen in the presence of danger signals produced by microbes or injured tissues (20). The second partner in this productive interaction is T cells. For an immune response to occur, these cells must recognize antigen displayed by the activated APC and then be instructed by the APC to mediate an appropriate immune response. During development, the immune system allows only helper T cells with low reactivity toward self to survive (21). Therefore, in most cases, the cognate T cells present do not permit the immune response to be self-directed. Thus, APC and T cell collaborations serve as a checkpoint in the promotion or restraint of immune responses. The CD40 molecule is central to this checkpoint, and serves as a pivotal decision point for the adaptive immune system (19).

CD40 signaling induces a variety of changes within APCs which influence the character of developing adaptive immune responses. CD40 shapes adaptive responses by inducing the secretion of many cytokines and chemokines, as well as by up-regulating the expression of costimulatory molecules on APCs (22, 23). In addition, CD40 signaling induces the rapid polyubiquitin-associated degradation of TRAFs 2 and 3 (24). This degradation serves to modulate the

signals of multiple TRAF2- or TRAF3-dependent receptors, not only those of the CD40 receptor itself (data shown below).

B cells have many important roles in the immune system, including antibody production and the generation of memory B cells that respond to specific antigens (25), as well as antigen presentation, T cell costimulation, and the production of many cytokines (25-27). B cell-produced cytokines activate dendritic cells (DCs), increasing their antigen presenting capability, and influence other immune cells, including T lymphocytes (25). CD40 is a critical transmembrane protein which mediates these important B cell functions. Interestingly, the Epstein-Barr virus (EBV) encodes a protein called latent membrane protein 1 (LMP1) that acts as a functional mimic of CD40, but LMP1 does so in a dysregulated manner (2, 28-30). Thus, comparing and contrasting these proteins provides valuable insight into both the normal regulatory controls of CD40 function, as well as the pathogenic effects of LMP1. As LMP1 activity can lead to direct B cell transformation (31-33), it is important to understand the similarities and differences between CD40 and LMP1 signaling to identify new therapeutic targets and aid in the development of new treatments.

CD40 in B cell activation

Proliferation

Human mutations in CD154 are responsible for the immunodeficiency X-linked hyper-IgM syndrome (HIGM) (34-36). HIGM patients have normal B cell

numbers, as do $CD40^{-/-}$ and $CD154^{-/-}$ mice, indicating that CD40 signaling is not required for B cell proliferation (37, 38). However, numerous *in vitro* studies demonstrate that the CD40-CD154 interaction induces B cell proliferation, particularly in the presence of IL-4 and/or signals through the B cell antigen receptor (BCR). Purified human tonsil or peripheral blood B cells, whether from normal controls or HIGM patients, or mouse spleen, are induced to proliferate by anti-CD40 agonistic Ab, soluble recombinant CD154, or CD154-expressing cells (10, 11, 39), and CD154 cooperates with IL-4 or LPS in this function (10, 35, 39-41). These results indicate that CD40-CD154 interaction may contribute to B cell proliferation, but is not absolutely required.

Early CD40-mediated activation events

Various studies have implicated CD40 in the activation of a variety of kinases, including p38, c-jun kinase (JNK), phosphatidyl inositol 3-kinase (PI3K), ERK, and the Src family kinases Syk, Lyn, and Fyk (7, 42). Mechanisms of pathway activation are discussed in detail below. CD40 signals also activate numerous transcription factors, including canonical and non-canonical members of the NF-κB family, AP-1, C/EBP, E2F, BSAP, STAT6, and NF-AT (28, 43).

Cytokine and Chemokine production

The production of cytokines and chemokines is an important function of B cells and other APCs, as these factors help regulate a number of processes,

including Ig isotype switching, antigen presentation, and T cell differentiation (26). CD40 signaling has been shown to induce the production of cytokines and chemokines, including IL-2, IL-6, IL-7, IL-10, IL-12, IL-15, IL-17, IFNγ, lymphotoxin-α (TNF-β), and TNF-α (3, 7, 26, 27). Collectively, these molecules participate in mediating CD40's effect on immunity.

CD40 in humoral immunity

Numerous studies have demonstrated an important role for CD40 in T cell-dependent (TD) humoral immune responses. $CD40^{-/-}$ mice immunized with the TD antigen DNP-OVA fail to develop primary and secondary DNP-OVA-specific antibody responses except for α-DNP-IgM (37). $CD154^{-/-}$ mice immunized with the TD antigens KLH, HEL, cytochrome-c, or sheep erythrocytes (SRBC) also lack antigen-specific responses (38, 44). These mice display significantly reduced levels of IL-4 and IFN-γ, supporting a key role for CD40 in TD antigen-specific antibody responses (44). These phenotypes mirror what is seen in HIGM patients, who also fail to respond to TD antigens (38, 45). Adoptively transferred activated $CD154^{-/-}$ T cells cannot expand in recipient mice upon antigen challenge (44). Furthermore, mice immunized with either SRBC or KLH and administered α-CD154 blocking Ab fail to respond to antigen challenge (46). Similar results were obtained in α-CD154 Ab-treated mice immunized with the TD Ag heterologous Ig (46). Together, these studies show that disruption of the CD40-CD154 interaction results in the inability to mount an antibody response to a wide variety of TD antigens.

In contrast to the above studies implicating a critical role for CD40 and CD154 in TD humoral immunity, CD40-mediated activation is not required to induce Ab responses to T cell-independent (TI) stimuli. $CD40^{-/-}$ mice immunized with the TI antigens TNP-LPS or TNP-Ficoll mount an α-TNP Ab response similar to that of WT mice (37), as do $CD154^{-/-}$ mice immunized with TNP-Ficoll (38). Similarily, mice immunized with TNP-Ficoll in the presence of blocking α-CD154 Ab develop a normal α-TNP Ab response (46). Thus, CD40-CD154 interactions are not required for Ab responses to TI antigens.

The CD40-CD154 interaction also plays a critical role in cell-mediated immune responses to infection. HIGM patients and $CD154^{-/-}$ mice have enhanced susceptibility to *Toxoplasma gondii* infection (47, 48). Both $CD40^{-/-}$ and $CD154^{-/-}$ mice exhibit impaired *Chlamydia muridarum* infection clearance compared to WT mice, as well as increased susceptibility to *Leishmania* infection (49, 50). $CD40^{-/-}$ mice also have increased susceptibility and mortality to West Nile virus infection (51). Thus, CD40 signaling is required for effective intracellular pathogen clearance.

CD40-mediated antibody production

HIGM patients have normal to elevated serum IgM (34, 36). $CD40^{-/-}$ and $CD154^{-/-}$ mice have normal to increased serum IgM levels (37, 38). Adoptively transferred T cells activated in WT and $CD40^{-/-}$ mice both drive IgM production in lightly irradiated $CD40^{-/-}$ hosts (52), as do T cells isolated from $CD154^{-/-}$ mice cultured with splenic B cells isolated from WT mice (38). Together, these studies

reveal that CD40 signals are not absolutely required for IgM production *in vivo.* It is likely that TI stimuli, which preferentially induce IgM, keep serum levels of this Ig isotype high, particularly as CD40-deficient B cells have a marked defect in switching to other Ig isotypes. However, it is clear that CD40 signals can enhance IgM production. Mouse B cells produce IgM *in vitro* when stimulated with fixed activated T cells expressing CD154, recombinant membrane-bound CD154, or insect cells expressing CD154 (41, 53, 54). Maximal IgM levels are obtained with the addition of additional signals, such as IL-4 and/or IL-5 (41, 53), or stimulation via the BCR or MHC class II (54). *In vivo* administration of α-CD154 Ab blocks IgM production in response to TD antigens (46), supporting the hypothesis that IgM produced in the absence of CD40 signals derives mainly from TI stimuli. Addition of a soluble CD40-Ig fusion protein at the initiation of culture with activated T cell plasma membranes inhibits IgM production even in the presence of IL-4 and IL-5 (13). However, addition of CD40-Ig at day 1 or 2 of culture is less inhibitory, suggesting that CD40 signaling plays most of its role in IgM induction early in the immune response (13).

Isotype switching

HIGM patients have significantly decreased levels of the IgG isotypes, IgA, and IgE (34, 36), indicating that the CD40-CD154 interaction is required for isotype switching, and $CD40^{-/-}$ or $CD154^{-/-}$ mice show this same defect (37, 38). Adoptively transferred T cells from $CD154^{-/-}$ mice are unable to induce the production of serum IgG1, IgG2a, and IgG2b in recipient animals (52).

Furthermore, the *in vivo* administration of α-CD154 Ab significantly reduces the levels of IgG1, IgG2a, IgG2b, IgG3, and IgE in response to KLH challenge (46). Additionally, purified mouse or human B cells cultured with IL-4, IL-10, or TGF-β and either α-CD40 Ab or CV-1/EBNA cells expressing CD154 can produce IgG, IgE, and IgA (10, 55, 56). This effect can be inhibited with the addition of soluble CD40-Ig (10). Collectively, these studies show that CD40 signaling induces and is required for Ig isotype switching.

Many events take place to induce isotype switching: Ig heavy chain gene germline transcript expression, proliferation, expression of activation-induced deaminase (AID), deletional switch recombination, and the expression of mature Ig transcripts (42, 57, 58). CD40 signaling has been demonstrated to play a role in each of these steps. Stimulation of purified human peripheral blood or tonsil B cells, mouse splenic B cells, or mouse B lymphoma cells with either α-CD40 Ab, soluble CD154, or insect cells expressing CD154 results in the expression of germline transcripts γ1, γ2, γ3, γ4, α1, α2, and ε (58-62). Induction of these transcripts is enhanced by the addition of IL-4 (58-62). Although germline transcription can occur in resting B cells, the other steps involved in isotype switching do not occur without the induction of cell proliferation (57, 63), to which CD40 contributes (discussed above). AID is critical for isotype switching (58, 63, 64). Mouse splenic B cells treated with α-CD40 Ab and IL-4 express AID (58, 62, 65). After AID induction, deletional switch recombination occurs, in which the IgM switch region (Sμ) is replaced by the switch region of a different Ig gene (57, 63). Sμ-Sγ1 and Sμ-Sε switch recombination occurs in mouse splenic B cells in

response to α-CD40 Ab and IL-4 (58, 62). The final step is the expression of mature transcripts. Mouse splenic B cells stimulated with α-CD40 Ab and IL-4 induce the expression of the mature transcripts Iμ-Cε and Iμ-Cγ1 (58, 62).

Germinal center formation and B cell memory

Both $CD40^{-/-}$ and $CD154^{-/-}$ mice lack GCs, and these mice cannot be induced to form GCs by immunization with a TD antigen (37, 38, 45). Similarly, immunized mice treated with α-CD154 antibody do not form GCs (45). GCs are the major site of memory B cell formation (38, 45), so it is not surprising that HIGM patients and $CD40^{-/-}$ and $CD154^{-/-}$ mice also lack memory B cells (2, 38, 45). A functional memory B cell response generates an isotype-switched, high affinity rapid Ig recall response to antigen challenge (45). If KLH-immunized recipient mice receive adoptive transfer of splenic B cells isolated from TNP immunized, α-CD154 Ab treated mice, they fail to respond to a subsequent TNP-KLH challenge, demonstrating that blocking CD40-CD154 interaction abrogates the generation of memory B cells (45). Prevention of GC B cell apoptosis has been suggested as important to the generation of B cell memory (45). Treatment of GC B cells or B cell lines in culture with agonistic α-CD40 antibody or CD154-expressing cells or membranes prevents apoptosis (45, 66, 67). This CD40-mediated function is dependent upon TRAF6-mediated PI3 kinase and Akt activation (67). Whether the generation of B cell memory depends upon CD40-mediated prevention of apoptosis or not remains to be determined.

Antigen Presentation

DCs are highly efficient at antigen uptake by nonspecific phagocytosis/endocytosis, and subsequent antigen presentation. However, while B cells are inefficient in nonspecific endocytosis, they are extremely effective in BCR-mediated antigen uptake, and can effectively process and present BCR-specific antigen to T cells (26, 68). Through its potent stimulation of B cells, CD40 signaling plays an important role in B cell antigen presentation. CD40 signals upregulate CD80 and CD86, as well as MHC class II and adhesion molecules (including CD23, CD30, CD54, Fas, ICAM, and LFA-1), enhancing T cell costimulation and T-B cell interactions (3, 7, 26, 42, 69). CD40-mediated production of cytokines also increases the effectiveness of B cell-mediated T cell activation (70).

The EBV-encoded CD40 mimic, LMP1

Epstein-Barr virus (EBV) is a double-stranded DNA virus of the γ-herpesvirus family that latently infects >90% of the global human population (33, 71-74). Transmitted orally (73, 75, 76), primary EBV infection is usually asymptomatic in children but often results in infectious mononucleosis in adolescents or adults (73, 76, 77). The primary target of EBV is B lymphocytes, but EBV can also infect T lymphocytes and epithelial cells (73, 78, 79), as well as monocytes and DCs (80-82). During primary infection, EBV infects resting B lymphocytes and expresses viral gene products driving proliferation of infected

cells (2, 33, 83, 84). EBV establishes latency with limited viral gene expression in resting memory B cells (74, 85, 86). Reactivation of the virus during the lytic cycle results in virus particle production and release, allowing viral transmission (73, 74, 79). Abnormal EBV reactivation in immunocompromised individuals is strongly associated with a number of human malignancies (79, 84, 87). In this situation, EBV expresses six nuclear EBNA proteins, three membrane proteins, including the key transforming protein LMP1, and two EBER RNAs (79, 84).

LMP1, an integral membrane protein (88), is expressed during infection and reactivation, but not during latency (73, 86). Experiments using a conditional LMP1 expression system demonstrate that LMP1 drives the proliferation of EBV-infected B cells (33, 89). Additional studies show that LMP1 is necessary for EBV entry into the lytic cycle and virus release into cultural supernatant. However, the loss of LMP1 does not affect expression of late-lytic proteins, EBV genome amplification, or the formation of the intranuclear nucleocapsid (84, 86).

Although EBV expresses a number of proteins during abnormal reactivation, LMP1 has drawn much attention over the years since it was established that LMP1 is an oncogene and is required for EBV-mediated B cell transformation (31-33). Thus, it is no surprise that LMP1 and components of LMP1-mediated signaling pathways have attracted interest as possible therapeutic targets. LMP1 is a functional mimic of CD40 effects in B cells (2, 28-30), so study of its signaling pathways also reveals new insights about the normal regulation of CD40 pathways (discussed in detail below).

LMP1 *in vivo*

Several groups have developed LMP1 transgenic (tg) mouse strains. LMP1 expression has been targeted to B cells using a transgene under the control of the IgH enhancer/promoter (90, 91). These LMP1 tg mice were created on both normal ($CD40^{+/+}$) and $CD40^{-/-}$ backgrounds (90, 91). LMP1 tg mice on a $CD40^{+/+}$ background have an increased incidence of lymphoma and enlarged spleens compared to control mice (90). However, these mice have normal ratios of mature to immature splenic B cells (91), and the lymphomas occur only in aged mice. LMP1 tg mice on a $CD40^{+/+}$ background also have increased production of IgM and IgG1 (91). When LMP1 tg mice on the $CD40^{+/+}$ and $CD40^{-/-}$ backgrounds are immunized with the TD antigen NP-CGG, both groups of mice produce α-NP IgM and IgG1, but only the LMP1 tg mice on the $CD40^{+/+}$ background produce high affinity α-NP IgG1 (91). Interestingly, both groups of mice fail to form GCs (91).

Another variety of LMP1 tg mouse expresses a chimera consisting of the extracellular and transmembrane domains of mouse CD40 (mCD40) and the cytoplasmic tail of LMP1 (mCD40-LMP1) (92). Expression of this chimera is controlled by the MHC class II promoter, and thus the chimeric molecule is expressed in all types of APC (92). The mCD40-LMP1 tg mouse was created on a $CD40^{-/-}$ background so that endogenous CD154 activates only mCD40-LMP1 (92). These mice have normal total lymphocyte numbers and B cell percentages, but form spontaneous GCs (92). The mCD40-LMP1 mice also exhibit normal isotype switching and TD antigen responses to TNP-KLH immunization, and

unlike the WT LMP1 tg mice, the mCD40-LMP1 mice show affinity maturation (92). This difference is most likely due to expression of the LMP1 molecules on DCs, as CD40 expression on DCs may contribute to optimal T cell activation and B cell help, and the WT LMP1 tg mice do not restore CD40 or CD40-like signals to non-B cells (90). Additionally, mCD40-LMP1 mice have increased spleen and lymph node size compared to controls and produce enhanced α-double stranded DNA and α-phospholipid Abs, indicative of autoimmunity (92).

Lastly, a mCD40-LMP1 mouse was developed in which mCD40-LMP1 is induced through CD19cre-mediated recombination (78). These mice have normal B cell development and differentiation, and on a CD40-deficient background have similar or higher levels of serum IgG1, IgG2a, and IgG2b compared to WT mice (78). Finally, in response to immunization with the TD antigen NP-CGG, these mice have an increase in GC formation and NP-specific Abs (78). Together, these models demonstrate that LMP1 can effectively mimic CD40 requirements for humoral immunity *in vivo*, but its dysregulated signals lead to an abnormal phenotype.

Mechanisms of CD40 and LMP1 Signal Transduction

The functions and signaling of CD40 and LMP1 have both been studied in epithelial cells and B lymphocytes. Initial studies focused on transiently transfected epithelial cells, as they are easier to manipulate experimentally than B lymphocytes. However, subsequent studies clearly indicate that CD40 and LMP1 signaling can vary depending on the cell type used. As the B cell is the

cell in which LMP1 establishes latency this section will focus on studies performed with B lymphocytes.

CD40 and LMP1 structure

CD40 is a single pass transmembrane receptor with a relatively short cytoplasmic (CY) tail and a cysteine rich extracellular domain (ECD). The ECD is engaged by its ligand, CD154, which is expressed on the surface of activated T cells (10). Engagement by CD154 clusters CD40 monomers into complexes (93). This clustering, and CD40's subsequent relocation to membrane lipid rafts, initiates CD40 signal transduction (94). Structurally, LMP1 is very different from CD40. LMP1 is a transmembrane protein with two CY tails and no ECD per se; LMP1 has 6 membrane-spanning domains with small portions that are extracellular (29, 95). LMP1 initiates constitutive, ligand-independent signaling via its self-oligomerizing membrane-spanning domains (29). As demonstrated in epithelial cells, the short amino-terminal CY tail of LMP1 anchors the protein to the cell membrane and mediates its proteasome-dependent degradation (96). The second CY domain of LMP1, the carboxy terminal (COOH) tail, is necessary and sufficient in mediating its signals to B cells (95, 97, 98). Both CD40 and LMP1 lack any known enzymatic activity (28). Hence, both rely on the adaptor molecules, TRAFs, for signaling (29, 99, 100) (Table 1). To date, TRAFs 1, 2, 3, 5, and 6 have been shown to play various roles in signals via both CD40 and LMP1 (70, 100, 101). CD40 has three known TRAF binding sites (TBS), including a membrane proximal region important for direct TRAF6 binding and a more

distal TBS that mediates the overlapping binding of TRAFs 1, 2, 3, and 5, as well as a second, more distal region important for TRAF2 association (102, 103). LMP1 also has an overlapping binding site for TRAFs 1, 2, 3, and 5 (101, 104, 105). However, while TRAF6 appears to play an important role in LMP1 functions in epithelial cells (106), whether it delivers signals by direct and/or indirect association with LMP1 in B cells has not been formally established.

The importance of the LMP1 COOH CY tail was first appreciated fifteen years ago, as a mutant EBV encoding an LMP1 with the COOH CY tail deleted is unable to transform primary human B cells (32). In a subsequent study, amino acids (aa) 188-231 of the C-terminal CY domain of LMP1 were shown to be critical for this process (107). The overlapping TRAF1, 2, 3 and 5 TBS was later reported to reside in this region, implicating the TRAFs as important to the process of transformation (108). Two regions of LMP1, now commonly called C-terminal activating region 1 (CTAR1) (residues 194-232) and CTAR2 (residues 351-386), were identified as important components in LMP1-mediated cellular transformation (109). Mutagenesis studies demonstrate that both CTAR1 and 2 regions mediate NF-κB activation, and both regions are required for LMP1-mediated CD40 and CD54 upregulation (109). CTAR1 was subsequently shown to be required for association of TRAFs 1 and 5 (104, 110). The LMP1 TBS, in contrast to that of CD40, has enhanced association with TRAF3 compared to TRAF2 (104).

Results of early studies noted that the CTAR1 and CTAR2 regions of LMP1 could independently activate NF-κB, and both CTARs cooperate to make

NF-κB signaling greater in amplitude than signaling stimulated by either CTAR alone (111). Later studies demonstrate that both regions cooperate to mediate upregulation of costimulatory and adhesion molecules, IgM secretion, JNK activation, and TRAF binding (97, 112). Interestingly, while CTAR2 does not directly associate with TRAF3, it cooperates with CTAR1 to regulate association of the TBS in CTAR1 with TRAF3 (97, 112). CTAR2 regulation of CTAR1-mediated TRAF binding might explain the differences seen between signaling by CTAR1 or CTAR2 alone, versus signals emanating from cooperation between the two regions, as CTAR1 and 2 cooperation in IgM secretion and JNK and NF-κB activation requires TRAF3. In the absence of CTAR2, there is increased TRAF1 and 2 binding to CTAR1, this might interfere with TRAF3-mediated cooperation between CTAR1 and CTAR2 (112). The finding that TRAF3 binding is regulated by regions outside of the TRAF2 and 3 TBS illustrates how separate regions of CD40 or LMP1 function together rather than independently. Thus, signaling molecules bound to the CTAR2 region of LMP1 could affect the binding of different molecules to the CTAR1 region. Hence, the CTAR2 region can influence signals emanating from the CTAR1 region. A question remains as to whether the influence of the CTAR2 region on TRAF binding is mediated directly or indirectly through uncharacterized CTAR2 binding molecules.

Unlike CD40, LMP1 lacks a typical TRAF6 binding sequence, and direct endogenous TRAF6 association with LMP1 is not detectable by typical immunoprecipitation techniques (112). However, *in vitro* studies using epithelial and fibroblastic cells suggest that LMP1 can utilize TRAF6 in signal transduction

(106, 113), and our own preliminary data indicate that LMP1 cannot signal in TRAF6$^{-/-}$ B cells (KMA, JPG, L. Stunz, GAB., unpublished). Interestingly, it was recently shown that although CD40 can bind TRAF6, not all TRAF6-dependent CD40 signals to B cells require this direct interaction. Restoration of a subset of CD40 signals in TRAF6$^{-/-}$ B cells with TRAF6 molecules lacking the receptor-binding TRAF-C domain, shows that cytoplasmic TRAF6 may suffice to deliver important signals, as it does for toll-like receptors (114).

Signaling pathways of CD40 and LMP1

As described above, LMP1 is a remarkable functional mimic of CD40, although LMP1 signals in an amplified and sustained manner in comparison to CD40 (28, 29). Thus, it is not surprising that both receptors activate many of the same signaling pathways. Both CD40 and LMP1 activate the kinases Akt, ERK, JNK, and the canonical and non-canonical NF-κB signaling pathways (109, 112, 115-123). As both molecules activate similar early molecular pathways, the different outcomes of CD40 and LMP1 signaling cannot simply be ascribed to a difference in pathways activated by each receptor. As described in an earlier section, the LMP1 COOH CY tail mediates immune dysregulation via its amplified signaling capacity, independently of constitutive signaling (92, 98). Thus, an important question is how LMP1 dysregulates the signaling pathways it shares with CD40 to produce the observed dramatic difference in downstream effects. As we discuss below, key to the answer is an understanding of the distinct ways in which the two molecules use the TRAF adaptor proteins.

CD40 vs. LMP1 in TRAF2 and 3 use

TRAF3 was initially identified through its ability to associate with mouse and human CD40 (124, 125). Subsequently, TRAFs 1, 2, and 3 were shown to associate with CD40 upon signaling in B cells (94, 126) and TRAFs 5 and 6 were also implicated in CD40 B cell signaling (127-129). As discussed above, LMP1 can also associate with and/or utilize each of these TRAFs in downstream signaling. However, although LMP1 is a CD40 functional mimic, and both can activate the signaling pathways described above, there are surprising, distinct, and sometimes sharply contrasting ways in which they use and regulate each of the TRAFs that make important contributions to the differences in their ultimate effects on B cells. These differences are helping to elucidate why LMP1 has pathogenic effects on B cells, and may suggest new therapeutic ways to exploit differences between the mechanisms used by CD40 and LMP1 to impact B cell functions.

Initial structure-function studies of the TBS indicated its importance in NF-κB and JNK activation by both CD40 and LMP1 (122, 130, 131). As TRAF2 was implicated by overexpression studies in epithelial cells as a potent receptor-independent activator of these pathways, while TRAF3 was not, it was long assumed that TRAF2 is the major positive regulator of both CD40 and LMP1 signals (132). However, subsequent studies in B cells reveal a different and more complex picture. Inducibly overexpressed TRAF2 enhances CD40 signaling in B cells, while inducible overexpressed TRAF3 inhibits these signals (133). However, the overlapping nature of the TRAF1, 2, 3 and 5 TBS of CD40 –

as well as LMP1 creates the unavoidable complication that mutations in this site, or exogenous provision of wild-type or mutant forms of these TRAFs, affects more than the association and function of just one type of TRAF. Thus, interpretation of such studies cannot be completely clear, even when performed in relevant cell types. To overcome these obstacles, and obstacles posed by the early lethality of mice totally deficient in TRAFs 2, 3, or 6, we created TRAF deficient B cell lines by targeting the TRAF genes via homologous recombination (100). Using this approach, TRAF2 was shown to play important roles in CD40, but not LMP1 signaling. A report characterizing the effects of TRAF2 deficiency on CD40 signaling in the mouse B cell line A20 demonstrated that TRAF2 is important for several facets of the CD40 signal (134). CD40-mediated JNK activation and CD40- BCR synergy are abolished with the loss of TRAF2 (134). Furthermore, substantial defects in CD40-mediated CD80 upregulation, TRAF3 degradation, and antibody secretion are detected in TRAF2 deficient cells (134). A subsequent study utilizing a B cell conditional TRAF2-deficient mouse demonstrated that TRAF2 is necessary for CD40-mediated IκBα phosphorylation and degradation and B cell proliferation (135). Surprisingly, TRAF2 is not required for LMP1-mediated JNK, p38, or NF-κB activation (136). TRAF2 is also dispensable for LMP1-mediated antibody secretion, and CD23 and CD80 upregulation (136). In a human lymphoma cell line, however, TRAF2 knockdown by siRNA abolishes LMP1-mediated protection from apoptosis and NF-κB reporter gene activity (137), which implies a role for TRAF2 in LMP1 signals. This study may reflect differences between mice and humans, but there is no

precedent for such differences in either CD40 or LMP1 signals to B cells. Both parameters used to judge the involvement of TRAF2 in the human cell line study occurred several days following LMP1 signaling. Because no early or intermediate events were measured, it is difficult to determine if the results can be directly attributed to the loss of TRAF2 during LMP1 signaling. These results may reflect the TRAF2 dependency of another factor, induced by LMP1 signals, which would be unable to mediate NF-κB activation and protect from apoptosis without TRAF2. A precedent for this type of indirect effect of TRAF2 is provided by the finding that TRAF2 is required for CD40-mediated IgM secretion indirectly, by transduction of signals through TNFR2, stimulated by CD40-induced TNF secretion (138).

TRAF3 deficient B cell lines reveal surprisingly disparate roles for TRAF3 in CD40 and LMP1 signaling. TRAF3 is an important negative regulator of CD40, but unexpectedly, a necessary positive mediator of LMP1 signals. Activation of JNK by LMP1 is nearly abolished in TRAF3 deficient B cells whereas CD40-mediated JNK activation is enhanced (136). Furthermore, LMP1, but not CD40-mediated p38 and NF-κB1 activation, CD23 and CD80 upregulation, and antibody secretion are dependent on TRAF3 (136). TRAF3 may serve as a negative regulator of CD40 signals by competing with TRAF2 for binding to the TBS or by interfering with the binding of other signaling proteins to separate regions of CD40 (138, 139). These results exemplify the receptor-dependent context of TRAF function.

Both CD40 and LMP1 have a TBS which binds TRAFs 1, 2, 3, and 5 (127, 130, 140). Although both molecules share a common core motif of PxQxT in this TBS, CD40 and LMP1 differ slightly in sequence around and within the variable residues of this core motif (141). As mentioned earlier, LMP1 binds more TRAF3 than CD40 whereas CD40 binds more TRAF2 (98, 112). Interestingly, analysis via X-ray crystallography demonstrates that both CD40 and LMP1 bind to the same molecular crevice of TRAF3, but LMP1 initiates more hydrogen bonds with TRAF3 than does CD40 (142). This finding suggests that the increased binding of LMP1 to TRAF3 compared to that of CD40 is driven by its unique TRAF1, 2, 3, and 5 TBS. Could these subtle structural differences contribute to the sharp functional contrasts described above? In a recent study, the common TBS for TRAFs 1, 2, 3 and 5 was swapped between CD40 and LMP1, creating a CD40 molecule with the TBS of LMP1 (CD40ADD) and an LMP1 molecule with the TBS of CD40 (LMP1AEDL) (141). Interestingly, the CD40ADD molecule does not display enhanced TRAF3 binding compared to normal CD40 in B cells. This result implies that regions outside of the TBS of LMP1 also make important contributions to the enhanced binding of TRAF3 to LMP1. Because the CTAR2 region of LMP1 affects CTAR1-mediated TRAF binding, the region most likely to influence LMP1-mediated TRAF3 binding, either directly or indirectly, is CTAR2. In support of this idea, the LMP1AEDL molecule displays the TRAF3 binding ability of normal LMP1, despite having the TBS of CD40 (141). These results are discussed in detail below.

The TBS does play a major role in mediating TRAF2 interactions with both CD40 and LMP1 (141). Upon signaling, the binding of TRAF2 to LMP1AEDL is enhanced to CD40 levels whereas the binding of TRAF2 to CD40ADD is reduced to LMP1 levels. Interestingly, the increased TRAF2 binding of LMP1AEDL is accompanied by acquisition of the ability to induce TRAF2 degradation upon signaling (141). Overall, these results demonstrate that the canonical TBS of CD40 versus LMP1 is functionally different, in ways additional to and more dramatic than might be expected from their subtle structural differences. One intriguing hypothesis is that the differences observed between each receptor's TBS-mediated signals is due to the ability of each receptor to induce the degradation of TRAFs 2 and 3 upon signaling.

TRAF/cIAP degradation

As noted above, much is known about the structural and molecular requirements of CD40 and LMP1 signaling. However, CD40-mediated activation is highly regulated, while LMP1 signaling is pathogenically dysregulated. It was initially noted that the amount of TRAF2 detected in B cell lysates decreases in proportion to the time a CD40 signal is allowed to persist (143). The authors reasoned that TRAF2, because it mediates CD40 signals, is a target for a signaling-induced negative regulatory mechanism. Hence they imagined that decreasing the amount of TRAF2 available to CD40 via CD40-induced TRAF2 degradation would dampen CD40 signals. Subsequently, Brown et al. discovered that CD40 signaling also induces the degradation of TRAF3 (98). Interestingly,

the authors noted that LMP1 signals do not similarly mediate TRAF2 and TRAF3 degradation. This led to the hypothesis that LMP1 displays an enhanced signaling capacity at least in part because it fails to induce TRAF2 and 3 degradation upon signaling. The process of CD40-induced TRAF2 and 3 degradation was subsequently shown to be dependent upon TRAF2 RING-mediated polyubiquitination, the E3 ubiquitin ligases cIAP1 and cIAP2, and proteasome-induced degradation (29, 98, 144, 145). Recently, we have also observed that cIAP1 degradation is induced in B cells within 30 minutes of CD40 signaling. cIAP2 may also play a role, as the two cIAP molecules have been reported to be redundant in many other ways (145). The degradation of the cIAPs, like TRAF2, may restrict the amplitude and duration of CD40 signals, because the cIAPs, like TRAF2, are themselves partial mediators of some CD40 signals (data shown below and (141 912).

Consistent with the hypothesis that degradation of TRAFs 2 and 3 is an important CD40-mediated homeostatic mechanism, blocking TRAF degradation with the use of a proteasome inhibitor amplifies CD40-mediated phosphorylation of c-Jun, a substrate of JNK (24). TRAF degradation initiated by signaling to mouse B cells through endogenous CD40, hours prior to signaling through a transfected human CD40, substantially blunts human CD40-mediated JNK and NF-κB activation (144). Phosphorylation of IκBα, a TRAF6 dependent event, is unaffected by the pre-stimulation, while two TRAF2 dependent events, (JNK activation and IκBα degradation) are reduced (144). CD40 signaling induces degradation of TRAFs 2 and 3 but not other TRAFs, and the signaling outcomes

affected by pre-stimulation have been demonstrated to depend upon TRAF2. Thus, these findings point to TRAF degradation as a direct negative regulatory mechanism for TRAF2-dependent CD40 signals. Interestingly, TRAF3 deficient mouse B cells retain enhanced CD40-mediated MAPK activation when pretreated with proteasome inhibitors, despite the absence of TRAF3 (data shown below). This demonstrates that TRAF3 (138, 139) is not required for TRAF2 degradation to serve as a negative regulator.

Recently, Matsuzawa et al. concluded that all receptors that activate MAPK/SAPK pathways require the translocation of multi-protein complexes from membrane bound receptors to the cytosol. The authors reason that because MAPK/SAPK substrates are located in the cytosol, this translocation is necessary for the membrane tethered proteins to gain access to their substrates (145). This predicts that TRAF3 regulates CD40 signaling by acting as a brake to slow the release of CD40-bound multi-subunit complexes, thus preventing premature CD40 signaling. According to this scenario, a possible role for the degradation of TRAF3, a negative regulator of CD40, is to allow release of the signaling protein complex from CD40. However, inhibiting TRAF degradation with proteasome inhibitors results in amplified CD40-mediated JNK and p38 activation in B cells (146) and JNK, p38, and ERK activation in primary human macrophages (data shown below), which is the opposite result from that predicted by the above hypothesis. If CD40-induced TRAF degradation mediates the protein complex translocation necessary for signaling, all TRAF-utilizing receptors should need to induce TRAF degradation to signal. However, LMP1, which doesn't initiate the

degradation of any TRAF, stimulates MAPK/SAPK activation more robustly than does CD40 (98), and this activation is not amplified by proteasome inhibitor treatment (data shown below).

TRAFs 1, 5, and 6 in CD40 and LMP1 signaling

TRAF1 deficient B cell lines demonstrate no discernible role for this TRAF in LMP1 signaling. However, in contrast, TRAF1 cooperates with TRAF2 to enhance CD40 signaling to B cells (123). Both CD40-mediated NF-κB (canonical and non-canonical) and JNK activation are abolished with combined TRAF1 and TRAF2 deficiency, whereas TRAF2 deficiency alone causes only a partial defect. However, TRAF1 deficiency alone does not affect CD40-mediated activation of these pathways, or CD40-mediated p38, Akt, and ERK activation (123). This is not entirely surprising, as TRAF1 is the only TRAF to lack a RING domain and Zn finger domains. The ability of TRAF1 to enhance TRAF2-dependent CD40 signals may be explained by TRAF1's ability to inhibit CD40 signaling-induced TRAF2 degradation (123).

The loss of TRAF5 affects LMP1 signals much than those delivered by CD40 (101, 147). In TRAF5 deficient primary mouse B cells, modest defects in CD40-mediated costimulatory and adhesion molecule upregulation, as well as proliferation, were noted. These effects were observed days after CD40 stimulation, so it is difficult to know if TRAF5 deficiency affects the CD40 signal directly, particularly as there is no evidence for a role for TRAF5 in the CD40-mediated early activation of NF-κB and JNK pathways (147).

In contrast, evidence has emerged for a major direct role of TRAF5 in LMP1 signaling. Kraus et al. observed that many phenotypes of the CD40LMP1 transgenic mouse described above. The phenotype associated with the CD40LMP1 transgenic mouse (92) is markedly reduced or ablated by the absence of TRAF5 (101). Defects were also noted in many immediate LMP1 signaling events, including the phosphorylation of JNK, Akt, and IκBα (101), pointing to a direct role for TRAF5 in LMP1 signals. TRAF5 does not mediate the degradation of IκBα, the activation of non-canonical NF-κB, and TAK1 phosphorylation. Because TRAF5 plays a major role in LMP1 signaling and at best only a very modest role in CD40 function, TRAF5 is an attractive therapeutic target for LMP1-driven pathogenesis. Targeting LMP1 signals via TRAF5 could spare key functions of CD40.

TRAF6 plays a central role in CD40 signals. CD40-mediated JNK activation, IκBα degradation, and CD80 upregulation are abolished in TRAF6-deficient B cells (114). Interestingly, a mutant TRAF6 which is unable to bind CD40 restores CD40-mediated JNK activation and CD80 upregulation, but not canonical NF-κB activation. This demonstrates that CD40 does not require direct TRAF6 binding to mediate some of its signals, similar to toll-like receptor signaling (114).

Much less is known about exactly how TRAF6 interacts with LMP1. Early studies in epithelial cells indicated that TRAF6 is important to LMP1 signals (106), and preliminary work from our lab indicates a necessary role for TRAF6 in LMP1 signaling to B cells (KMA, JPG and GAB, unpublished). In this work we

have also found that TRAF6 association with LMP1, whether direct or indirect, requires the TBS within the CTAR1 region of LMP1 (KMA, JPG and GAB, unpublished and data).

In vivo structural studies

Almost a decade ago, two groups published work describing the *in vivo* roles of the TRAF1/2/3/5 and TRAF6 TBS in CD40 functions (62, 148). In each study, transgenic mouse CD40 CY tails were mutated at the putative TRAF1, 2, 3, and 5 TBS, the TRAF6 TBS, or both to abrogate the binding of various TRAF molecules. A caveat was noted earlier; this mutation was defined by overexpression in epithelial cells, and when expressed in B cells, this CD40 mutant actually binds reduced but detectable levels of TRAF2 and normal amounts of TRAF3 (149); TRAF1 and 5 binding was never tested in B cells. While both laboratories expressed these molecules in CD40 deficient mice, Ahonen et al. drove transgene expression with a MHC class II promoter while Jabara et al. made use of a B cell specific promoter. When expressed via an MHC class II promoter, all wildtype and mutant CD40s are expressed in macrophages and DCs, as well as B cells. These other APC types can influence the behavior of immune cells, via the elaboration of cytokines/chemokines and through direct interactions of costimulatory molecule ligand-receptor pairs. CD40 signaling is an important driver of cytokine/chemokine secretion and surface molecule upregulation in macrophages and DCs, as well as B cells. In addition to affecting CD40 signals in B cells, the mutant CD40 molecules also alter the

activity of the other APCs in which they are expressed. Hence, considering that APCs interact with and contribute to B cell activation, mutant CD40 molecules may indirectly affect B cell function by disrupting normal APC activity. Jabara et al. avoided this complexity with the use of a B cell specific promoter. However, because these mice are from a CD40 deficient background, all other cell types, including macrophages and DCs, lack CD40. The lack of CD40 expression by macrophages and DCs may affect APC ability to properly influence B cells during immune responses.

Another important difference between the two sets of transgenic mice is that Ahonen et al. utilized a human CD40 ECD in place of the mouse CD40 ECD. The human CD40 ECD may affect CD40 signals as mouse CD154 does not engage human CD40 as effectively as it does mouse CD40 (150). Thus, differences between each group's findings may be explained by the differences in how each transgene is expressed and which ECD the transgenic molecule contains.

TBS mutagenesis studies are useful, but have an Achilles' heel, inasmuch as other as yet uncharacterized proteins important in CD40 and/or LMP1 signaling may also be affected. These unknown proteins may bind directly or indirectly to the receptors. Mutation of a TBS can possibly affect both direct and indirect interactions of a molecule with CD40 or LMP1 in many ways. First, an unknown molecule could bind directly to the TBS region. Hence, a mutation to the TBS may abrogate both the binding of the uncharacterized molecule as well as the respective TRAF. Second, a TBS mutation may affect the conformation of

the receptor as a whole. This conformational change could affect the binding of uncharacterized proteins to regions outside of the TBS. Third, signals which rely on cooperation of the TRAF1, 2, 3, and 5 TBS and the TRAF6 TBS would be abrogated by mutagenesis of one or the other TBS.

While the TRAF1, 2, 3, and 5 TBS mutation had no effect on CD40's ability to mediate GC formation in one study (148), the TRAF1, 2, 3, and 5 TBS mutant CD40 only partially restores this response in CD40 deficient mice in another (62). Mice expressing CD40 with the TRAF1, 2, 3, and 5 TBS mutation have increased antigen specific serum IgG1 following vaccination in one (148), while this mutation completely abolishes the response in another (62). Furthermore, while the TRAF6 TBS mutation in the mice of Ahonen et al. decreases antigen specific serum IgG1, this mutation increases this response in the mice of Jabara et. al. (62, 148). Both studies share similar conclusions in regards to *in vitro* signaling of B cells. This suggests that either early CD40-mediated signaling events do not completely determine antigen specific immune responses *in vivo* or that the differences in regard to mutant CD40 expression in other APCs affect antigen specific immune responses. Hence, the differences noted may be explained by the differences in CD40 expression, as well as strength of CD154-CD40 binding between the differently designed transgenic mice.

More recently, a potential role for a non-canonical TRAF2 binding site C terminal to the canonical TRAF1, 2, 3, and 5 TBS was suggested. A human CD40 ECD, with murine CD40 CY tail truncation mutant containing only the non-

canonical TRAF2 TBS was found to substitute for normal CD40 in some *in vivo* functions (151). CD40 deficient mice expressing this mutant CD40 transgene under the control of the MHC class II promoter show effective antigen specific antibody responses to vaccination. This CD40 mutant transgene is also able to restore B cell CD40-mediated proliferation, CD23 and CD80 upregulation, plasma cell differentiation, and antibody secretion. The non-canonical TRAF2 TBS is unable to mediate CD40-induced GC formation, however. Interestingly, mice expressing CD40 containing only the non-canonical TRAF2 TBS display enhanced high affinity antigen specific antibody responses over that of the almost non-existent response induced by vaccination of mice expressing a double TRAF1, 2, 3, and 5 TBS and TRAF6 TBS mutant CD40 with an intact non-canonical TRAF2 TBS. Thus, while the mutant CD40 which contains only the non-canonical TBS can mediate CD40 signals in isolation, it remains to be determined if it can when other regions, such as the TRAF6 TBS and canonical TRAF1, 2, 3, and 5 TBS of CD40 are present.

Recently, a role for TRAFs 2 and 3 in mediating CD40 signaling induced class switch recombination was proposed (42). Using CD40 deficient mice expressing CD40 mutant molecules which selectively lack either the binding of TRAF2 or TRAF3, Jabara et al. demonstrate that serum IgG1 and IgE, GC number, and serum antigen specific IgG1 induced by vaccination are decreased in comparison to normal CD40 transgenic controls. *In vitro,* CD40-mediated B cell proliferation and IgG1 and IgE secretion are also reduced when either TRAF2 or TRAF3 are prevented from binding CD40. Furthermore, markers of

class switch recombination, such as CD40-induced expression of AID, are decreased in mice expressing CD40 molecules unable to bind TRAF2 or TRAF3. Mice expressing CD40 molecules unable to bind TRAF3 display the most severe defect in this regard. Collectively, these data suggest that TRAF2 and TRAF3 play overlapping roles in mediating some CD40 functions *in vivo.* A pro-signaling role for TRAF3 in CD40 signaling was not observed in *in vitro* studies utilizing TRAF3 deficient B cells, but these studies did not examine isotype switching (136). Alternatively, the mutations used by Jabara et al. may disrupt interactions of both characterized and uncharacterized CD40 binding proteins. In support of this notion, the amounts of TRAF6 that each mutant CD40 is able to co-immunoprecipitate varies noticeably(42). This further demonstrates that mutations to a receptor can affect the binding of proteins to regions separate from that which was mutated.

To date, *in vivo* structure-function analyses for LMP1 have not been conducted. It may be possible to approach these questions using the transgenic mouse models described above, although the same caveats in data interpretation discussed above would apply.

Additional molecules in CD40 and LMP1 signaling

Though many CD40 and LMP1 studies have focused on the TRAFs, other molecules have also been implicated in mediating signals (Fig. 1). In the human B cell line, BJAB, the Janus kinase JAK3 is phosphorylated within 10 minutes of CD40 signaling and small amounts of JAK3 can be co-immunoprecipitated with

CD40 (152). However, JAK3 is not phosphorylated following CD40 stimulation of normal human resting B cells (153). JAK3-LMP1 associations and/or JAK3 activation has not been demonstrated to occur for LMP1 (154).

NF-κB inducing kinase (NIK) has been implicated in CD40-mediated proliferation, antibody secretion, and the phosphorylation of IκBα by studies of a naturally occurring strain of mice expressing a spontaneous mutant of NIK (aly) (155). In the aly strain, CD40-mediated non-canonical NF-κB activation is reduced (121). However, all B cell activation signals are decreased in aly mice, including those delivered via the BCR (155). Additionally, subsequent studies in $NIK^{-/-}$ mice reveal defects primarily in signaling by other receptors (156). Using NIK deficient Ramos cell lines and lymphoblastoid cells treated with siRNA against NIK, both CD40-mediated canonical and non-canonical NF-κB pathways are compromised (157). Thus, the physiologic role played by NIK in CD40 and LMP1 signaling to B cells is unresolved. Future experiments focusing upon primary human B cells could be helpful in resolving this question.

More than a decade ago, the kinases IKKα and IKKβ were shown to mediate CD40 induced IκBα phosphorylation in a Burkitt lymphoma cell line (158). Overexpression of TRAF2 in these cells induces IKK activation, suggesting that CD40 might mediate this activation through TRAF2 (158). Results from mouse B cell lines contradict this hypothesis by demonstrating CD40-mediated IKK phosphorylation is intact in TRAF2-deficient but not TRAF6-deficient B cells (114). No such studies have been performed for LMP1. It would be interesting to determine if LMP1 activates the IKKs and, if so, the TRAF

requirements of LMP1-mediated IKK activation. These studies would provide important therapeutically relevant information as to how LMP1 mediates canonical NF-κB activation.

Act1, BANK, and Cbl-b have all been identified as negative regulators of CD40 signaling. Primary mouse B cells from spleens of one strain of Act1 deficient mice display increased CD40-mediated ERK and NF-κB activation (159). Immunoprecipitation of Act1 in a human B cell line demonstrates an association of Act1 with CD40, TRAF2, and TRAF3 following stimulation (159). But a subsequent, different Act1-deficient mouse showed only an increase in IL-17 production with no effects on CD40 signaling (160). As with NIK, the story of Act1's role in CD40 signaling is not clear. Complementary approaches, such as studies of CD40 signaling with siRNA-mediated Act1 depletion in primary human tonsil or peripheral blood B cells, may help in resolving this confusing picture. Whether Act1 plays any role in LMP1 signaling to B cells is unknown.

Splenic B cells from BANK deficient mice display enhanced CD40-mediated Akt phosphorylation. This enhanced Akt activation results in enhanced proliferation and survival of BANK-deficient B cells (161). Cbl-b deficient splenic B cells show decreased TRAF2 and TRAF3 ubiquitination and degradation, and Cbl-b is recruited to CD40 via TRAF2 (162). In this study, the authors suggest that the lack of CD40-induced TRAF degradation in the Cbl-b deficient mouse results in enhanced CD40-mediated JNK and canonical NF-κB activation.

The ubiquitin ligases, cIAP1, cIAP2, and Ubc13, associate with CD40 via TRAF2 and participate in CD40 signals. Ubc13 is required for the CD40-

mediated activation of the JNK and canonical NF-κB pathways, and the cIAP molecules are reportedly required for CD40 signaling-induced TRAF2 and TRAF3 K48-linked polyubiquitination and degradation, as well as for CD40-mediated activation of JNK and p38 (145). An association of these molecules with LMP1 in B cells has not been reported.

CD40 utilizes a variety of proteins in MAPK/SAPK pathways. MAPKAPK-2, a factor implicated in the ERK and p38 pathways, is activated by CD40 signaling (131, 163). CD40-mediated MEK1/2 phosphorylation, an event upstream of ERK activation, requires Tpl2, and may associate with CD40 via TRAF6 (164). Tpl2 complexes with ABIN-2, another molecule necessary for CD40-mediated ERK activation (165). TAK1 is necessary for CD40 mediated B cell proliferation, and is phosphorylated by CD40 signals in a TRAF6-dependent manner, and MEKK1 co-immunoprecipitates with CD40 and plays a necessary role in CD40-mediated JNK, p38, and ERK activation (145, 166). IKKγ, MKK4, and MKK3/6 have all been demonstrated to participate in CD40 signaling (145). Germinal center kinase, (GCK), is activated by phosphorylation upon CD40 signaling and correlates with the activation of JNK, suggesting that GCK mediates the activation of JNK induced by CD40 (167).

In a TRADD deficient Burkitt lymphoma cell line, DG75, LMP1 cannot activate the canonical NF-κB pathway mediator IKKβ. In DG75, TRADD was demonstrated to weakly immunoprecipitate with LMP1 (168). However, no LMP1-TRADD association was detected in mouse B cell lines (112), despite clear demonstration of TRADD-TNFR1 association. It remains to be determined

whether LMP1 associates with TRADD in primary B cells. No role for TRADD in CD40 signaling has been reported.

Though many of the MAPK/SAPK intermediates and TRADD have been implicated in CD40 and/or LMP1 signaling, their unique roles in the ultimate outcomes of the receptor signal remain to be determined. More work is needed to delineate the exact role of these molecules in CD40 and LMP1 functions. It is of clinical interest to determine if both receptors utilize the same molecules in the same ways, given the precedent of differing ways of utilizing TRAFs. If differences are found, these distinct substrates may be useful in therapies targeting LMP1.

In summary, CD40 signals are carefully restricted, whereas those of LMP1 are amplified and sustained. CD40 signals need to be tightly regulated because CD40 plays a major and central role in activation of multiple arms of immune responses. Dysregulated CD40 signals, and LMP1 signals, can result in autoimmunity and/or B cell malignancies. As the phenotype of the CD40LMP1 transgenic mouse illustrates, pathogenic effects induced by LMP1 require the difference in kinetics and amplitude that are characteristic of signaling through this molecule. Though CD40 and LMP1 use TRAFs in signaling, they use them differently. This differential use contributes to the functional differences of the CD40 and LMP1 signals. CD40 signaling also tightly regulates itself by inducing the degradation of TRAF2, TRAF3, cIAP1, and, most likely, cIAP2. This mechanism may have effects on subsequent signals from other receptors which require these molecules for signaling. It is thus interesting to speculate that CD40

signaling induces TRAF/cIAP degradation, not only to regulate itself, but to regulate other TRAF/cIAP-dependent pathways important in immune cell activation. Though CD40 and LMP1 signaling promotes similar downstream events, distinct signaling regulatory mechanisms and molecules used by LMP1 or CD40 make all the difference between critical normal immune activating signals, and pathogenic outcomes.

The purpose of the work described in this dissertation is to further explore how differences in TRAF degradation and the TRAF binding sites of CD40 and LMP1 impact the functional outcomes mediated by each receptor's signaling. This dissertation addresses four distinct questions: 1. How does CD40 signaling-induced TRAF degradation negatively regulate CD40 signaling? 2. What is the impact of CD40-mediated TRAF degradation upon other TRAF-dependent pathways? 3. What is the role of the TBS in CD40 and LMP1 signaling and TRAF degradation? 4. Why does CD40 signaling induce TRAF degradation whereas LMP1 signaling does not?

We have found that CD40-mediated TRAF2 and TRAF3 degradation can subsequently affect the signals of other TRAF2 or 3 utilizing pathways. Furthermore, we found that the specific TBS of CD40 and LMP1 make important contributions to the differences observed in regards to each receptor's signaling. Lastly, we find that the mechanism for TRAF degradation has redundancy, which further supports my idea about the importance of TRAF degradation in immunity.

	TRAF1	TRAF2	TRAF3	TRAF5	TRAF6
LMP1	NOT REQUIRED	NOT REQUIRED	JNK, P38, NFKB1, NFKB2?	JNK, NFKB1, AKT	JNK, NFKB1, P38, AKT?, NFKB2?
CD40	ENHANCES TRAF2 SIGNALS BY INHIBITING TRAF DEGRADATION	JNK, TRAF DEGRADATION, NFKB1&2, P38	NEGATIVE REGULATOR	LITTLE TO NO ROLE IN SIGNALING	JNK, ERK? NFKB1&2, AKT?

Table 1. Roles of TRAF molecules in CD40 versus LMP1 signaling pathways to B cells.

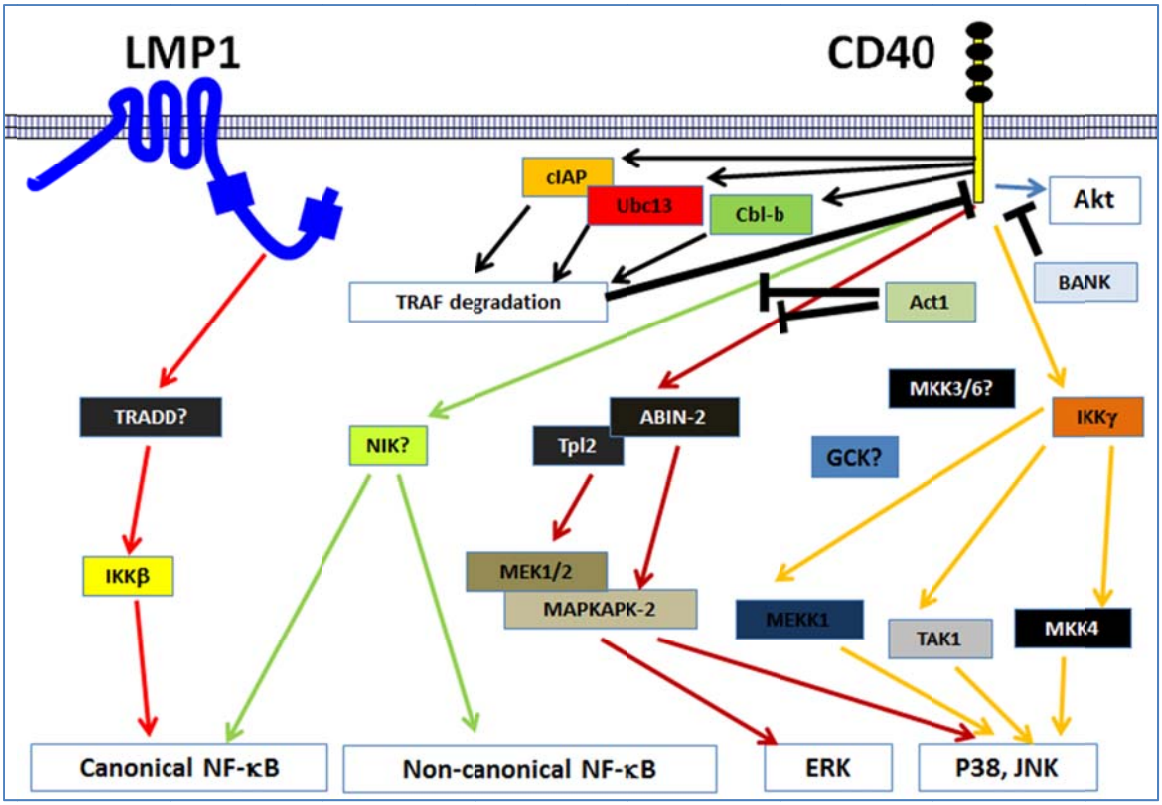


Figure 1. Non-TRAF molecules used in the activation of CD40 and LMP1 signaling pathways.
Outlined here are molecules reportedly used in the transduction of CD40 or LMP1 signals in B cells. The absence of a molecule on this diagram does not indicate that it has no role in signaling; only those molecules verified to contribute to CD40 or LMP1 signaling to B cells are shown. Molecules denoted with a question mark indicate either that the exact role in a pathway has yet to be determined or that the role in signaling is not yet established, due to contradictory findings among different reports.

CHAPTER II

MATERIALS AND METHODS

Cells

The mouse B cell lines M12.4.1, CH12.LX, TRAF3-/- CH12.LX and A20 have been previously described (169-171). B cell lines were maintained in RPMI 1640, 10 μM 2–mercaptoethanol (GIBCO, Grand Island, NY) with 10% heat-inactivated FCS (Atlanta Biologicals, Lawrenceville, GA) and antibiotics (medium referred to as BCM-10). Cells transfected with either WT human CD40 (hCD40), hCD40LMP1, hCD40LMP1AEDL, or hCD40ADD were maintained in 400 μg/mL G418 disulfate (Research Products International, Mt. Prospect, IL). All stably transfected subclones were generated by electroporation as previously described (172). The human monocytic leukemia cell line THP-1 has been previously described (173). Normal human peripheral blood monocytes, obtained from the DeGowin Blood Center (University of Iowa Hospitals & Clinics, Iowa City, IA) and isolated from leukocyte reduction system cones as described (174), were differentiated into macrophages by culture with BCM-10 supplemented with GM-CSF (50 ng/ml) (Peprotech, Rocky Hill, NJ) as described (175, 176). Primary mouse splenic B cells were isolated as previously described (101).

Antibodies and Reagents

Rabbit anti-TRAF2 Ab was purchased from Medical and Biological Laboratories Co. Ltd. (Nagoya, Japan). Rabbit anti-TRAF3 (H122) and rabbit

anti-hCD40 (H-120) Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-actin mAb (MAB150R) was purchased from Chemicon Int. (Temecula, CA). Rabbit anti-IκBα and anti-pJNK1/2 Abs were purchased from Cell Signaling Technology, Inc. (Beverly, MA) Cells were stimulated through hCD40 using the anti-hCD40 mAb G28.5 (mouse IgG1) produced from a hybridoma obtained from the ATCC (Manassas, VA). MOPC21 isotype control mouse IgG1 used for mouse experiments was purchased from eBiosciences and the mIgG1 isotype control mAb (20F11.3) for use in human experiments was made in-house. The hamster anti-mouse (m)CD40 IgM mAb, HM40.3, was purchased from eBiosciences (San Diego, CA). The isotype control Armenian Hamster IgM was also purchased from eBiosciences. Poly I:C, LPS, and R848 were obtained from Invivogen (San Diego, CA). Human GM-CSF and TNFα were obtained from Peprotech (Rocky Hill, NJ).

Co-immunoprecipitation

Ten x 10^6 B cells were stimulated with G28.5 (10μg/10 μl Dynal beads, Invitrogen) for 15 minutes and receptors immunoprecipitated (IP) as previously described (114, 136). Amounts of TRAF2 and TRAF3 co-immunoprecipitated with the various receptors were detected by Western blotting as described below.

DNA constructs

WT-hCD40 and the hCD40LMP1 chimeric DNA constructs have been previously described (98). The hCD40ADD and hCD40LMP1AEDL molecules

were created from the WT-hCD40 and hCD40LMP1 constructs by PCR SOEing (177). The primers for the hCD40LMP1AEDL joint were 5'-caagagaccttagattctggc and 5'-aatctaaggtctcttgttgagg. The hCD40ADD mutation was made with the primer 5'- gtgcaggcgactgatgat using a construct containing the WT hCD40 CY domain into which a noncoding change had been engineered to create a new SacI restriction enzyme recognition sequence. This construct (hCD40TSS) allows removal of just the CY domain of CD40. The WT hCD40 CY domain in the hCD40TSS construct was then replaced with the mutated ADD CY sequence via standard cloning techniques.

TRAF degradation

The TRAF degradation assay has been described previously (98, 138, 146). Briefly, 3 x 10^6 cells were washed in RPMI 1640, resuspended in 2ml of BCM-10 and added to a 6 well tissue culture plate. The cells were stimulated with 10ug/ml G28.5, 1C10, or isotype control mAbs (EM95 + MOPC31c), then incubated for the indicated time periods at 37^o C. After chilling plates to 4 ^{0}C, cells and medium were transferred into 1.5 ml Eppendorf tubes and centrifuged at 4^{o}C for 2 min at 200 x g. Whole cell lysates were prepared by removing the supernatant and adding 200 μl of 2X SDS-PAGE loading dye to the pellet. The lysates were sonicated with 15 pulses at 90% duty cycle, output 1.5. The samples were boiled for 5 min at 95^{o}C and kept on ice prior to gel loading.

MAPK phosphorylation and NFkB1 activation

1 x 10^6 cells were washed in RPMI 1640, resuspended in 1ml of BCM-10 in 1.5ml Eppendorf tubes, and rested for 1hr. at 37^{o}C. The cells were then stimulated for 5, 10, 15, 30, 45, or 75 minutes with α-hCD40 (G28.5), α-mCD40 (1C10), isotype control (EM95 + MOPC31c) mAbs, or medium alone. Whole cell lysates were prepared as described for the TRAF degradation assay. The presence of total IKBα or phospho-JNK (pJNK), pP38, or pERK in lysates was detected using a phospho-specific or total specific Abs on Western blots of samples subjected to SDS-PAGE, as described (144).

Western blotting

5-10 μL of sample were resolved on 10% SDS-PAGE. Proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA), and membranes were blocked with 10% non-fat dried milk in 20mM TRIS buffered saline with 0.1 % TWEEN (TBST) for 1 hour. The membranes were washed 3 times in TBST and incubated overnight at 4^{o}C with one of the above Abs. Blots were incubated with secondary Abs for 1 hour and developed with an enhanced chemiluminescence system (Supersignal West Pico; Pierce Biotechnology, Rockford, IL). To accurately compare and quantify the amount of protein analyzed, Western blot chemiluminescence was read on a low-light digital

camera (LAS-1000 or LAS-3000, Fujifilm Medical Systems USA, Stamford, CT), using the Image Gauge program (Fujifilm Medical Systems).

IgM secretion

Quantitation of IgM-secreting transfected CH12.LX cells stimulated through WT hCD40, hCD40LMP1, hCD40LMP1AEDL, or hCD40ADD was accomplished as previously described (134, 178). Briefly, CH12.LX transfected subclones were cultured in 96-well plates (1.5 $x10^3$/well) with various stimuli. Anti-CD40 and isotype control mAbs were used at a final concentration of 2 μg/ml. SRBC (Elmira Biologicals, Iowa City, IA) at a final concentration of 0.1% were used as a source of the Ag for which the Ig of CH12.LX is specific (phosphatidylcholine) (179). Triplicate cultures were incubated for 72 h, and viable cells were counted by Trypan blue exclusion. IgM-secreting cells were enumerated as SRBC hemolytic plaques/million recovered viable cells, as previously described (178).

ELISA

To quantify IL-6 production, transfected subclones of CH12.LX cells (1 x 10^5 cells/ml, 1ml total volume in a 24 well plate) were co-cultured with anti-CD40 or isotype control mAbs (2μg/ml) for 48 h in BCM-10, and the supernatants were examined for IL-6 by ELISA as previously described (150). To measure TNF-

α production, transfected subclones of M12.4.1 B cells were resuspended in BCM-10 (5 x 10^5 cells/ml, 200μl/well) and placed in an anti-TNF-coated 96-well flat-bottom plate with anti-CD40 or isotype control Abs. We have found that this 'in plate' assay is necessary because B cells rapidly bind the TNFα that they secrete (31). Cells were stimulated with the various Ab for 4h, after which culture supernatants were assayed for TNF-α by ELISA as previously described (24).

NFκB luciferase reporter assay

The NF-κB Luciferase Reporter Assay has been described previously (136). M12.4.1 B cell subclones (2 x 10^7 cells) expressing hCD40, hCD40LMP1, hCD40LMP1AEDL, or hCD40ADD were electroporated at 225 V and 50 mS with 38 μg 4X NF-κB *firefly* luciferase (a gift from Dr. Edward Clark, University of Washington, Seattle, WA) and 2 μg *renilla* (null) luciferase reporter plasmids (Promega). After transfection, cells were rested in medium containing 15% FCS overnight at 37°C. Cells were washed and resuspended in BCM-10, aliquoted into 24-well plates (2 ml/well), and stimulated with 10 μg of anti-mCD40, anti-hCD40, or isotype control mAbs for 6 h at 37°C. Cell lysates were analyzed for the *firefly* and *renilla* luciferase activities with the Dual Luciferase Reporter Assay kit (Promega) on a TD-20/20 Luminometer (Turner Designs) following the manufacturer's protocol.

CHAPTER III

CD40-MEDIATED TRAF DEGRADATION IN IMMUNE REGULATION

Rationale

TRAF2 recruitment to CD40 induces TRAF2 RING domain-dependent K48 polyubiquitination and rapid proteasome-dependent degradation of both itself and TRAF3, but this does not occur upon LMP1 signaling (24, 98, 134). It has thus been proposed that CD40-induced TRAF2 and/or TRAF3 degradation is an important mechanism for restraining CD40 signaling cascades (24, 180). Consistent with this hypothesis, inhibition of proteasome-mediated TRAF degradation during CD40 signaling leads to amplified c-Jun phosphorylation in as little as 15 minutes (24). Furthermore, the initial phases of TRAF2 degradation following CD40 engagement appear to promote the dissolution of the CD40 signaling complex (145). TRAF2 can be co-immunoprecipitated with CD40 following 10, but not 30 minutes of CD40 stimulation. Interestingly, CD40-TRAF2 associations at 30 minutes post-signaling are observed in proteasome-inhibited B cells. Because TRAF degradation regulates the CD40 signal within minutes (24) and its effects last for hours (180), we suggest that TRAF degradation inhibits receptor signaling at several points. In its initial phase, TRAF degradation may rapidly break up CD40-TRAF signaling complexes, terminating a burst of CD40 signaling. To further test this idea, we wished to determine the effects of proteasome inhibition on CD40 and hCD40LMP1-mediated MAPK activation. If CD40 mediated TRAF degradation rapidly negatively regulates signal delivery by

breaking up CD40-TRAF signaling complexes then we would expect CD40, but not hCD40LMP1-mediated MAPK activation to be prolonged by proteasome inhibition. Because CD40LMP1 signaling does not induce TRAF degradation we would expect proteasome-inhibited LMP1 signals to be unaltered in comparison to controls. Progressive TRAF degradation might also regulate CD40-dependent and CD40-independent but TRAF-dependent pathways mediated by other receptors, by significantly lowering the total cytoplasmic amount of TRAF available. As TRAF2 is a major positive regulator of CD40 signaling, it has been assumed that CD40-mediated TRAF2 degradation limits the CD40 signal (24). However, CD40 signaling also induces the degradation of its negative regulator, TRAF3 (98), which is seemingly paradoxical as a mechanism for restraining CD40 signaling. We thus tested the hypothesis that CD40-mediated TRAF degradation can restrain the function of other TRAF3-utilizing receptors by investigating the effects of CD40 pre-stimulation on subsequent TRAF-dependent and independent pathways. If CD40 signaling can regulate other pathways via TRAF degradation, we would expect that only the TRAF-dependent pathways would be affected by CD40 signaling. Furthermore, we predict that any effects mediated by CD40 pre-stimulation would depend on the proteasome, as does TRAF degradation.

Results

Effect of proteasome inhibition on CD40-mediated MAPK activation

CD40-induced TRAF degradation is a proteasome-dependent process (24, 98). CD40-TRAF2 complexes can be detected at 10 minutes, but not 30 minutes following CD40 engagement. If the activity of the proteasome is inhibited, however, CD40-TRAF2 complexes remain intact for at least 30 minutes (145). Interestingly, we noticed that the peak of CD40-mediated JNK, p38, and ERK activation correlated with the 10 minute point of maximal CD40-TRAF2 association (Fig. 2A and 1B). To determine if the extended CD40-TRAF2 association observed with proteasome inhibition led to sustained kinase activation upon proteasome inhibition, we assessed the effects of proteasome inhibition on CD40-mediated MAPK activation (Fig. 2A). We chose to examine primary human macrophages because they are representative of the myeloid lineage, they express CD40, and can be feasibly derived in large numbers from PBMCs. To test the possibility that the proteasome inhibitor MG132 non-specifically amplifies MAPK activation in a TRAF degradation-independent manner, we also tested the effects of MG132 treatment on CD40LMP1-mediated JNK activation in mouse B cells (Fig. 2B). Signaling via the LMP1 cytoplasmic C-terminus mimics CD40 in an amplified and sustained manner, and this is associated with a failure of LMP1 to induce TRAF degradation (98). Because hCD40LMP1 signaling does not induce TRAF degradation (98), hCD40LMP1 signals should not be enhanced by proteasome inhibition.

As shown in Figure 2A and 1B, MG132 treatment of mouse B cells and human macrophages inhibited the proteasome-dependent degradation of IκBα in response to endogenous mCD40. This result demonstrates the efficacy of MG132 in proteasome inhibition. Figure 2A demonstrates that CD40-mediated JNK, p38, and ERK activation in primary human macrophages was amplified and sustained with MG132 treatment. Also, in B cells, mCD40-mediated JNK and p38 phosphorylation was amplified and sustained in the MG132-treated group in comparison to vehicle-only controls (Fig. 2B). As predicted, MG132 treatment did not amplify hCD40LMP1-mediated MAPK signals (Fig. 2B), in the same clone of cells, demonstrating the specificity of MG132. Thus, inhibition of proteasome activity resulted in extension and amplification of CD40-mediated MAPK signals, congruent with the extended CD40-TRAF2 associations previously observed (145).

Effect of TRAF3 degradation on the CD40 signal

CD40 requires TRAF2 for a portion of its signals (134), whereas TRAF3 is a negative regulator of CD40 signaling to B cells (134, 136). Because CD40 uses TRAF2 as a positive mediator for signaling, it could be assumed that CD40 signaling is constrained primarily via the degradation of TRAF2, as TRAF6, the other major positive mediator of CD40 signals, is not degraded (180). However, as CD40 signaling also leads to TRAF3 degradation (98), this event may also be involved in regulating CD40 signaling via various direct or indirect mechanisms. If TRAF3 degradation plays an important role in restraining the CD40 signal, CD40

signals in TRAF3-deficient B cells should not be amplified and sustained by MG132 treatment. Endogenous CD40-induced TRAF degradation in mouse B cells abrogates the ability of a transfected hCD40 to subsequently activate the JNK and NFκB1 pathways (144). If TRAF3 degradation plays no role in restraining subsequent hCD40 signals, then mCD40 signaling in TRAF3-deficient B cells should inhibit any subsequent signals through transfected hCD40.

As expected, TRAF3-deficient B cells displayed amplified and sustained CD40-mediated JNK activation in comparison to TRAF3-sufficient counterparts ((136) and Fig. 3A versus Fig. 2B). Following proteasome inhibition, CD40 signals in TRAF3-deficient B cells were further amplified and sustained in comparison to the vehicle-only control samples (Fig. 3A). Furthermore, mCD40 pre-stimulation of TRAF3 deficient B cells was still able to abrogate subsequent hCD40-mediated JNK activation (Fig. 3B). These results indicate that neither the presence of TRAF3 nor TRAF3 degradation is required for the proteasome-dependent restraint of CD40 signaling.

Effect of CD40-mediated TRAF3 degradation on LMP1 signals

TRAF3 is a powerful negative regulator of the CD40 signal (Fig. 3A vs. Fig. 2B and (133, 136)). As demonstrated above, TRAF3 degradation has no role in the proteasome-dependent restraint of the CD40 signal (Fig. 3). Yet, CD40 signaling also induces the degradation of its negative signaling regulator, TRAF3. (134). I thus hypothesized that CD40-mediated TRAF3 degradation may regulate

other TRAF3-dependent pathways. To test this hypothesis, we examined signaling mediated to B cells by the C-terminal cytoplasmic domain of LMP1, as it has been demonstrated that LMP1-mediated B cell activation is independent of TRAF2, but instead requires TRAF3 as a positive mediator (134, 136). To study early LMP1-mediated signals, which cannot be done with the unpredictably intermittent signaling mediated by the self-aggregating Wt LMP1(95), we used the chimeric CD40LMP1 receptor that has proved valuable in prior studies of LMP1-mediated signaling events (98, 136).

Mouse B cells expressing hCD40LMP1 were stimulated for 1 hour through either endogenous mCD40 (which induces TRAF degradation), hCD40LMP1 (which does not induce TRAF degradation), or with a control stimulus. After washing and resting, the cells were re-stimulated via either endogenous Wt mCD40 or hCD40LMP1 (Fig. 4A). As shown in Figure 4B, pre-stimulation through mCD40 abrogated the ability of hCD40LMP1 to activate JNK. This inhibition was specific to CD40 pre-stimulation, because conversely, hCD40LMP1 signaling did not preclude the ability of subsequent mCD40 signals to activate JNK (Fig. 4C).

If CD40 pre-stimulation regulates the signals of other receptors via TRAF degradation we predict that the signals of a TRAF2 or TRAF3-independent receptor would not be lessened. In support of this notion, CD40 pre-stimulation of mouse splenic B cells amplified - not abrogated - the TRAF-independent signals of the B cell receptor (BCR) (Fig. 9). Taken together, these results demonstrate that CD40 signaling can regulate the signals of a CD40-independent, TRAF3-

dependent pathway.

Based upon results presented in Figure 4, we hypothesized that receptor-mediated TRAF degradation significantly shapes immune responses by regulating the availability of TRAFs for use in signal propagation. This predicts that the inhibiting effects of TRAF2/3 degradation will last for at least several hours following CD40 signaling. We thus addressed the duration of signal inhibition mediated by CD40-induced TRAF degradation, as well as the correlation between the extent of TRAF loss and the degree of inhibition achieved. hCD40LMP1^{+} mouse B cells were pre-stimulated through mCD40 or with isotype control Ab as in Figure 4A. At various times following the rest period, cells were stimulated through hCD40LMP1 for 30 minutes, which is the point of maximal hCD40LMP1-mediated JNK activation. In comparison to lysates from unstimulated cells in the far left lane of Figure 5A, lysates from cells receiving CD40 pre-stimulation displayed drastic reductions in the amount of TRAF3. When comparing samples from cells stimulated through hCD40LMP1 at 2, 4, 6, and 8 hours post-rest, a partial restoration of TRAF3 levels was observed (Fig.5A). mCD40 pre-stimulation drastically reduced the level of hCD40LMP1-mediated JNK activation, for up to at least 8 hours following the rest period (Fig.5A). Importantly, there was a tight correlation between the slight recovery in hCD40LMP1-mediated JNK activation at later times and the increase of TRAF3 levels following the rest period (Fig.5A). Together, these data demonstrate that the effect of CD40-mediated TRAF3 degradation on the TRAF3-dependent hCD40LMP1 signaling pathway was robust and durable. Notably, the ability of

CD40 signaling to regulate a TRAF3-dependent pathway was tightly correlated with the amount of TRAF3 degradation induced by CD40 signaling.

To further test whether the effects of CD40 pre-stimulation on subsequent hCD40LMP1 signaling were due to TRAF degradation, we performed the experiment outlined in Figure 4A, using cells treated with or without a proteasome inhibitor during mCD40 pre-stimulation. Figure 5B demonstrates that MG132 treatment during mCD40 pre-stimulation significantly inhibited the ability of CD40 signaling to induce TRAF3 degradation. This inhibition was partial, because a less than maximally effective concentration of MG132 was used to minimize stress-mediated JNK activation. Despite this, hCD40LMP1-mediated JNK activation was significantly restored with MG132 treatment (Fig. 5B). This effect on JNK activation was specific to proteasome inhibition, because MG132 treatment during hCD40LMP1 signaling did not amplify CD40LMP1-mediated JNK activation (Fig. 2B). Taken together, these results demonstrate that CD40 signaling, via TRAF3 degradation, directly and robustly regulated the subsequent signals of the TRAF3-dependent pathway mediated by a different receptor.

CD40-mediated TRAF degradation and cytokine production

To investigate the effect of CD40-mediated TRAF degradation on downstream functional outcomes of CD40 and hCD40LMP1 signaling, B cells were stimulated through CD40, hCD40LMP1, or with isotype control Ab; washed and rested as before, then re-stimulated through mCD40, hCD40, or hCD40LMP1. As shown in Figure 6A, B cells stimulated through endogenous

mCD40 secreted TNFα, consistent with previous studies (138, 181). However, cells pre-stimulated through transfected hCD40 displayed a substantially reduced capacity to secrete TNFα in response to subsequent mCD40 signaling, further supporting the idea that TRAF2-dependent CD40 signaling can limit itself via TRAF2 degradation. Figure 6B and 6C show, as previously demonstrated (138, 141, 181), that mCD40 or hCD40LMP1 signals induce TNFα production. When cells were pre-stimulated through mCD40, subsequent hCD40LMP1-induced TNFα secretion was reduced (Fig. 6B). However, hCD40LMP1 pre-stimulation enhanced, rather than reduced, mCD40-induced TNFα production (Fig. 6C).

We also investigated the effects of CD40 pre-stimulation on subsequent hCD40LMP1-mediated IL-6 production, an important mediator of LMP1's biological effects (22), as well as implicated in the pathogenesis of various types of B cell lymphomas (22). Figure 6D shows that, consistent with published reports (112, 141), engagement of hCD40LMP1 induced B cell IL-6 production. CD40 pre-stimulation reduced, by approximately half, the ability of hCD40LMP1 to induce IL-6 secretion (Fig. 6D). As with TNFα, the order in which receptor signals were received was important, as hCD40LMP1 pre-stimulation greatly *increased* the ability of mCD40 to induce IL-6. These results demonstrate that CD40 signaling negatively regulated not only early signaling pathways, but also downstream cytokine secretion induced by itself and that of the TRAF3-dependent LMP1-mediated activation pathway.

Modulation of TNFR/CD120b signals by CD40-mediated TRAF2 degradation

We wanted to determine whether CD40-mediated TRAF degradation could regulate the signals of the CD40-independent, TRAF2-dependent activating receptor, TNFR (182), and whether cell types in addition to B lymphocytes use this regulatory mechanism . Cells of the human monocytic line, THP-1, were pre-stimulated with anti-hCD40 or isotype control Abs, and then subjected to a secondary stimulation with recombinant TNFα. As seen in Figure 7a, cells pretreated with isotype control Ab activated the JNK and NFκB1 pathways in response to TNFα. However, cells pretreated with hCD40 agonistic Ab were unresponsive to the TNF stimulus. A similar result was noted with primary human macrophages (Fig. 7b). To determine if CD40 pre-stimulation affected later TNFR-mediated events, we pre-stimulated primary human macrophages through CD40 and measured subsequent TNFR-mediated IL-6 secretion. Figure 7c demonstrates that TNFR-mediated IL-6 secretion was inhibited by CD40 pre-stimulation. Thus, CD40-mediated TRAF2 degradation can modulate the signaling of an endogenous TRAF2-dependent cytokine receptor of monocytes and macrophages.

CD40 and innate immune receptor signaling

In humans, TRAF3 mediates TLR3 and TLR4-induced NF-κB activation and TNFα secretion (183). Unfortunately, investigations delving into the role of TRAF2 in TLR signaling have yet to be performed. We hypothesized that CD40-

mediated TRAF degradation affects subsequent TLR signals. To begin to test this hypothesis, macrophages were stimulated through endogenous CD40 for 1 hour, washed and rested for 1 hour, and then stimulated with the TLR agonists poly I:C (TLR3), LPS (TLR4), or R848 (TLR7). As shown in Figure 8a and 7b, CD40 pre-stimulation ablated TLR3-mediated TNFα secretion and drastically reduced that mediated by TLR4. A similar trend was noted for CD40 pre-stimulation and TLR3-mediated TNFα secretion by mouse splenic B cells (Fig. 10). Again, the order of stimulation was important because poly I:C pre-stimulation of primary human macrophages increased, not lessened CD40-mediated TNFα secretion (Fig. 11). Interestingly, TLR7-mediated TNF secretion was not affected by CD40 pre-stimulation (Fig. 8c). Thus, CD40 signaling can modulate the responsiveness of macrophages and B cells to some, but not all innate immune signals.

Conclusions

The results of this study demonstrate that CD40-mediated TRAF degradation is a rapid and robust regulatory mechanism for both the CD40 signal itself and TRAF2/3-dependent signals delivered by other receptors on B lymphocytes and macrophages (Figures 4-11). TRAF2 degradation regulated the CD40 signal in several ways. The initial phases of TRAF2 degradation following CD40 engagement promote the dissolution of the CD40 signaling complex ((145) and Figure 2). Also, CD40-mediated decreases in the total

cellular pool of TRAF2 or TRAF3 dampened subsequent TRAF2- or TRAF3-dependent signaling (Figure 2-8).

Here I demonstrate that the proteasome-dependent mechanism mediating rapid negative regulation of CD40 signals was TRAF3 and TRAF3 degradation-independent. Furthermore, TRAF3 was not required for the ability of CD40 pre-stimulation to abrogate subsequent CD40 signals in mouse B cells (Figure 3B). The results presented here indicate that the CD40-mediated degradation of TRAF3 is important in regulation of CD40-independent, TRAF3-dependent pathways in the CD40-expressing cell. Evidence supporting this concept is presented in Figs. 4-6, and 8.

CD40 is central in the transition from innate to adaptive immunity (19). Not only can CD40 signaling directly promote the expression of factors which shape an immune response, this work shows that it can also work indirectly by modulating the responsiveness of an APC to host cytokines (Fig. 7) and pathogen-associated molecular patterns (Fig. 8).

Overall, these findings reveal a potentially important, powerful and newly appreciated function for CD40 in regulating immune responses. In addition to acting as a powerful stimulator of APC activation, CD40 signaling can also shape immune responses via the induction of TRAF degradation, which significantly modulated the signals of other TRAF2- or TRAF3-dependent immune receptors.

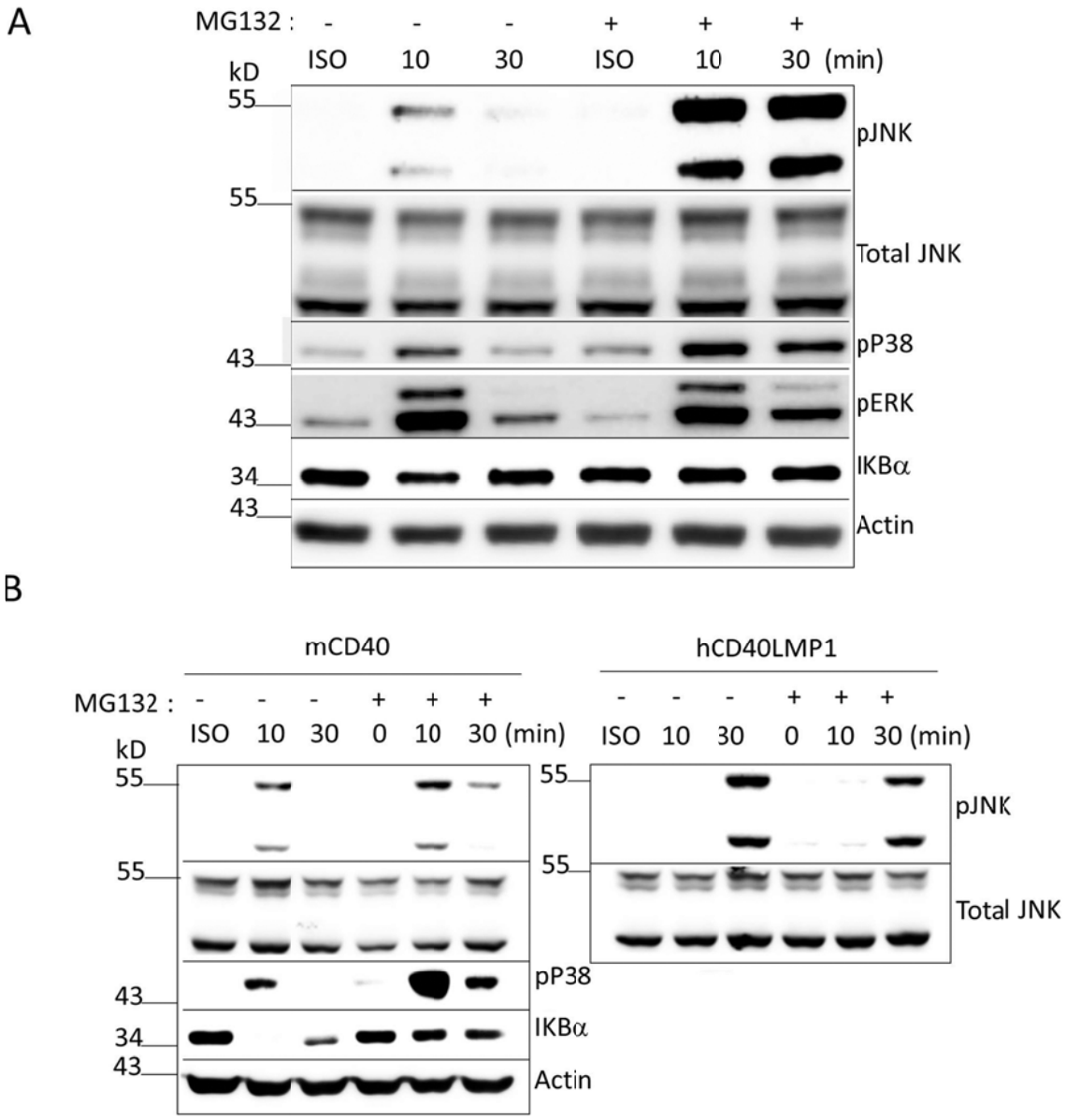


Figure 2. Proteasome inhibition during the CD40 signal.
(A) Human macrophages were derived from peripheral blood monocytes as described in Methods. Before experiments, cells were pretreated with 20μM MG132 or DMSO (vehicle) for 30 minutes and then stimulated with G28.5 or isotype control mAbs at indicated times. Data shown are representative of two independent experiments. **(B)** M12.4.1 mouse B cells expressing hCD40LMP1 were pretreated with 25μM MG132 or DMSO for 30 minutes and then stimulated with HM40.3 (αmCD40), G28.5 (αhCD40), or isotype control mAbs at indicated times. Samples were then subjected to Western blotting for pJNK, total JNK, p-p38, total IκBα and actin. Data shown are representative of more than 5 independent experiments in mouse A20 B cells, M12.4.1 B cells, or CH12.LX B cells.

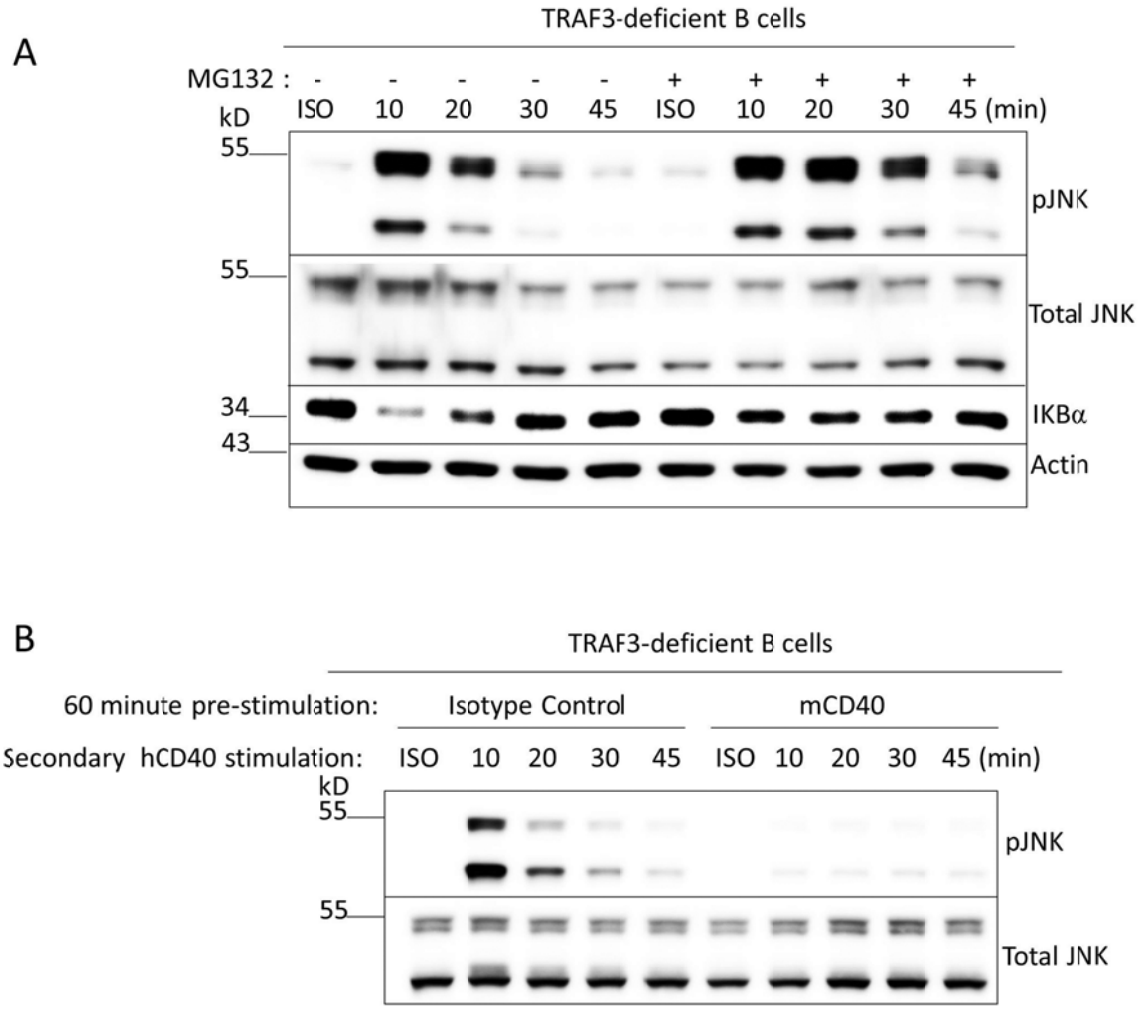
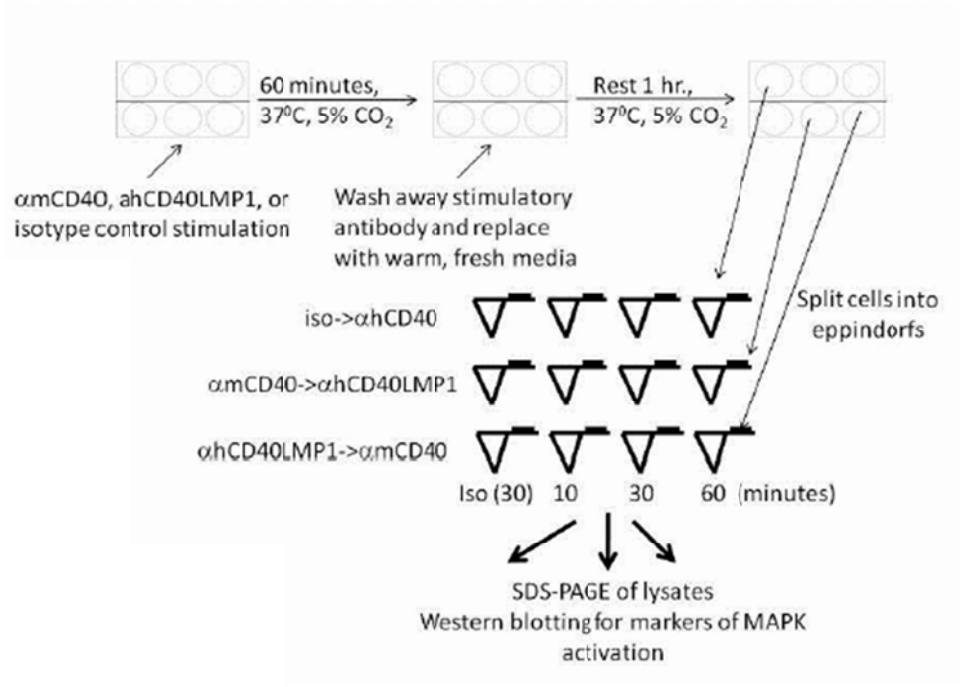


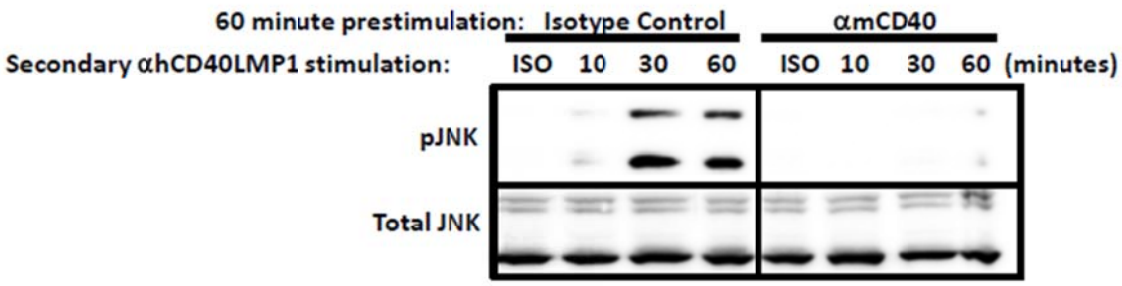
Figure 3. Proteasome inhibition during CD40 signaling in TRAF3-deficient B lymphocytes.
(A) TRAF3-deficient CH12.LX mouse B cells were treated as described for Figure 1*A*. Data shown are representative of two independent experiments.**(B)** CH12.LX TRAF3-deficient mouse B cells were pre-stimulated with anti-mCD40 HM40.3 mAb for 1 hour, washed and resuspended in fresh medium, rested for 1 hour, and stimulated with anti-hCD40 G28.5 mAb at indicated times. Data shown are representative of two independent experiments in both CH12.LX and A20 B cell lines.

Figure 4. CD40 effects upon LMP1 signaling.
(A) Diagram depicting an experimental scheme relevant to Figures 3B, 3C, 4A, and 4B is presented. Cells are stimulated as indicated for 1 hour, washed and resuspended in fresh medium, then rested for 1 hour. Following the rest, cells are re-stimulated as indicated. **(B)** M12.4.1 mouse B cells expressing hCD40LMP1 were stimulated with anti-mCD40 or isotype control mAbs. Both groups of cells were then stimulated with anti-hCD40 mAb at the indicated times, or with isotype control mAb for 30 minutes. Samples were then subjected to Western blotting for pJNK and total JNK. Data are representative of 4 independent experiments in either mouse M12.4.1 B cells or CH12.LX B cells. **(C)** A similar experiment as in *B* was performed, except cells were stimulated with either anti-mCD40 or anti-hCD40 mAbs. Data are representative of 3 independent experiments in mouse M12.4.1 B cells or CH12.LX B cells.

A


60 minutes,
37°C, 5% CO2
Rest 1 hr.,
37°C, 5% CO2
αmCD40, ahCD40LMP1, or
isotype control stimulation
Wash away stimulatory
antibody and replace
with warm, fresh media
iso->αhCD40
αmCD40->αhCD40LMP1
αhCD40LMP1->αmCD40
Split cells into
eppindorfs
Iso (30) 10 30 60 (minutes)
SDS-PAGE of lysates
Western blotting for markers of MAPK
activation

B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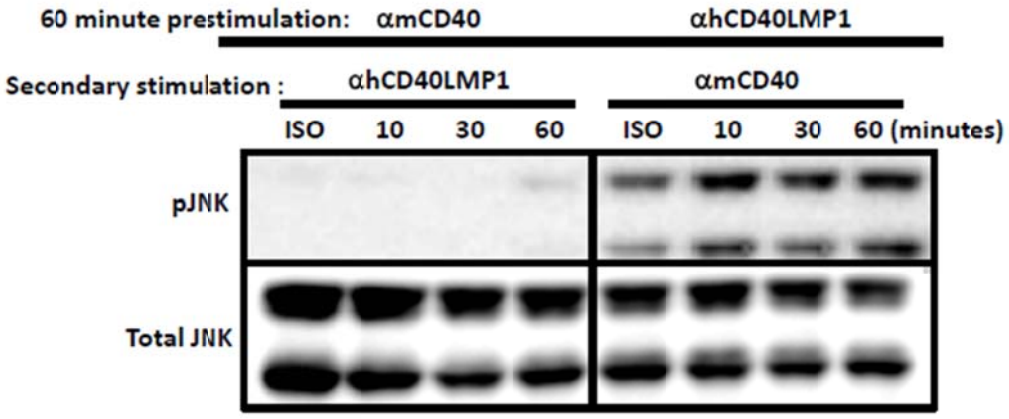
αmCD40
Secondary αhCD40LMP1 stimulation:
ISO 10 30 60 ISO 10 30 60 (minutes)
pJNK
Total JNK

C


60 minute prestimulation: αmCD40
αhCD40LMP1
Secondary stimulation :
αhCD40LMP1
αmCD40
ISO 10 30 60 ISO 10 30 60 (minutes)
pJNK
Total JNK

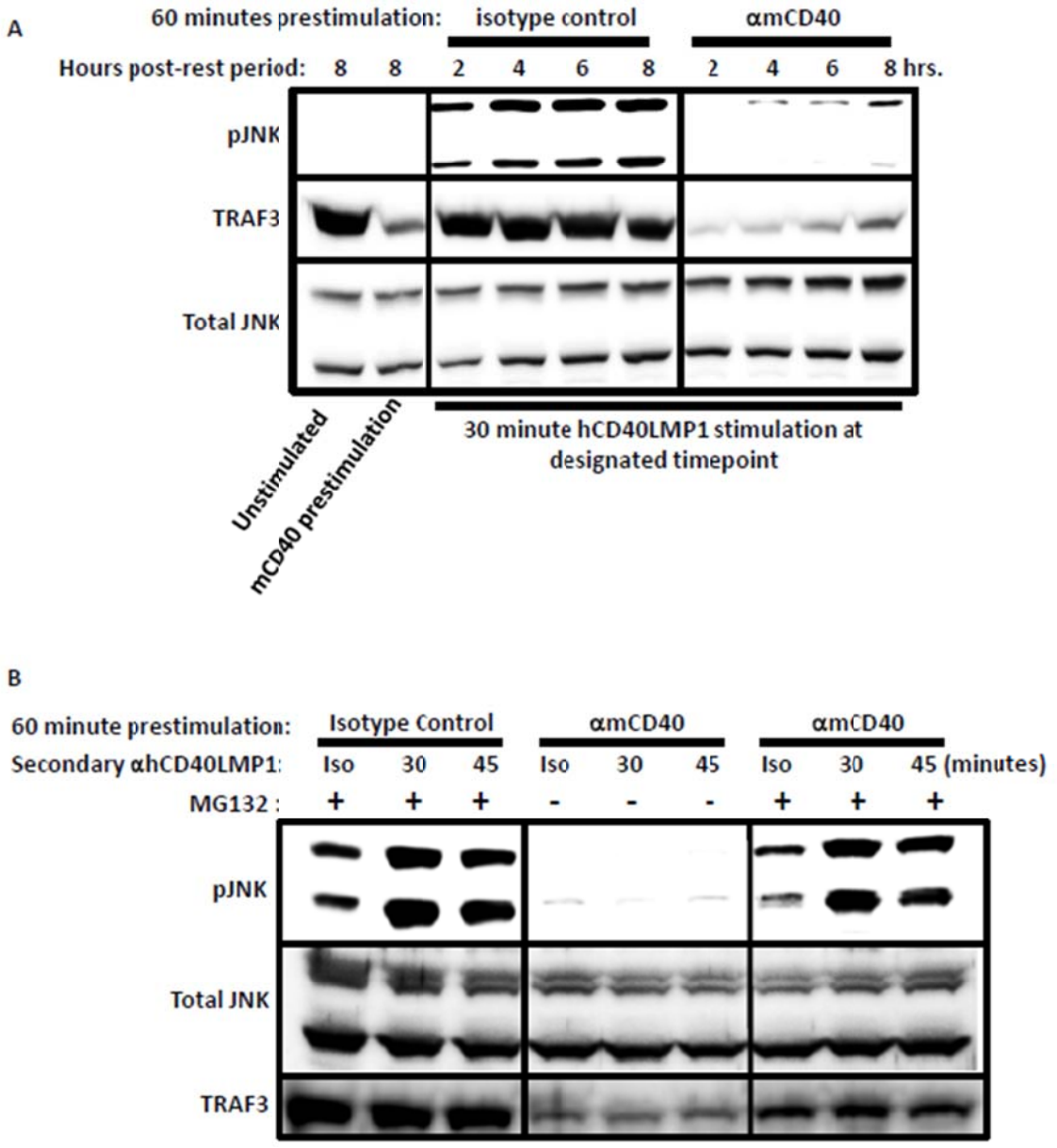


Figure 5. Effects of proteasome inhibition during CD40 signaling and subsequent CD40LMP1 activation.
(A) An experiment as described in Figure 3A was performed with M12.4.1 B cells, except cells were re-stimulated through hCD40LMP1 for 30 minutes at 2, 4, 6, and 8 hours following the rest period. Samples were then subject to Western blotting for pJNK, total JNK, and TRAF3. Data are representative of 2 independent experiments. **(B)** M12.4.1 B cells were used in the protocol described for Figure 3B. , Cells treated with isotype control or anti-mCD40 mAbs were incubated with 25μM MG132 or DMSO during CD40 stimulation. Western blots of samples for pJNK, total JNK, and TRAF3 are representative of 2 independent experiments.

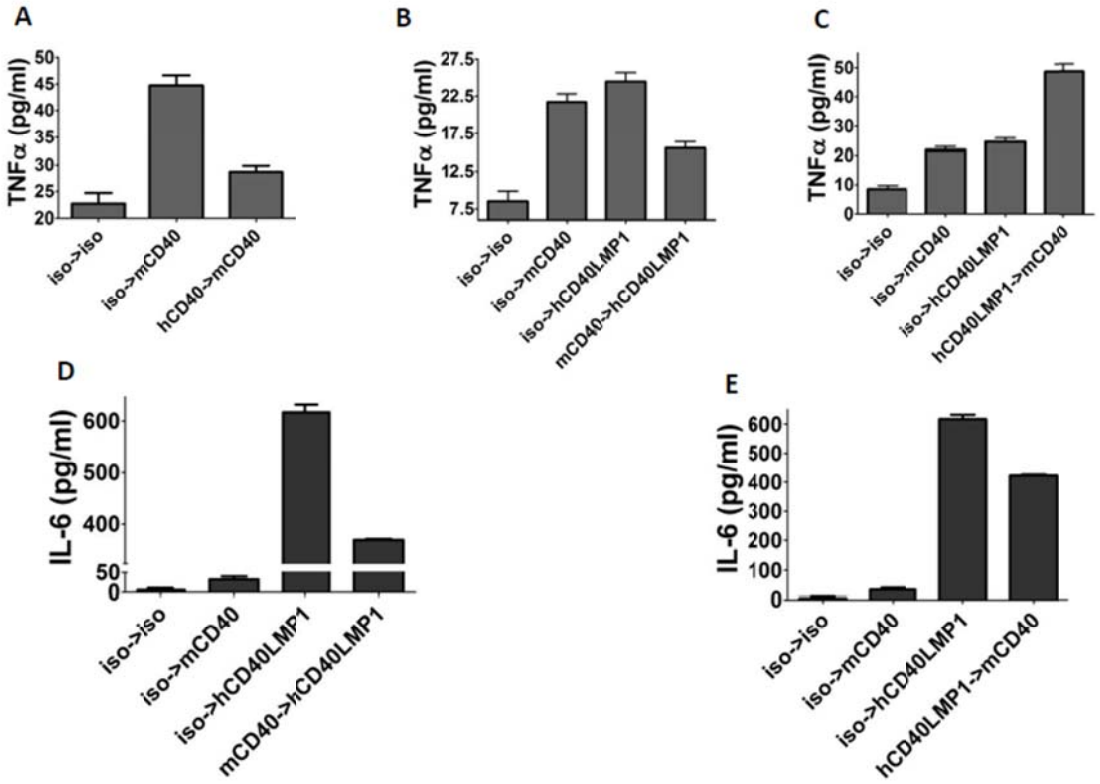


Figure 6. Effects of CD40 pre-stimulation upon CD40LMP1-mediated cytokine secretion.
(A) M12.4.1 mouse B cells expressing transfected hCD40 were stimulated with anti-hCD40 or isotype control mAbs, washed and resuspended in fresh medium, rested for 1 hour, and re-stimulated with anti-mCD40 or isotype control mAbs. TNFα was detected in the cultures by ELISA as described in *Methods*. Results shown are typical of 3 independent experiments. **(B and C)**. M12.4.1 or CH12.LX mouse B cells transfected with hCD40LMP1 were treated as in *A* except cells were stimulated with either isotype control, anti-hCD40, or anti-mCD40 mAbs, and re-stimulated as indicated. Results shown are typical of 3 independent experiments. **(D and E)** The experiment was performed exactly as in *B and C,* except an IL-6 ELISA was performed on the culture supernatants as described in *Methods.* Data are representative of 2 independent experiments.

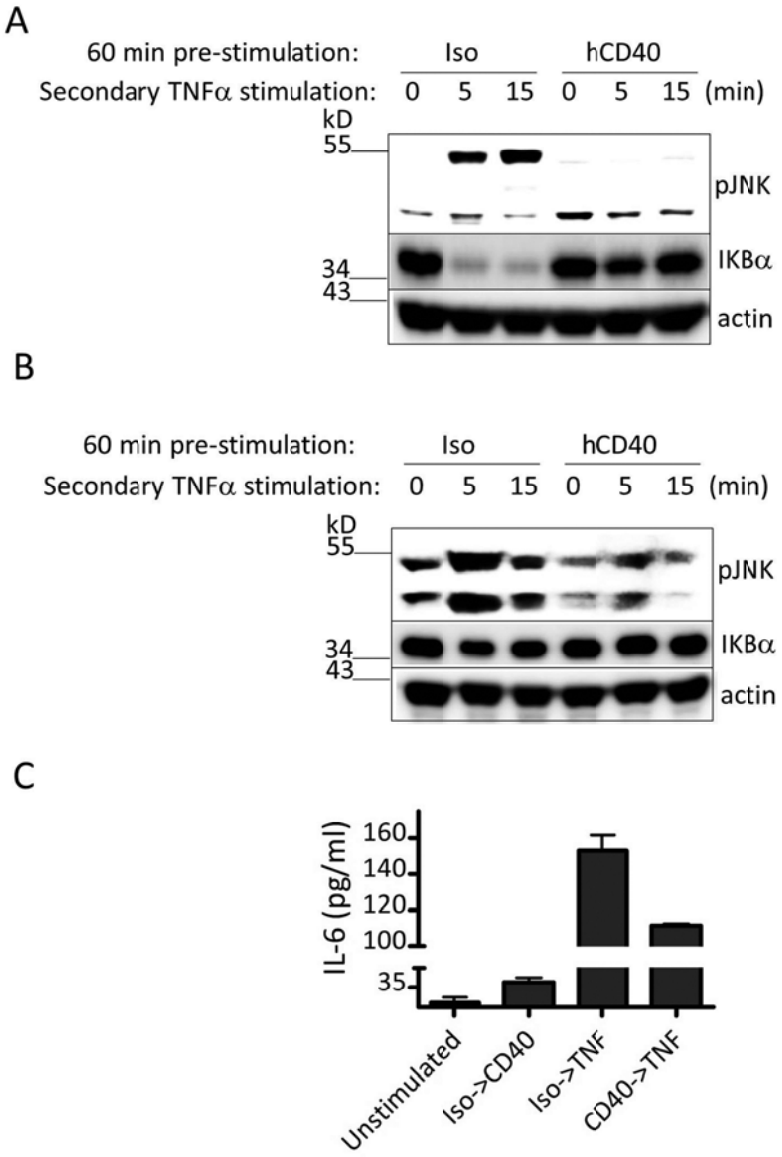


Figure 7. Effect of CD40 stimulation upon subsequent TNFR2 signaling. **(A)** THP-1 human monocytes were stimulated with anti-hCD40 or isotype control mAbs for 1 hour, washed and resuspended in fresh medium and allowed to rest for 1 hour. Following this rest period, cells from both pre-stimulation groups were re-stimulated with recombinant TNFα at the indicated times. Western blots of samples for pJNK, total JNK, total IκBα, and actin are representative of 2 independent experiments. **(B)** A similar experiment as in *A* was performed except human primary macrophages were used. **(C)** Human primary macrophages were stimulated with anti-hCD40 or isotype control mAbs for 1 hour, washed and resuspended in fresh medium and allowed to rest for 1 hour. Following this rest period, cells from both pre-stimulation groups were re-stimulated with recombinant TNFα at the indicated times. IL-6 secretion into culture supernatants was then assessed by ELISA as noted in the methods section.

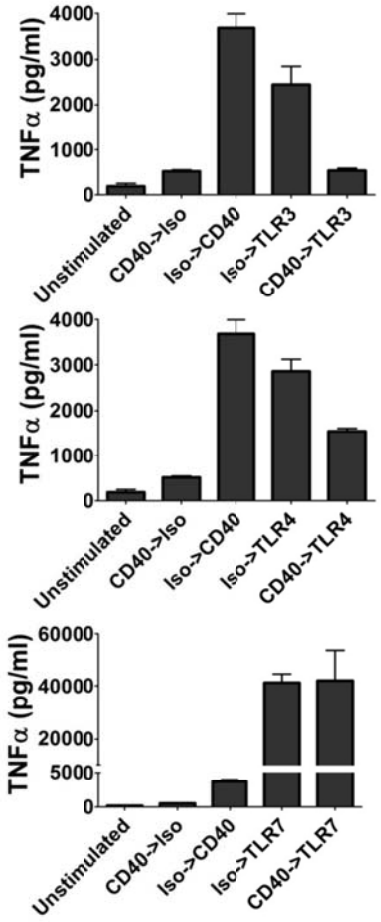


Figure 8. CD40 pre-stimulation and TLR-mediated TNFα secretion.
Human primary macrophages were stimulated for a total of 18 hours as in figure 5 except in **(A)** poly I:C (30 μg/ml) was used to stimulate TLR3, **(B)** LPS (5μg/ml) was used to stimulate TLR4, and **(C)** R848 (1μg/ml) was used to stimulate TLR7. TNFα in culture supernatants was determined by ELISA. Results shown are typical of two or more experiments.

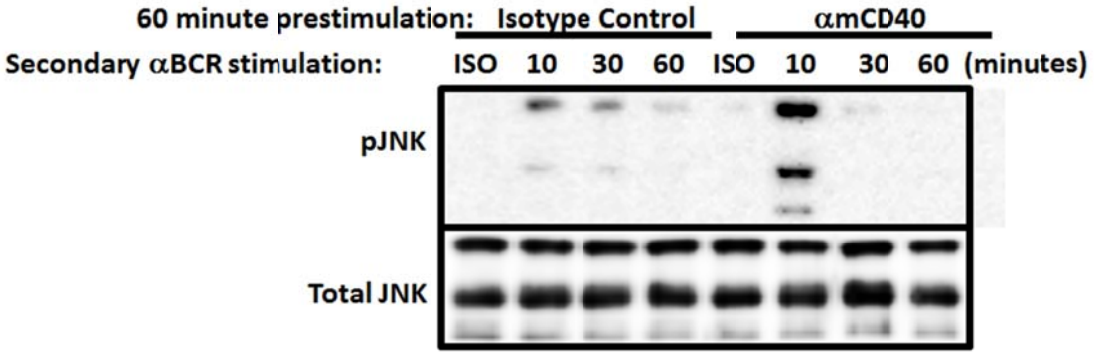


Figure 9. CD40 pre-stimulation and BCR-mediated JNK activation.
Primary splenic mouse B cells were pre-stimulated with agonistic anti-mCD40 or isotype control Ab for 1 hour. Cells were then washed and rested for 1 hour before they were subsequently stimulated with anti-mouse IgM antibody for the indicated time points. Samples were then subjected to Western blotting for pJNK and total JNK. Data are representative of two independent experiments.

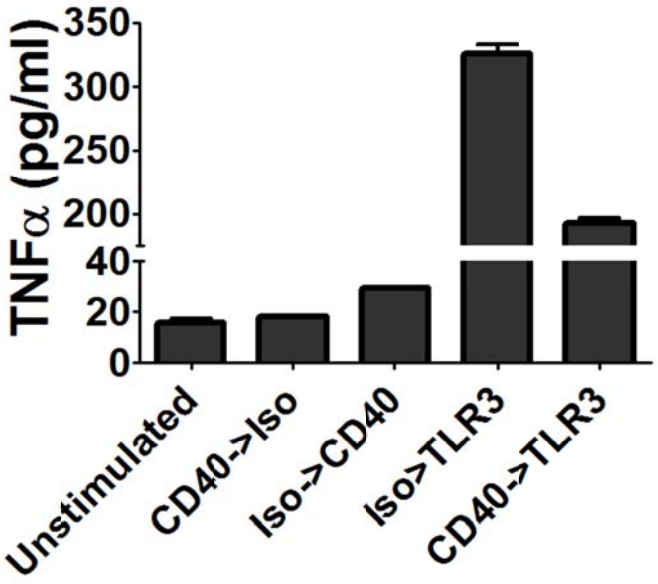


Figure 10. Effects of CD40 pre-stimulation on TLR3-mediated TNFα secretion in B cells.
Primary splenic mouse B cells were pre-stimulated with agonistic anti-mCD40, isotype control Ab for 1 hour. Cells were then washed and rested for 1 hour before they were subsequently stimulated with agonistic anti-mCD40 Ab or poly I:C (30 μg/ml) for 16 hours. TNFα in culture supernatants was determined by ELISA. Results shown are typical of two or more experiments.

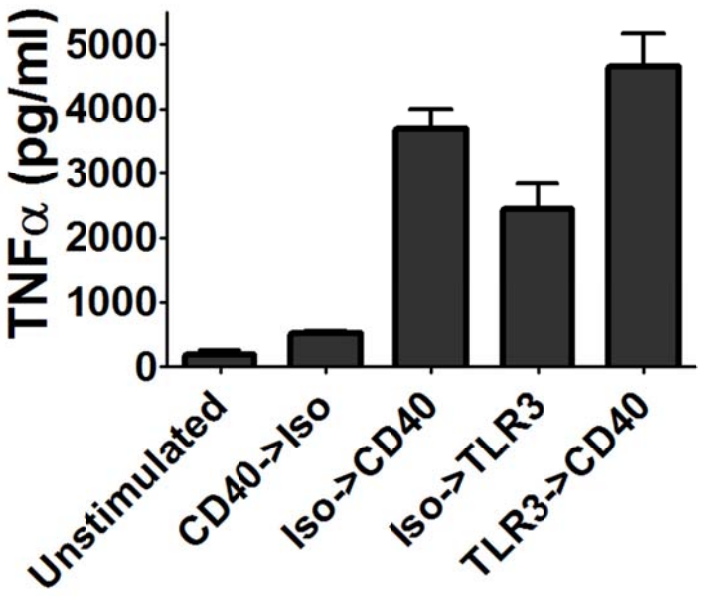


Figure 11. TLR3 pre-stimulation and CD40 stimulation of human macrophages.
Human primary macrophages were pre-stimulated with either agonistic anti-hCD40 Ab or poly I:C (30 μg/ml) for 1 hour, washed and then rested for 1 hour, and then stimulated with isotype control Ab or anti-hCD40 Ab for 16 hours. TNFα in culture supernatants was determined by ELISA. Results shown are typical of two or more experiments.

CHAPTER IV

ROLES OF THE TNF RECEPTOR-ASSOCIATED FACTOR (TRAF)2/3 BINDING SITE IN DIFFERENTIAL B CELL SIGNALING BY CD40 AND ITS ONCOGENIC MIMIC, LMP1

Rationale

LMP1 consists of a short N-terminal and long COOH CY domain, separated by 6 membrane-spanning domains, which aggregate to initiate ligand-independent signaling (29). LMP1 and CD40 share a short COOH CY domain motif which allows binding to members of the TRAF family of signaling adaptor proteins. Binding of TRAFs 1, 2, 3, and 5 is mediated by the general motif PxQxT, commonly referred to as the TRAF binding site (TBS) (Fig. 12)(142). Each TRAF binds the TBS in a distinct but overlapping manner (184). The TBS of CD40 is considered a 'major' TRAF2-binding motif of PVQ**E**T**L**, while the TBS of LMP1 has been called a 'minor' TRAF2 binding motif (PQQ**A**T**D)** (185). CD40 and LMP1 associate with the same binding crevice of TRAF3, but LMP1 has additional binding contacts that may contribute to its more robust association with TRAF3 (142, 186). For both CD40 and LMP1, the TBS influences NF-κB and JNK activation, surface molecule upregulation, and IgM secretion (97, 109, 112, 131, 133, 136, 138, 140, 187-190).

TRAFs interact with LMP1 in several unexpected and different ways, compared to their interactions with CD40 (142). TRAFs 1 and 2 cooperate to promote a subset of CD40-mediated signals, while deficiencies of either or both these TRAFs have no major effect on *in vitro* LMP1 signaling to B cells (123). Conversely, TRAF3 is a negative regulator of CD40-induced B cell activation, but

an important positive element of LMP1-induced signaling (133, 134, 136). TRAF2 recruitment to CD40 induces TRAF- dependent polyubiquitination and proteasome-dependent degradation of both itself and TRAF3, but this doesn't occur upon LMP1 signaling (24, 70, 98, 134). Differential TRAF usage and regulation by LMP1 in comparison to CD40 contributes to its unique signaling nature.

To further our understanding of how CD40 and LMP1 differentially regulate the TRAFs, we devised a novel and complementary approach to build upon mutational analysis of the TBS. This approach retains the normal sequence of each TBS while placing it in the context of the CY domain of the complementary receptor. This allows a direct comparison of the contributions of the TBS in signaling by CD40 and LMP1 to B lymphocytes, while retaining the normal overall structure of each receptor's CY domain. To this end, we created recombinant human CD40 and chimeric hCD40LMP1 molecules in which the major TRAF binding site of CD40 (PVQETLH) and the minor TRAF binding motif of LMP1 (PQQATDD) were swapped. The signaling characteristics and TRAF binding potentials of each of these molecules compared to their WT counterparts were examined. As mentioned above, the cytoplasmic (CY) domain of LMP1 binds the signaling adaptor TRAF2 with lower avidity than the CY domain of CD40, and TRAF2 is needed for CD40-mediated degradation of TRAFs 2 and 3. LMP1 doesn't induce TRAF degradation, and employs TRAF3 as a positive mediator of cell signaling, whereas CD40 signals are inhibited by TRAF3. Through these experiments we thus tested the hypothesis that relative

affinity for TRAF2, and/or distinct sequence differences in the TRAF2/3 binding sites of CD40 vs. LMP1, controls the disparate ways in which CD40 and LMP1 use TRAFs 2 and 3, and their distinct signaling characteristics. CD40 and LMP1 mutants in which the TRAF binding site sequences were swapped were examined, testing TRAF binding and degradation, and induction of B cell activation. Results revealed that TRAF binding affinity and TRAF binding site sequence dictate a distinct subset of CD40 vs. LMP1 signaling properties. Examination of TRAF binding, degradation, cytokine production, IgM secretion, and the activation of c-Jun kinase and NF-κB revealed that some events are dictated by TRAF binding site sequences, others partially regulated, and still others are independent of the TRAF binding site sequence.

Results

Effects of differences in CD40 vs. LMP1 TRAF binding site sequence onTRAF2/3 recruitment

TRAF2 and TRAF3 associate with CD40 via a PxQxT motif located in a region separate from the TRAF6 binding site in the CD40 CY domain (70). These TRAFs associate with LMP1 in a region of similar sequence necessary for LMP1-mediated B cell transformation, commonly referred to as CY activating region 1 (CTAR1) (97, 187). However, the specific amino acid residues within and immediately flanking this site differ between CD40 and LMP1 (185). The major TBS of hCD40 binds more TRAF2 than hCD40LMP1, whereas hCD40LMP1's minor TBS binds more TRAF3 than hCD40 (Fig.12) (112, 136,

185).

To begin this study, we examined whether these particular TBS sequences dictate preferential binding of hCD40 or hCD40LMP1 to TRAF2 and/or TRAF3. To do so, we expression matched multiple sets of clones in two different cell lines by immunofluorescence flow cytometry. A “set” is a grouping of four cell line clones expressing either hCD40, hCD40LMP1, hCD40ADD, or hCD40LMP1AEDL at similar levels. To begin a study, all experimental comparisons were performed within one set of clones. Subsequent experiments using additional expression matched sets of clones were used to confirm initial findings. Comparisons of each receptor were made within additional expression matched sets and not between (an example of an expression matched set of M12 B cell clones can be viewed in Fig. 12B). Abs specific for the hCD40 extracellular domains of each molecule were used to immunoprecipitate the receptors from cell lysates 15 minutes post-stimulation with agonistic Ab. Western blotting of precipitates subject to SDS-PAGE was employed to detect the relative amounts of TRAF2/3 co-immunoprecipitated with each receptor. Conversion of the sequence of the hCD40 TBS to that of hCD40LMP1 (hCD40ADD) reduced by approximately two-fold the ability of hCD40ADD to bind TRAF2, compared to hCD40 (Fig. 12). However, the binding of hCD40ADD to TRAF3 was unchanged. Conversely, converting the TBS of hCD40LMP1 to that of hCD40 gave hCD40LMP1AEDL the ability to bind comparatively increased amounts of TRAF2. Similar to the hCD40ADD molecule, the ability of hCD40LMP1AEDL to bind TRAF3 was unaltered from that of its parent receptor.

Thus, the TBS sequences of both CD40 and LMP1 predicted the relative ability to bind TRAF2 but not TRAF3.

Effects of differences in CD40 vs. LMP1 TRAF binding site sequence onTRAF2/3 degradation

TRAF2 and 3 association with CD40 but not LMP1 induces their polyubiquitination and proteasome-dependent degradation (98). TRAF degradation is dependent upon the RING domain of TRAF2 (24, 134, 144). We thus asked if hCD40LMP1 acquires TRAF-degrading ability if its binding site is altered to allow more robust TRAF2 binding. The increased TRAF2 binding ability of hCD40LMP1AEDL (Fig. 12) correlated with the ability of hCD40LMP1 to induce TRAF2, but not TRAF3 degradation upon signaling (Fig. 13). However, the decreased TRAF2 binding ability of hCD40ADD did not preclude this molecule's induction of TRAF2 and TRAF3 degradation (Fig. 12 and 13). There was a trend towards lower ultimate hCD40ADD-induced TRAF2 degradation in comparison to hCD40 after 3 hours of stimulation, but degradation still occurred (Fig. 13). Interestingly, hCD40ADD signaling seemed more efficient at inducing TRAF3 degradation compared to hCD40. Taken together, these results suggest that the TBS of CD40 can positively influence the ability to induce TRAF2 degradation. However, CD40 uses regions outside of the TBS to mediate signaling induced TRAF3 degradation.

Effects of differences in CD40 vs. LMP1 TRAF binding site sequence on Ig, IL-6, and TNF-α secretion

The CY domain of LMP1 mediates amplified production of Ig, IL-6, and TNF-α, compared to that of CD40 (98, 181). hCD40LMP1AEDL-induced Ig secretion was reduced to CD40-like levels, whereas that induced by hCD40ADD signaling was increased to levels similar to hCD40LMP1 (Fig.14C). However, TNF-α secretion and CD23/CD80 upregulation induced by hCD40ADD and hCD40LMP1AEDL were not altered from the levels mediated by their parental counterparts (Fig. 14A and data not shown). These findings indicate that induction of Ig is principally regulated by the TBS sequence, but additional or alternate factors regulate differences in TNFα production and CD23/CD80 upregulation.

CD40 signaling induced by agonistic anti-CD40 Ab is insufficient to induce B cells to secrete IL-6; membrane bound CD154 stimulation is required for this event (150). Signaling initiated by agonistic Ab to hCD40LMP1, however, is sufficient to induce B cells to secrete IL-6 (92). Therefore, we wished to determine if the TBS sequence is relevant to the more robust stimulation required by CD40 to induce IL-6 secretion. hCD40LMP1AEDL signaling induced IL-6 secretion in response to agonistic Ab. Thus, this ability was not lost by converting its TBS to that of CD40. However, hCD40ADD gained the ability to induce IL-6 production in response to anti-CD40 Ab, similar to molecules with a LMP1 CY domain (Fig.14B). Like TRAF degradation, regulation of IL-6 production was partially, but not solely, regulated by the TBS sequence.

Effects of differences in CD40 vs. LMP1 TRAF binding site sequence on early signaling events

TRAF2 is necessary for CD40-mediated, but not LMP1-mediated, JNK activation in B cells (134). In contrast, whereas TRAF3$^{-/-}$ mouse B cells are markedly defective in hCD40LMP1-mediated JNK activation, TRAF3 deficiency results in an increase in CD40-mediated JNK activation (134). TRAF3 has also been shown to negatively regulate CD40-mediated Ig production and CD40-BCR synergy (134, 149), while promoting LMP1-mediated Ig production (134). Thus, JNK is a very important early signaling pathway connected to multiple downstream effector functions of CD40 and LMP1. Similar to NF-κB activation (Fig. 16), the pattern of JNK activation differs between hCD40 and hCD40LMP1, with hCD40LMP1 mediating a slower but more sustained activation (Fig. 15) (98, 112, 136). Fig. 15 shows that hCD40LMP1AEDL-induced JNK activation converted to the rapid, transient pattern mediated by hCD40. hCD40ADD-mediated JNK activation showed a pattern intermediate to that of hCD40LMP1 and hCD40. Like hCD40, hCD40ADD signaling induced an early peak of JNK activation. However hCD40ADD-mediated JNK activation was also sustained, similar to hCD40LMP1. These results show that the TBS played important roles in the nature of both hCD40 and hCD40LMP1 mediated JNK activation.

CD40 and LMP1 both activate the canonical and non-canonical NF-κB pathways (33, 100). However, hCD40LMP1-mediated canonical pathway induction is slower and more sustained than hCD40 (Fig. 15 and (98). Fig. 16

shows that both hCD40LMP1AEDL and hCD40ADD induced the degradation of IκBα with the same kinetics as stimulation through endogenous mCD40 and hCD40. These results indicate that regions additional to the TBS of CD40 were important in regulating the process of IκBα degradation, but the delayed and sustained pattern of canonical NF-κB activation typical of hCD40LMP1 depended upon its unique TBS.

Fig. 17 illustrates that the sum of activation of NF-κB pathways at 6 hours post-stimulation is greater with both hCD40LMP1AEDL and hCD40LMP1 than in the CD40 counterparts. Whereas the hCD40 and hCD40ADD molecules induced roughly the same amount of NF-κB activation, hCD40LMP1AEDL signaling trended toward higher levels of NF-κB activation than did hCD40LMP1 signaling (Fig. 17). These results show that the sum of later activation of combined canonical and non-canonical NF-κB pathways is primarily dictated by regions outside of the TRAF binding site, although early activation of the canonical pathway is sensitive to TBS differences (Fig. 17).

Conclusions

Here we show that hCD40 and hCD40LMP1-induced TNF-α secretion, CD80 upregulation, and TRAF3 binding were unaltered by TBS swapping. This indicates that any pathways of hCD40 and hCD40LMP1 which rely on cooperativity of the TBS and other regions of the receptor are intact in hCD40ADD and hCD40LMP1AEDL molecules.

Interestingly, the respective TBS of CD40 and LMP1 regulated the strength of TRAF2 but not TRAF3 binding (Fig. 12 and (112, 185, 187). Most likely, the COOH-terminal portion CTAR2 is influencing TRAF3 binding, this will be discussed in detail later.

The present report illustrates that a receptor's ability to recruit TRAF2 cannot fully explain whether TRAF2/3 degradation will occur upon signaling. Despite lowering the TRAF2 binding of hCD40ADD compared to hCD40, hCD40ADD retained its ability to induce TRAF2/3 degradation, although TRAF2 degradation was at a lower level than that induced by hCD40 at later times. This result, together with the finding that hCD40LMP1AEDL initiated TRAF2 degradation, suggests that the CD40 TBS is most important in enabling TRAF2 but not TRAF3 degradation. The rate of TRAF3 degradation was improved by the replacement of the hCD40 TBS with that of hCD40LMP1.

These results also illustrate differences in the way the TBS sequences of CD40 and LMP1 interact with and regulate TRAFs for signaling. While both CD40 and LMP1 bind TRAF2 via their respective TBS, the sequence of the CD40 TBS induces TRAF2 to perform distinct functions compared to the TBS of LMP1.

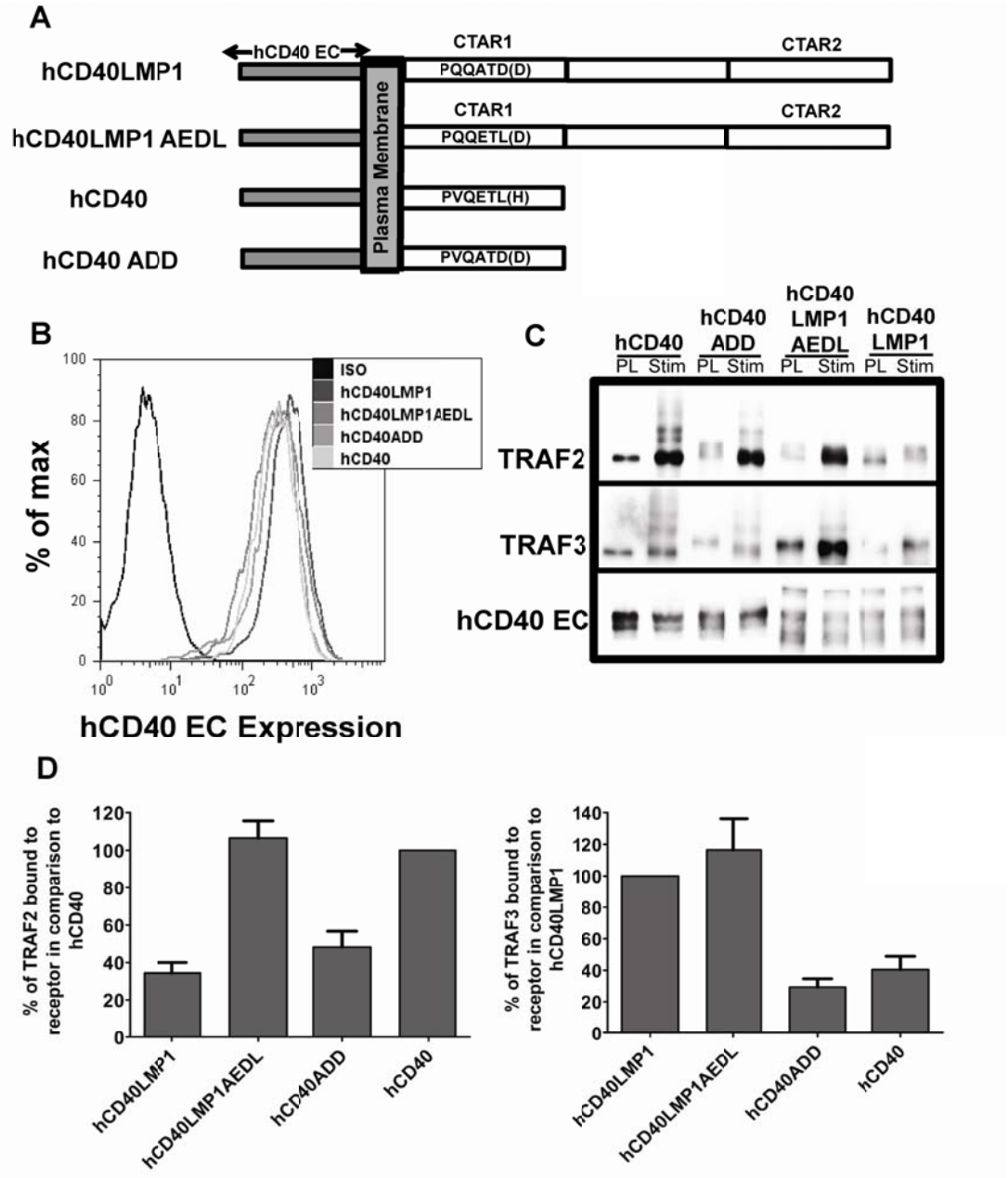
The TBS of LMP1 clearly influences the exaggerated signaling nature of hCD40LMP1. The TBS of hCD40LMP1 imparted to hCD40ADD the ability to induce IL-6 in response to agonistic Ab, in contrast to hCD40 (98, 150). This ability was not merely the result of loss of a negative regulatory mechanism associated with the TBS of CD40, because hCD40LMP1AEDL retained the

ability to induce IL-6 secretion in response to agonistic Ab.

In the case of signals activating the canonical NF-κB pathway, an interesting intermediate pattern of TBS influence emerged for both hCD40ADD and hCD40LMP1AEDL (Fig. 16). This suggests that, like IL-6 production, both TBS properties as well as additional factors regulate the ability to activate the canonical NF-κB pathway. Despite the effects of the TBS on activation of the canonical NF-κB pathway, the overall sum of both NF-κB activation pathways at later time points correlated with regions outside of the TBS in both hCD40 and hCD40LMP1 (Fig. 17). hCD40LMP1AEDL's maintenance of an hCD40LMP1-like NF-κB activation profile is most likely due to its ability to maintain IκBα degradation at later times, similar to hCD40LMP1.

The activation of JNK showed a stronger dependence upon the distinct TBS, and this was also reflected in a downstream function of hCD40ADD, IL-6 production, heavily influenced by the JNK pathway (191). hCD40LMP1AEDL signaling, however, maintained the hCD40LMP1-like ability of inducing IL-6 secretion in response to agonistic Ab despite its inability to activate the JNK pathway for prolonged periods of time. This suggests that other LMP1 TBS-independent pathways are capable of a compensatory contribution to this signaling outcome.

Figure 12. Recruitment of TRAF2 and TRAF3 by hCD40LMP1AEDL and hCD40ADD in B cells.
(A) Domain composition of hCD40LMP1, hCD40LMP1AEDL, hCD40, and hCD40ADD. The LMP1 chimeric receptors are composed of the extracellular and transmembrane domains of hCD40 and either the full length CY domain of LMP1 (aa 187-386 of LMP1) or a TBS mutant version of this LMP1 CY domain (hCD40LMP1AEDL) where the sequence of the TBS has been changed from PQQATDD to PQQETLD (the CD40 TBS sequence). hCD40 is WT hCD40 while hCD40ADD has had its TBS mutated from that of CD40 (PVQETLH) to that of LMP1 (PVQATDD). **(B)** M12 and M12 B cell clones were expression matched into sets expressing similar levels of hCD40, hCD40LMP1, hCD40LMP1AEDL, and hCD40ADD as determined by immunofluorescence flow cytometry. Similar results were obtained for CH12.LX subclones (not shown). **(C)** M12.4.1 B cells were stably transfected with hCD40LMP1, hCD40, hCD40ADD, or hCD40LMP1AEDL and stimulated with 10 μl of Dynabeads coated with anti-hCD40 Ab for 15 min. The post lysis (PL) control sample was lysed before addition of anti-hCD40 Ab coated Dynabeads as in Methods. Samples were blotted for TRAF2, TRAF3, and hCD40. *D,* Quantification of TRAF binding in B cells was performed by measuring the bands with a low-light imaging system. The desitometric quantification of the TRAF band was normalized to the desitometric quantification of the corresponding CD40 band. The normalized densitometric quantification of TRAFs co-immunoprecipitated with a receptor following cell lysis (“PL”) was subtracted from the normalized value of the stimulated (“Stim”) samples. The normalized values for hCD40 ADD and hCD40LMP1AEDL are represented graphically as percentages of the normalized values of hCD40 and hCD40LMP1. Data shown are representative of six or more independent experiments utilizing two or more clones of each transfectant.


A
hCD40 EC
CTAR1
CTAR2
hCD40LMP1
PQQATD(D)
hCD40LMP1 AEDL
PQQETL(D)
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hCD40 ADD
PVQATD(D)
Plasma Membrane
B
% of max
ISO
hCD40LMP1
hCD40LMP1AEDL
hCD40ADD
hCD40
hCD40 EC Expression
C
hCD40
hCD40 ADD
hCD40 LMP1 AEDL
hCD40 LMP1
PL Stim
TRAF2
TRAF3
hCD40 EC
D
% of TRAF2 bound to receptor in comparison to hCD40
hCD40LMP1
hCD40LMP1AEDL
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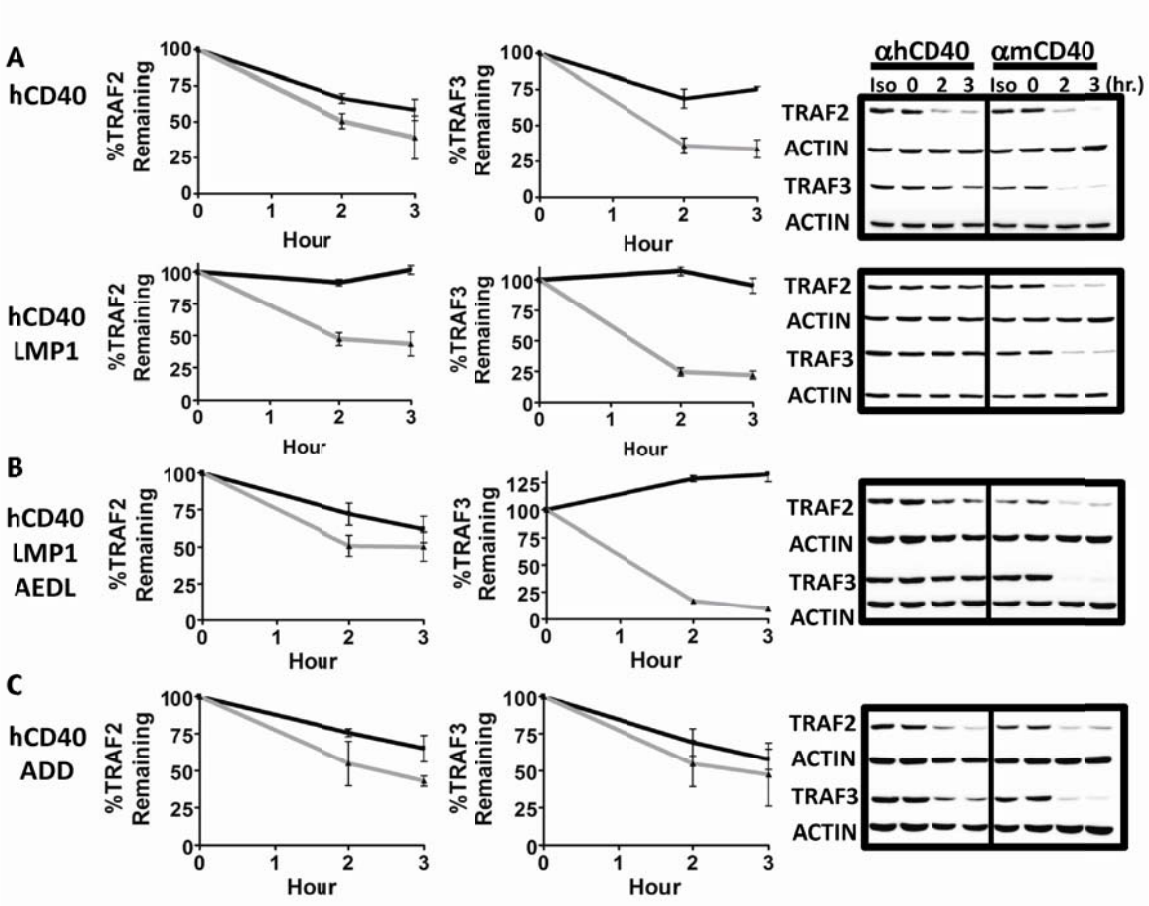


Figure 13. Differential abilities of CD40, CD40LMP1, CD40ADD, and CD40LMP1AEDL CY domains to induce TRAF degradation.
Whole cell lysates from M12.4.1 B cells transfected with hCD40 or hCD40LMP1 **(*A*)**, hCD40LMP1AEDL **(*B*)** or hCD40ADD **(*C*)** and stimulated with isotype control Ab, anti-mCD40 Ab, or anti-hCD40 Ab were analyzed for TRAF2 and TRAF3 degradation. Quantification of TRAFs **(*A-C*)** was performed by measuring the intensities of TRAF2, TRAF3, and actin bands as in Methods. The amount of each TRAF band was normalized to the corresponding actin band. These values were then normalized to the 0 hour time, which was set as 100% of either TRAF2 or TRAF3. Black solid lines represent TRAF degradation induced by hCD40 agonistic mAb, grey lines represent TRAF degradation induced by anti-mCD40 agonistic mAb, and serves as an endogenous control for each transfected subclone. Data shown are representative of three or more experiments performed with two or more clones of each transfectant.

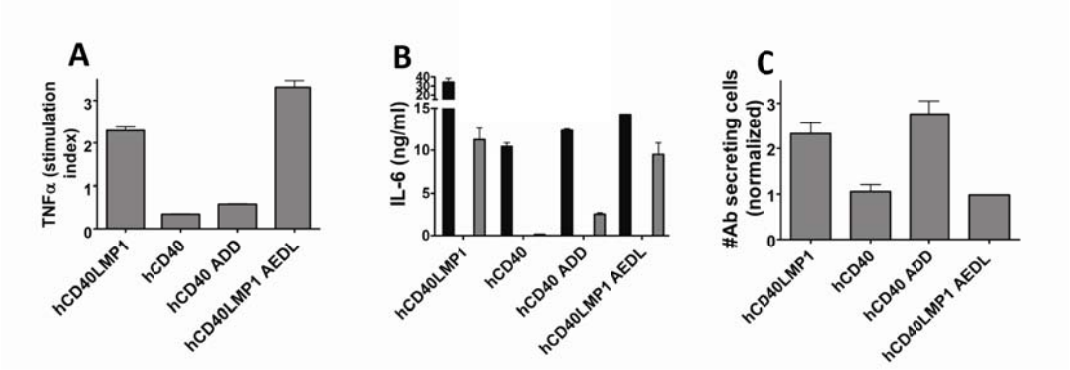


Figure 14. Effects of TBS mutation on TNF-α, IL-6, and IgM production. **(A)** M12.4.1 B cells stably transfected with hCD40LMP1, hCD40, hCD40ADD, or hCD40LMP1AEDL were stimulated for 4 hours with agonistic mouse or human CD40-specific mAbs or isotype control mAbs. TNF-α secreted in response to stimulation was assayed from cell culture supernatants by ELISA. Data are presented as hCD40-induced TNF-α normalized to mCD40-induced TNF-α . TNF-α secreted in response to isotype control mAbs was subtracted from CD40 stimulated groups prior to normalization. **(B)** CH12.LX B cells stably transfected with hCD40LMP1, hCD40, hCD40ADD, or hCD40LMP1AEDL were stimulated for 2 days with Hi5 insect cells expressing hCD154, agonistic anti-mouse or human anti-CD40 mAbs, or isotype control mAbs. IL-6 was assayed from cell culture supernatants by ELISA. Black bars represent cells stimulated by Hi5 insect cells expressing hCD154, open bars represent cells stimulated by agonistic anti-mCD40 mAb, gray bars represent cells stimulated by agonistic anti-hCD40 mAb. Open bars are generally below the level of detection because anti-CD40 mAb does not induce IL-6 production via hCD40 (150). IL-6 secreted in cultures containing isotype control mAbs or untransformed Hi5 insect cells was subtracted from values obtained from cultures stimulated via hCD40 extracellular domains. **(C)** CH12.LX B cells stably transfected with hCD40LMP1, hCD40, hCD40ADD, or hCD40LMP1AEDL were stimulated for 3 days with agonistic mouse or human anti-CD40 mAbs or isotype control mAbs. Data are presented as the mean number of Ab secreting cells ± S.E. of replicate cultures induced in response to hCD40 stimulation normalized to the number of Ab secreting cells induced in response to mCD40 stimulation. Ab secreted by cells cultured with isotype control mAbs was subtracted from CD40 stimulated groups prior to normalization. Data shown are representative of three or more experiments performed with two or more clones of each transfectant.

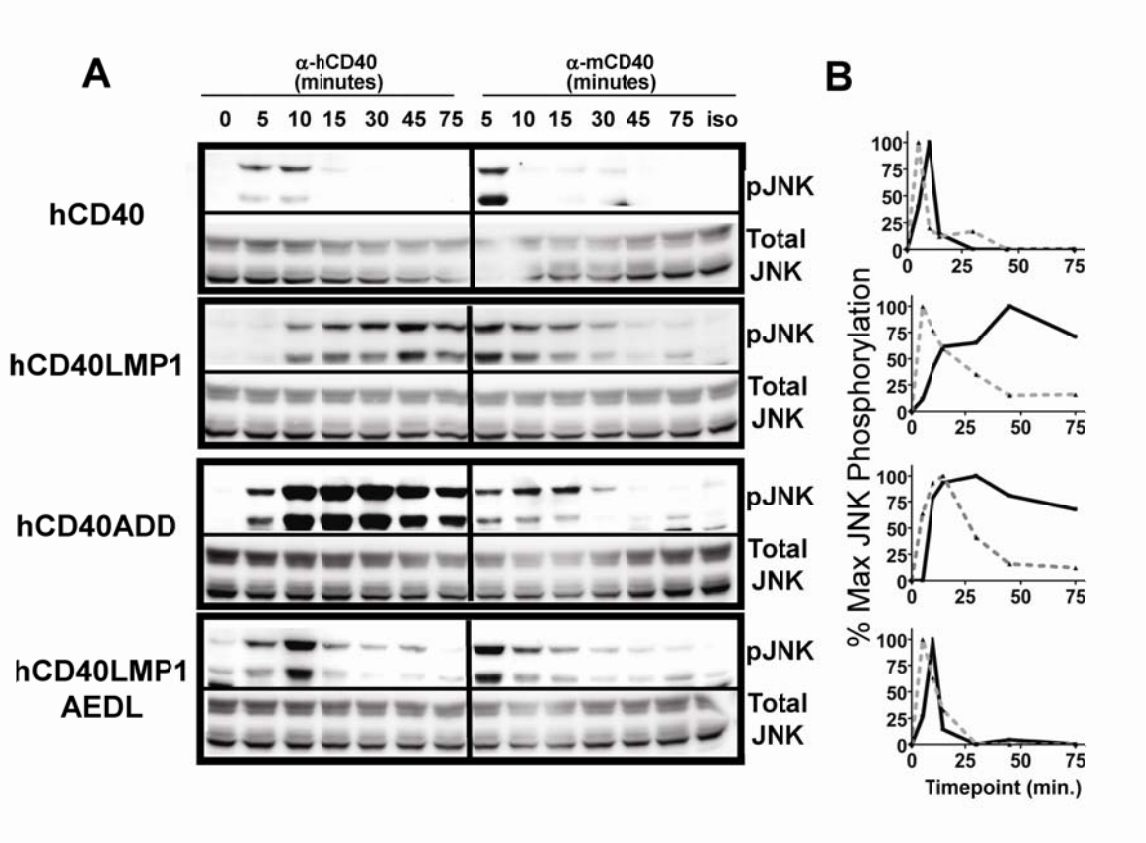


Figure 15. Effect of TBS mutation on JNK activation.
(A) Whole cell lysates from M12.4.1 B cell transfectants stimulated for the indicated times with agonistic anti-mouse or human CD40 mAbs or isotype control mAbs were analyzed by immunoblotting for phosphorylated JNK (pJNK) or total JNK. **(B)** Quantification of JNK activation was performed by measuring pJNK and the corresponding total JNK bands with a low-light imaging system. The amount of each pJNK band was normalized to the corresponding total JNK band. These values were then normalized to the point of maximal JNK activation (greatest pJNK/total JNK), which was set as 100%. Black solid lines represent JNK activation induced by anti-hCD40 agonistic mAb, grey dashed lines represent JNK activation induced by anti-mCD40 agonistic mAb. Data shown are representative of three or more experiments performed with two or more clones of each transfectant.

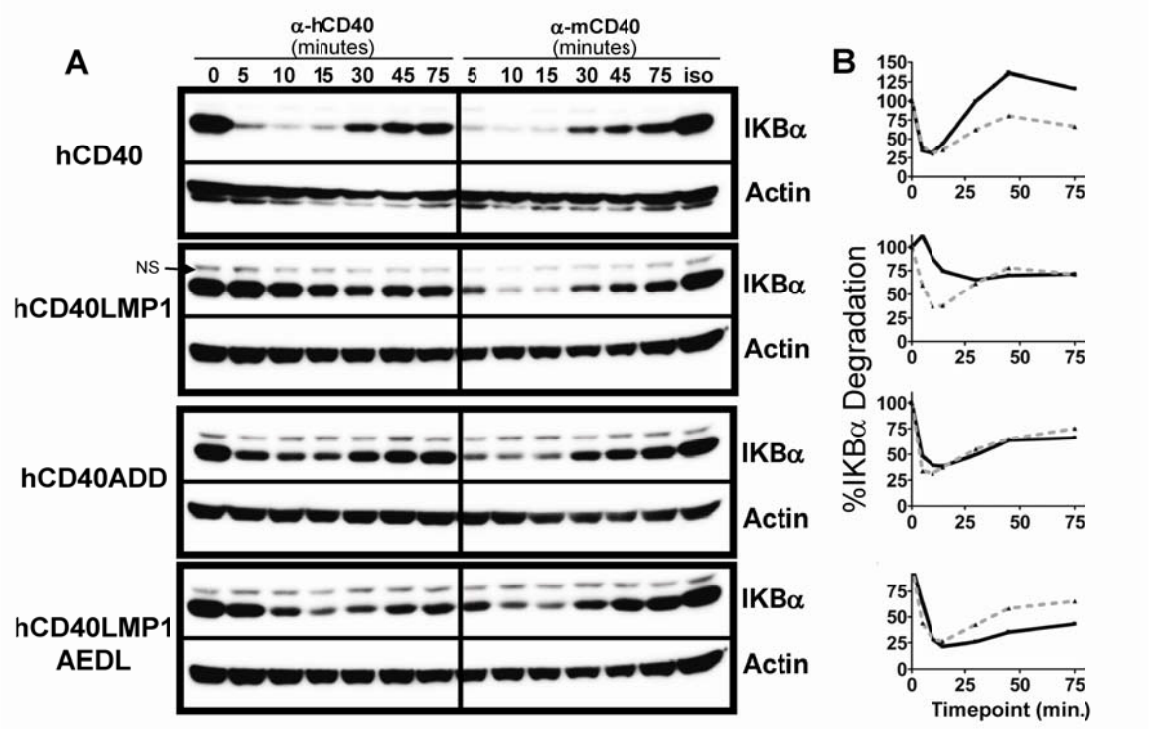


Figure 16. Effect of TBS mutation on canonical NF-κB activation.
(A) Whole cell lysates from M12.4.1 B cell transfectants stimulated for indicated times with agonistic anti-mouse or human CD40 mAb or isotype control mAbs were analyzed by immunoblotting for total IκBα or actin. **(B)** Quantification of IκBα degradation was performed by measuring total IκBα and actin bands with a low-light imaging system. The amount of IκBα was normalized to the corresponding actin band. These values were then normalized to the 0 time (0 min. IκBα/actin), which was set as 100%. Black solid lines represent IκBα degradation induced by anti-hCD40 agonistic mAb, grey dashed lines represent IκBα degradation induced by anti-mCD40 agonistic mAb. Data shown are representative of three or more experiments performed with two or more clones of each transfectant.

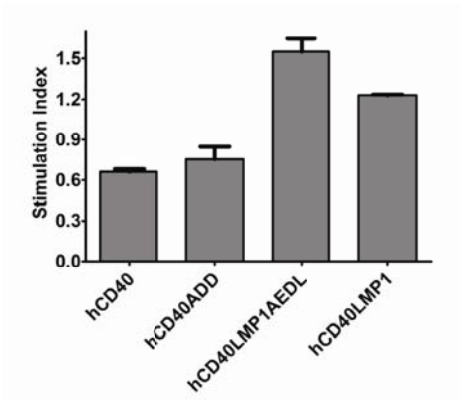


Figure 17. Effect of TBS mutation on total NF-κB activation.
M12 B cells expressing either hCD40, hCD40LMP1, hCD40LMP1AEDL, or hCD40ADD were transiently transfected with a NF-κB *firefly* reporter plasmid and a *renilla* control plasmid, stimulated with α-mouse or human CD40 or isotype control Abs for 6h. Values from each sample were normalized for transfection efficiency by dividing the *renilla* luciferase activity by the *firefly* luciferase activity in each sample. The stimulation index for each receptor was determined by dividing the normalized values of each sample as: (αhCD40 mAb stimulated-isotype control mAb stimulated) / (αmCD40 mAb stimulated- isotype control mAb stimulated). Data shown are representative of three or more experiments performed with two or more clones of each transfectant.

CHAPTER V

COMPARISONS OF CD40 AND LMP1 IN CIAP USAGE AND TRAF DEGRADATION

Rationale

The E3 ubiquitin ligases, cIAP1 and cIAP2, have been reported to play a crucial role in CD40 signaling (145). In primary mouse splenic B cells, the cIAPs are recruited to CD40 via TRAF2 and are required for CD40-mediated signaling and TRAF degradation (145). The cIAPs are redundant and can substitute for each other in cases of a single deficiency (145). Because LMP1 is a mimic of CD40 signals, we hypothesized that LMP1 may require the cIAPs for signaling. To investigate this possibility, we used Western blotting-based signaling assays and immunoprecipitation techniques to elucidate the role of the cIAPs in CD40 and LMP1 signaling. During these investigations we also made use of Smac mimetics (SM). SM are small molecule mimics of the pro-apototic protein Smac, that specifically bind to cIAP1 and 2 and induce their rapid degradation (145). To also further our understanding of the molecular underpinnings of why CD40 but not LMP1 induces TRAF2 and TRAF3 degradation upon signaling, we sought to understand the requirements for the cIAPs in CD40 and CD40LMP1AEDL-mediated TRAF degradation.

CD40LMP1AEDL-mediated TRAF2 degradation (Fig. 13) was associated with an increased CD40-like TRAF2 association upon signaling (Fig. 12). A CD40LMP1 molecule containing only the CTAR1 region displays an increased TRAF2 association in comparison to WT CD40LMP1 (112). Because of these

findings we wanted to test the hypothesis that the higher TRAF2 binding ability of CD40LMP1CTAR1 compared to CD40LMP1 allows induction of TRAF degradation upon signaling.

TRAF6 plays an integral role in many facets of the CD40 signal (114). Therefore, TRAF6 may play a role in the mechanism leading to signaling-induced TRAF degradation. To test the hypothesis that TRAF6 contributes to CD40-mediated TRAF degradation, we performed TRAF degradation assays (described in Methods) using $TRAF6^{-/-}$ mouse B cells.

Here, I show that CD40 and LMP1 differentially utilized the cIAPs in signaling. Also, the mechanism driving TRAF degradation depends on more than a high TRAF2 binding ability or the cIAPs. The results presented here suggest that the machinery mediating TRAF degradation has many redundancies, highlighting the importance of TRAF degradation to normal immune function.

Results

cIAP association with CD40, CD40LMP1, CD40ADD, and CD40LMP1AEDL

CD40 has been reported to associate with cIAP1 and cIAP2 in mouse splenic B cells (145). To determine if the cIAPs associate with LMP1, and what role the TBS plays in LMP1 and or CD40-cIAP associations, we immunoprecipitated hCD40, hCD40ADD, hCD40LMP1AEDL, and hCD40LMP1 from mouse B cells 15 minutes following the initiation of signaling. We then Western blotted for the presence of cIAP-receptor associations in immune

precipitates.

As seen in Figure 18, cIAP1 and cIAP2 co-immunoprecipitate with hCD40, hCD40ADD, and hCD40LMP1AEDL but not hCD40LMP1. CD40 and CD40ADD bind similar amounts of the cIAPs, while CD40LMP1AEDL binds less. Because CD40ADD binds similar levels of the cIAPs in comparison to hCD40, regions outside of the TBS of CD40 must be important in mediating CD40-cIAP interactions. However, because the TBS of CD40 imparts upon CD40LMP1AEDL the ability to bind the cIAPs, the TBS is also an important component to CD40-cIAP interactions. These results demonstrate that the TBS of CD40 is redundant with and may cooperate with other regions of CD40 in the recruitment of the cIAPs.

The cIAPs in CD40 and LMP1 signaling

To investigate the role of the cIAPs in CD40 and LMP1 signaling we used SMs to deplete the cIAPs. Figures 19 and 20 demonstrate the effectiveness of SM-mediated cIAP depletion in both mouse and human cells. To directly compare the effects of cIAP depletion on CD40 and CD40LMP1 signals, we used a mouse B cell line expressing endogenous mCD40 and transfected CD40LMP1. Using this line we found that CD40 and CD40LMP1 signals are differentially affected by cIAP depletion (Fig. 19). CD40-mediated JNK and p38 activation was decreased by SM pretreatment, while LMP1-mediated JNK activation was increased and p38 activation was decreased. Furthermore, CD40-mediated IKBα phosphorylation and degradation was enhanced whereas LMP1-mediated

IKBα phosphorylation was decreased. LMP1-mediated IKBα degradation appeared largely unaffected by SM pretreatment. For CD40, these findings were recapitulated in primary human macrophages (Fig. 20). SM pretreatment of human macrophages drastically reduced CD40-mediated p38 activation while it more modestly decreased JNK and ERK activation. Similar to mouse B cells, SM pretreatment of human macrophages boosted the ability of CD40 to mediate IKBα phosphorylation and degradation. In conclusion, both CD40 and LMP1 utilize the cIAPs in mediation of their signals. However, the results discussed here and those showing differential recruitment to CD40 and LMP1 (Fig. 18) suggest that CD40 and LMP1 use the cIAPs in different ways.

The cIAPs and TRAF degradation

The cIAPs are reportedly required for CD40-mediated TRAF degradation in cells of murine origin (145). Hence, we wished to determine if they were required for CD40-mediated TRAF degradation in human cells. To do so, we subjected human primary macrophages to TRAF degradation assays as detailed in Methods. Upon CD40-signaling, both TRAF2 and TRAF3 are induced to degrade (Fig. 21). With SM pretreatment there is a defect in TRAF degradation at earlier times. However, in contrast to the earlier study (145), CD40-mediated TRAF degradation occurred in the absence of the cIAPs. The amount of TRAF degradation by the SM pretreated group reached control group levels at later times (Fig. 21). Furthermore, we found that the cIAPs were not required for the initiation of CD40-mediated TRAF degradation in primary human B cells (Fig.

22). We therefore conclude that the cIAPs contribute, but are not absolutely required for CD40-mediated TRAF degradation.

cIAPs and CD40LMP1AEDL-mediated TRAF2 degradation

The cIAPs are not absolutely required for CD40-mediated TRAF degradation. Despite this, and because the cIAPs are recruited to LMP1AEDL (Fig. 18), we were curious about the involvement of the cIAPs in LMP1AEDL-mediated TRAF2 degradation. Unlike CD40, SM pretreatment abrogated the ability of LMP1AEDL to induce TRAF2 degradation (Fig. 23). Similarly to CD40, LMP1AEDL signaling induced the degradation of cIAP1 in control cells (Figures 19, 23, and 27). These results suggest that the TBS of CD40 mediates the ability to induce TRAF2 and cIAP degradation through the cIAP molecules. However, other regions of CD40 may facilitate interactions with additional E3 ubiquitin ligases that function redundantly with the cIAPs.

LMP1CTAR1 and TRAF degradation

CD40LMP1AEDL-mediated TRAF2 degradation (Fig. 13) was associated with an increased CD40-like TRAF2 association upon signaling (Fig. 12). In comparison to CD40LMP1, CD40LMP1CTAR1 displayed an increased TRAF2 association (112). To determine if a high TRAF2 binding capacity is sufficient for a receptor to induce TRAF2 degradation, we investigated the ability of CD40LMP1CTAR1 signaling to induce TRAF degradation in mouse B cells.

Figure 24 demonstrates that endogenous mCD40 signaling induced TRAF2 and TRAF3 degradation. However, like WT CD40LMP1 (Fig. 13), CD40LMP1CTAR1 was unable to induce TRAF degradation (Fig. 24).

In addition to showing increased TRAF2 binding, CD40LMP1CTAR1 also binds increased amounts of TRAF1 in comparison to WT CD40LMP1 (112). TRAF1 cooperates with TRAF2 and increases CD40 signaling by decreasing CD40-mediated TRAF degradation (123). CD40LMP1CTAR1 binds similar amounts of TRAF3 in comparison to WT CD40LMP1 (112), and both molecules bind much greater amounts of TRAF3 than CD40 (Fig. 11 and 210). Therefore, TRAF1 or TRAF3 may interfere with the ability of CD40LMP1CTAR1 signaling to induce TRAF degradation. To test this, we subjected TRAF1 or TRAF3 deficient mouse B cells expressing CD40LMP1CTAR1 to TRAF degradation assays. As seen in Figure 25, the deficiency of either of these TRAFs did not give CD40LMP1CTAR1 the ability to induce TRAF degradation. Together these results suggest that merely having a high TRAF2 binding capacity is not sufficient for a receptor's signals to induce TRAF2 or 3 degradation.

TRAF6 and CD40-mediated TRAF degradation

CD40ADD, a CD40 molecule with the LMP1 TBS in place of the WT TBS (Fig. 12), induced TRAF degradation upon signaling (Fig. 13). CD40 did not require the cIAPs to initiate TRAF degradation, whereas LMP1AEDL did (Fig. 21-23). These results suggest that the TBS of CD40 mediates TRAF degradation via the cIAPs, but regions outside of the CD40 TBS may mediate both TRAF2 and

TRAF3 degradation in their absence. TRAF6 binds to a region separate from the TBS (114). Therefore, TRAF6 may facilitate TBS-independent TRAF degradation. To test this possibility, we performed TRAF degradation assays with TRAF6 deficient mouse B cells. Interestingly, despite the dramatic effect TRAF6 deficiency has on most CD40 signals (114), its deficiency did not abrogate CD40-mediated TRAF degradation (Fig. 26). Furthermore, the levels of TRAF degradation were similar to those induced in WT mouse B cells (Fig. 26 versus Fig. 13).

CD40 versus TLR-mediated cIAP and TRAF degradation

The TRAFs and the E3 ubiquitin ligases cIAP1 and cIAP2 have been implicated in mediating TLR signals. To determine if these molecules induce TRAF and cIAP degradation upon signaling, we subjected primary mouse splenic B cells to TRAF degradation assays following stimulation through CD40, TLR3, TLR4, and TLR7. Figure 27 shows that CD40 was the only receptor that induced significant TRAF2 and TRAF3 degradation. Interestingly, upon signaling all receptors induced cIAP1 degradation. TLR3 and TLR4, however, mediated sustained CD40-like cIAP1 degradation, whereas TLR7-mediated cIAP degradation was more transient. Overall, these results reveal that CD40 is unique in its ability to induce TRAF2, TRAF3, and cIAP1 degradation.

Conclusions

The TRAF binding site controls some but not all aspects of the CD40 or LMP1 signal (141). Interestingly, the TBS only partially affected the recruitment of the cIAPs to CD40 (Fig. 18). Because the cIAPs are important positive regulators of CD40-mediated MAPK activation and negative regulators of NFκB activation (Fig. 19 and 20), the influence of the CD40 TBS on CD40 and LMP1AEDL signals (Figures 15 and 16) is likely mediated in-part by these factors.

Interestingly, CD40LMP1 used the cIAPs differently than CD40. Upon signaling, CD40 recruited the cIAPs whereas CD40LMP1 did not (Fig. 18). This implies that CD40LMP1 utilizes the cIAPs in a cytosolic manner, in a fashion similar to CD40's use of cytosolic TRAF6 in signaling (114). Whereas all CD40-mediated MAPK signals were decreased by cIAP depletion, CD40LMP1-mediated JNK activation was increased (Fig. 19). Furthermore, CD40LMP1 had decreased IKBα phosphorylation following cIAP depletion, suggesting that the cIAPs are positive regulators of CD40LMP1-mediated IKBα phosphorylation (Fig. 19).

Contrary to a previous report, we found that the cIAPs were not absolutely required for CD40-mediated TRAF degradation, though they might be important for maximal TRAF degradation at earlier times (Fig. 20). However, CD40LMP1AEDL required the cIAPs to induce TRAF2 degradation (Fig.19). These results imply that the TBS of CD40 mediates TRAF degradation via the cIAPs, but other regions of CD40 may foster interactions with cIAP-redundant molecules that can mediate TRAF degradation. If this is true, it is likely that

TRAF6 plays no role in the recruitment of cIAP-redundant, TRAF degradation promoting factors, as there were no noticeable defects in CD40-mediated TRAF degradation in TRAF6 deficient mouse B cells (Fig. 26).

To determine if a high TRAF2 binding ability is sufficient for a receptor's signals to induce TRAF2 degradation, we investigated the ability of CD40LMP1CTAR1 signaling to induce TRAF degradation. However, like WT CD40LMP1 (Fig. 13), CD40LMP1CTAR1 was unable to induce TRAF degradation (Fig. 24). The deficiency of TRAF1 or TRAF3 did not allow CD40LMP1CTAR1 to induce TRAF degradation (Fig. 25). These results indicate that high TRAF2 binding is not sufficient for a receptor to induce TRAF degradation, even in the absence of any possible negative influences of TRAF1 or TRAF3. Because LMP1AEDL induced cIAP-dependent TRAF2 degradation (Fig. 13), we surmise that the unique sequence of the CD40 TBS imparts upon TRAF2 the ability to initiate TRAF degradation in a cIAP-dependent manner. LMP1 signaling is unable to induce TRAF degradation because the sequence of its TBS does not foster TRAF2-cIAP interactions or interactions with cIAP-redundant E3 ubiquitin ligases.

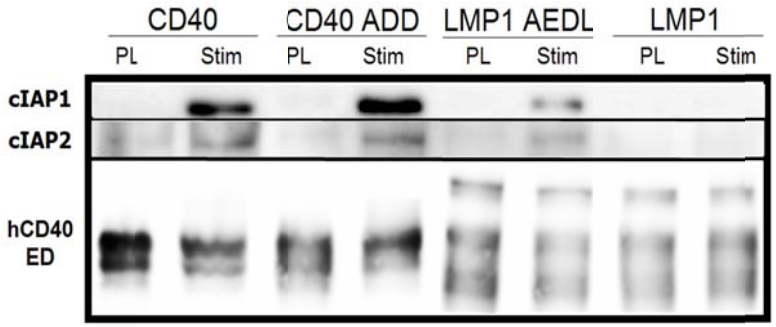
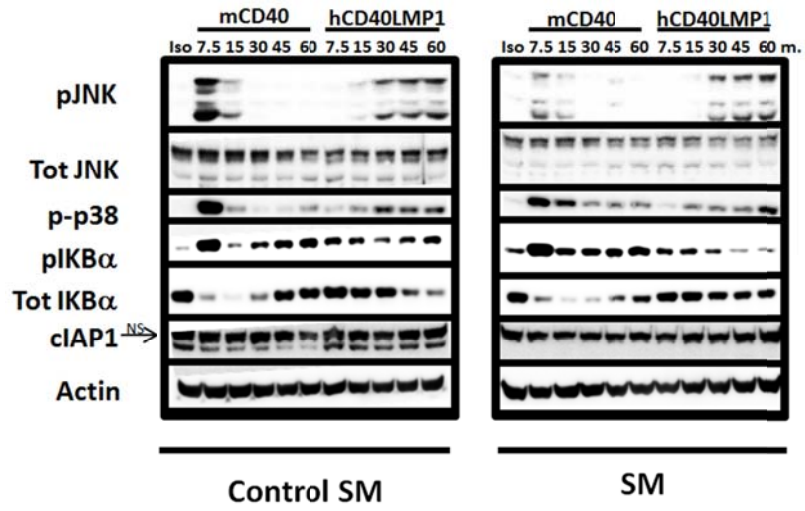


Figure 18. The TBS and cIAP association. Samples created during experiments described in Fig. 11 were subjected to SDS-PAGE electrophoresis and Western blotting for cIAP1, cIAP2, and the hCD40 extracellular domain (EC).

Figure 19. The effects of cIAP depletion on CD40 and CD40LMP1 signals. **(A)** Whole cell lysates from A20 B cells transfected with hCD40LMP1 were analyzed for MAPK and NFκB activation upon mCD40 or hCD40LMP1 stimulation by Western blotting as described in Methods. Quantification of signaling **(*B*)** was performed by measuring the intensities of pJNK, pP38, pIKBα, total IKBα and actin or total JNK bands as in Methods. The amount of each signaling band was normalized to the corresponding actin or total JNK band. These values were then normalized to the 0 time point. Black solid lines represent control, non-cIAP depleting peptide, grey lines represent cIAP-depleting SM peptide.

A


mCD40
hCD40LMP1
Iso 7.5 15 30 45 60 7.5 15 30 45 60
mCD40
hCD40LMP1
Iso 7.5 15 30 45 60 7.5 15 30 45 60 m.
pJNK
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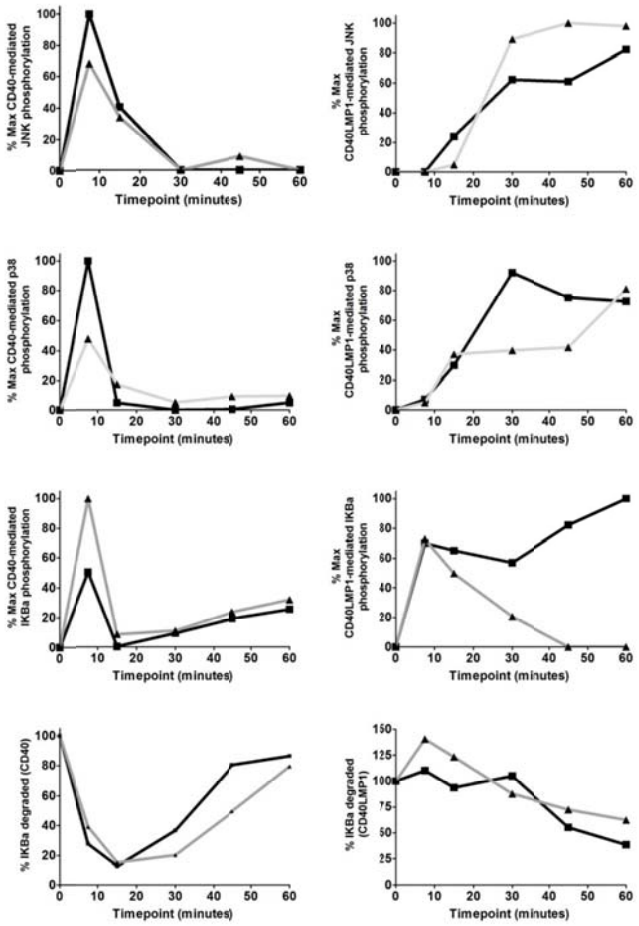
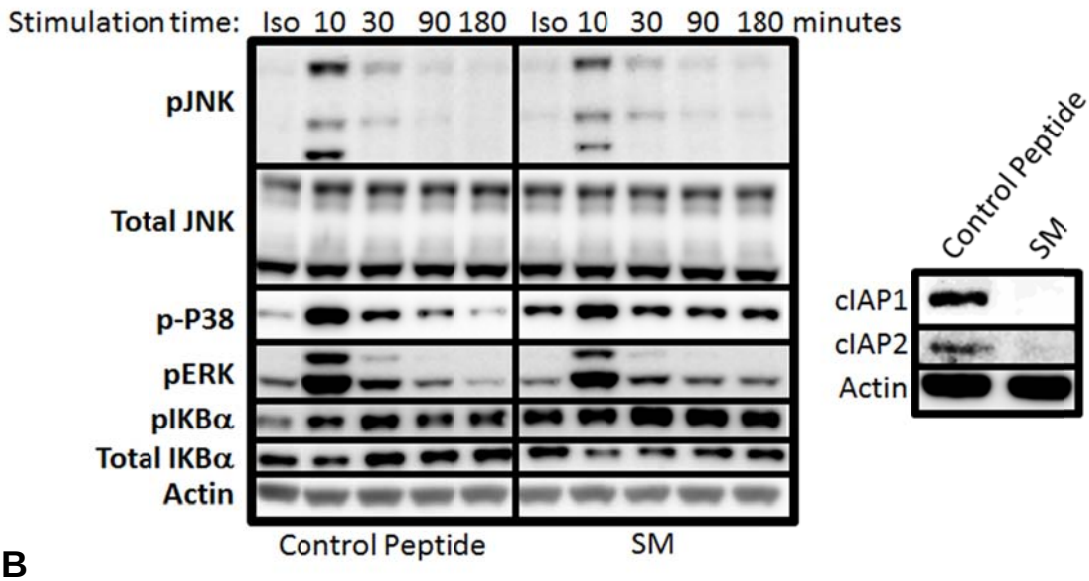
pIKBα
Tot IKBα
cIAP1
NS
Actin
Control SM
SM

B


% Max CD40-mediated JNK phosphorylation
% Max CD40LMP1-mediated JNK phosphorylation
% Max CD40-mediated p38 phosphorylation
% Max CD40LMP1-mediated p38 phosphorylation
% Max CD40-mediated IKBa phosphorylation
% Max CD40LMP1-mediated IKBa phosphorylation
% IKBa degraded (CD40)
% IKBa degraded (CD40LMP1)
Timepoint (minutes)

A



B

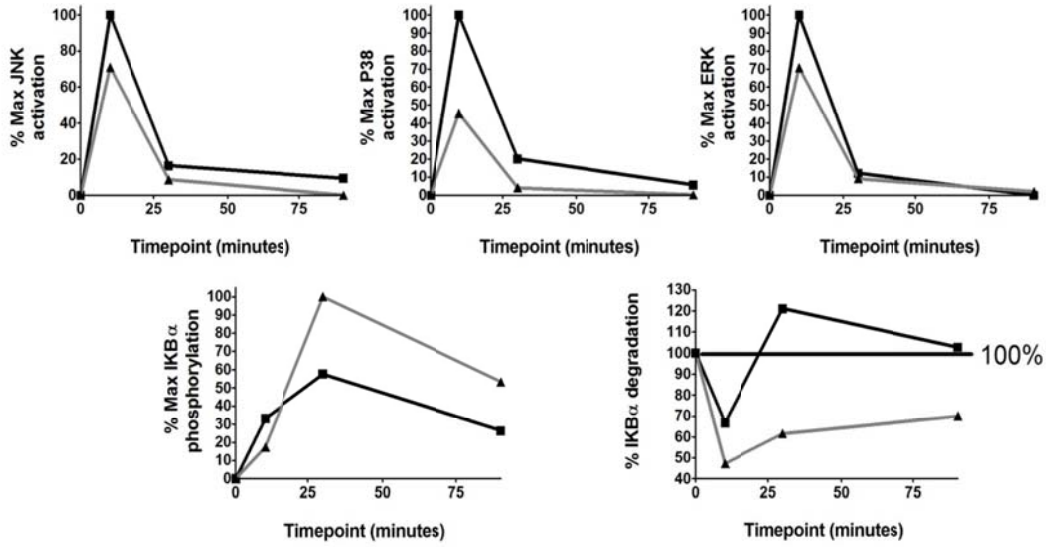


Figure 20. cIAP depletion and human primary macrophage CD40 signals. **(A)** Whole cell lysates from human macrophages were analyzed by Western blotting for MAPK and NFκB activation upon hCD40 stimulation by agonistic Ab. Quantification of signaling **(*B*)** was performed by measuring the intensities of pJNK, pP38, pIKBα, total IKBα and actin or total JNK bands as in Methods. The amount of each signaling band was normalized to the corresponding actin or total JNK band. These values were then normalized to the 0 time point. Black solid lines represent control, non-cIAP depleting peptide, grey lines represent cIAP-depleting SM peptide.

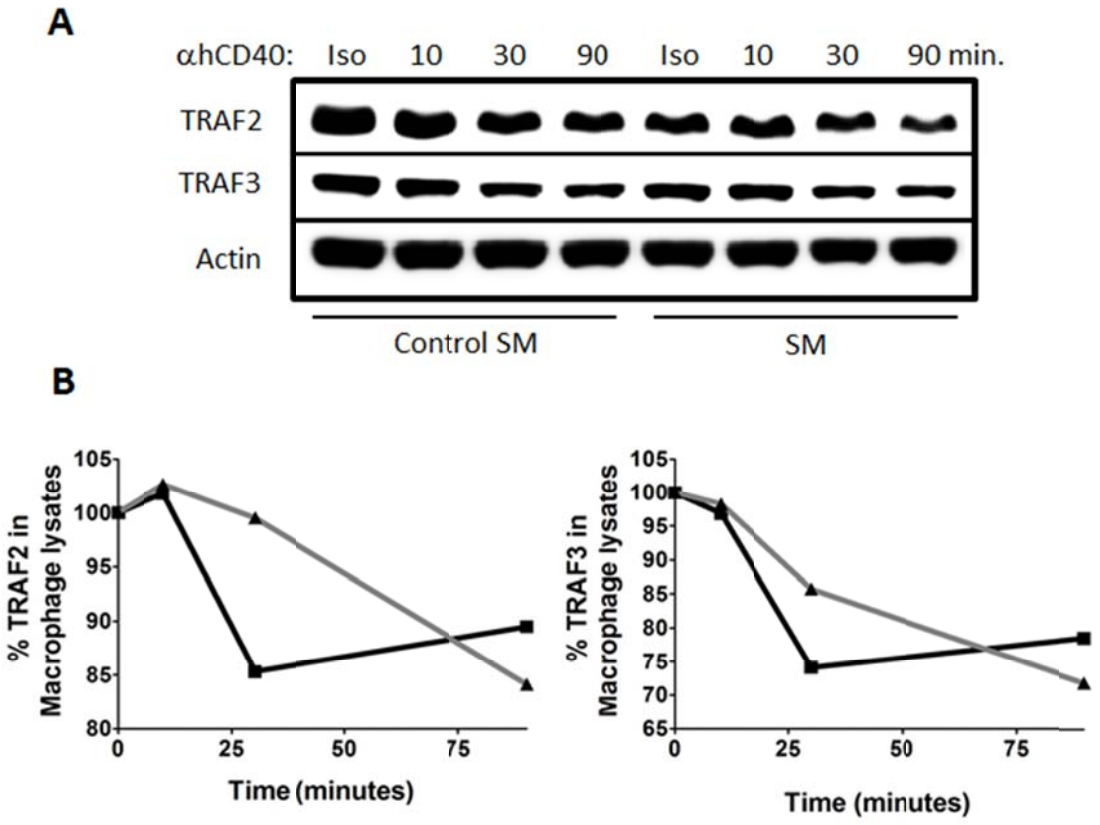


Figure 21. cIAP depletion and CD40-mediated TRAF degradation in human macrophages.
(A) Whole cell lysates from primary human macrophages were stimulated for the indicated times with agonistic human CD40 mAbs or isotype control mAbs analyzed by immunoblotting for TRAF2, TRAF3, and actin. **(B)** Quantification was performed by measuring TRAF and the corresponding actin bands with a low-light imaging system. The amount of each TRAF band was normalized to the corresponding actin band. These values were then normalized to isotype control treated group, which was set as 100%. Black solid lines represent control peptide treated cells whereas grey lines represent SM treated cells.

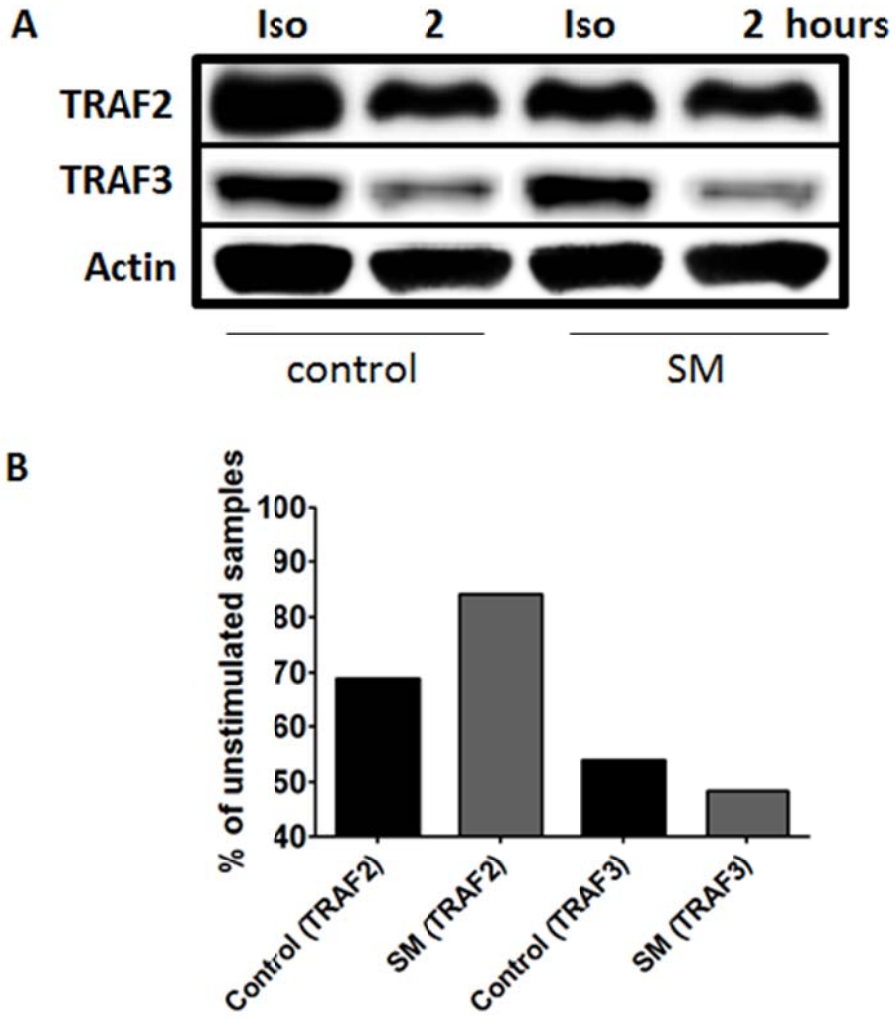


Figure 22. TRAF degradation and cIAP depletion in human B cells.
(A) Whole cell lysates from human primary B cells were stimulated for 2 hours with agonistic anti- human CD40 mAb and analyzed by Western blotting for TRAF2, TRAF3, or actin. **(B)** Quantification of TRAF degradation was performed by measuring TRAF and actin bands with a low-light imaging system. The amount of TRAF was normalized to the corresponding actin band. These values were then normalized to the 0 time (0 min. TRAF/actin), which was set as 100%. Black solid bars represent TRAF degradation induced by cells treated with control peptide, grey bars represent TRAF degradation induced by cells treated with Smac Mimetics.

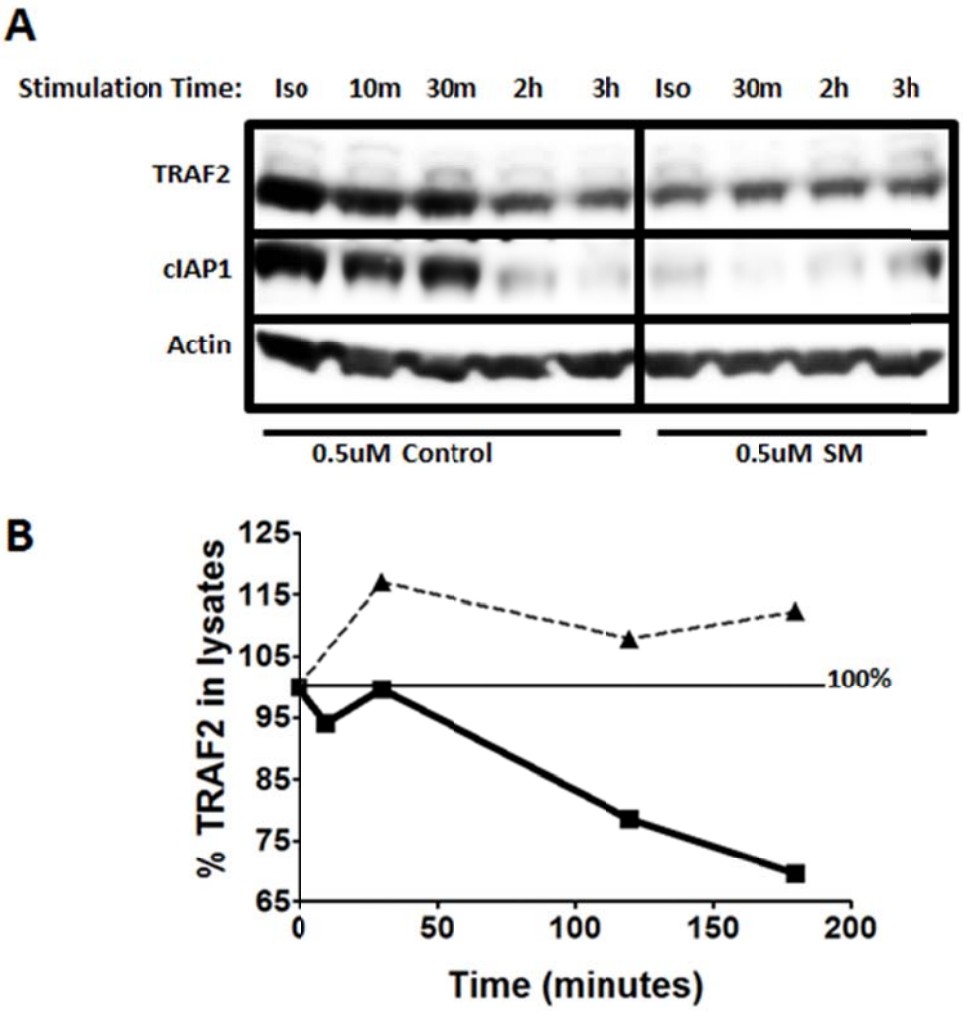


Figure 23. cIAP depletion and CD40LMP1AEDL-mediated TRAF2 degradation.
(A) Whole cell lysates from CH12.LX B cells expressing hCD40LMP1AEDL were stimulated at the indicated times with agonistic anti- human CD40 mAb or isotype control mAbs and were analyzed by immunoblotting for TRAF2 or actin. **(B)** Quantification of TRAF2 degradation was performed by measuring TRAF2 and actin bands with a low-light imaging system. The amount of TRAF2 was normalized to the corresponding actin band. These values were then normalized to the 0 time (0 min. TRAF2/actin), which was set as 100%. Black solid lines represent TRAF2 degradation induced by anti-hCD40 agonistic mAb in the presence of control peptide, grey dashed lines represent TRAF2 degradation induced by anti-hCD40 agonistic mAb in the presence of SM.

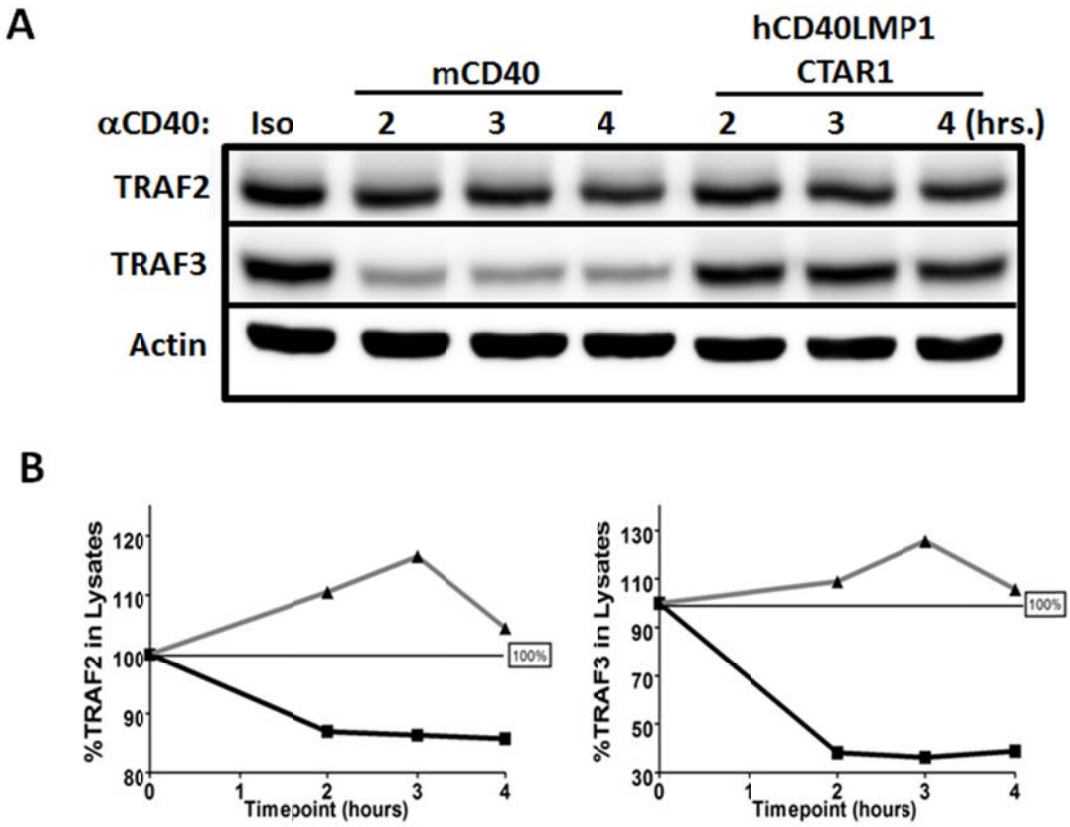


Figure 24. Role of LMP1 CTAR1 in TRAF degradation.
(A) Whole cell lysates from CH12.LX B expressing hCD40LMP1CTAR1 were stimulated at the indicated times with agonistic anti- human CD40 mAb or anti-mouse CD40 mAbs and were analyzed by immunoblotting for TRAF2, TRAF3, or actin. **(B)** Quantification of TRAF degradation was performed by measuring TRAF and actin bands with a low-light imaging system. The amount of TRAF was normalized to the corresponding actin band. These values were then normalized to the 0 time (0 min. TRAF/actin), which was set as 100%. Black solid lines represent TRAF degradation induced by anti-mCD40 agonistic mAb grey lines represent TRAF degradation induced by anti-hCD40 agonistic mAb (hCD40LMP1CTAR1).

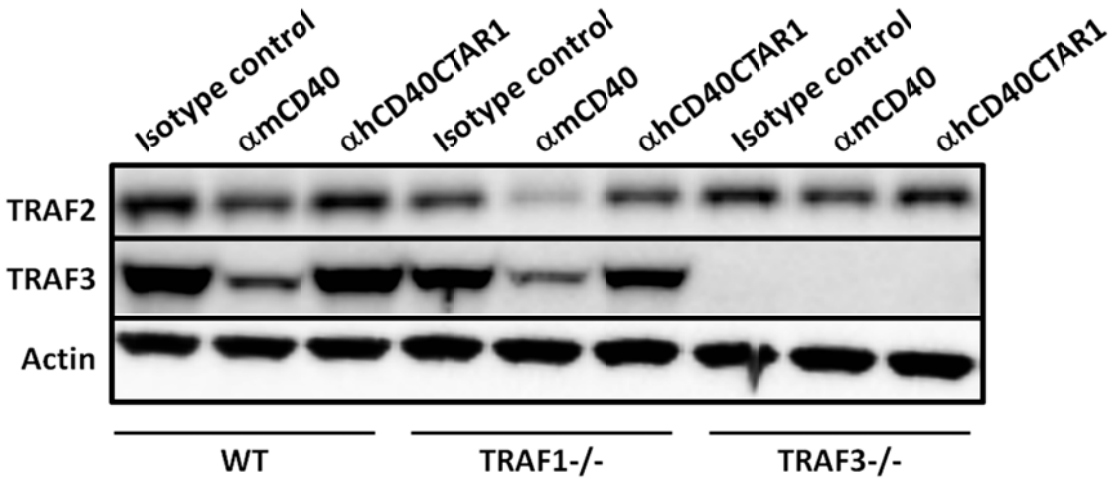


Figure 25. hCD40LMP1CTAR1 does not induce TRAF degradation in TRAF1 or 3 knock-out B cells.
Whole cell lysates from WT, TRAF1-/-, or TRAF3-/- CH12.LX B cells expressing hCD40LMP1CTAR1 were stimulated for 3 hours with agonistic anti-human CD40 mAb or anti- mouse CD40 mAbs and were analyzed by immunoblotting for TRAF2, TRAF3, or actin.

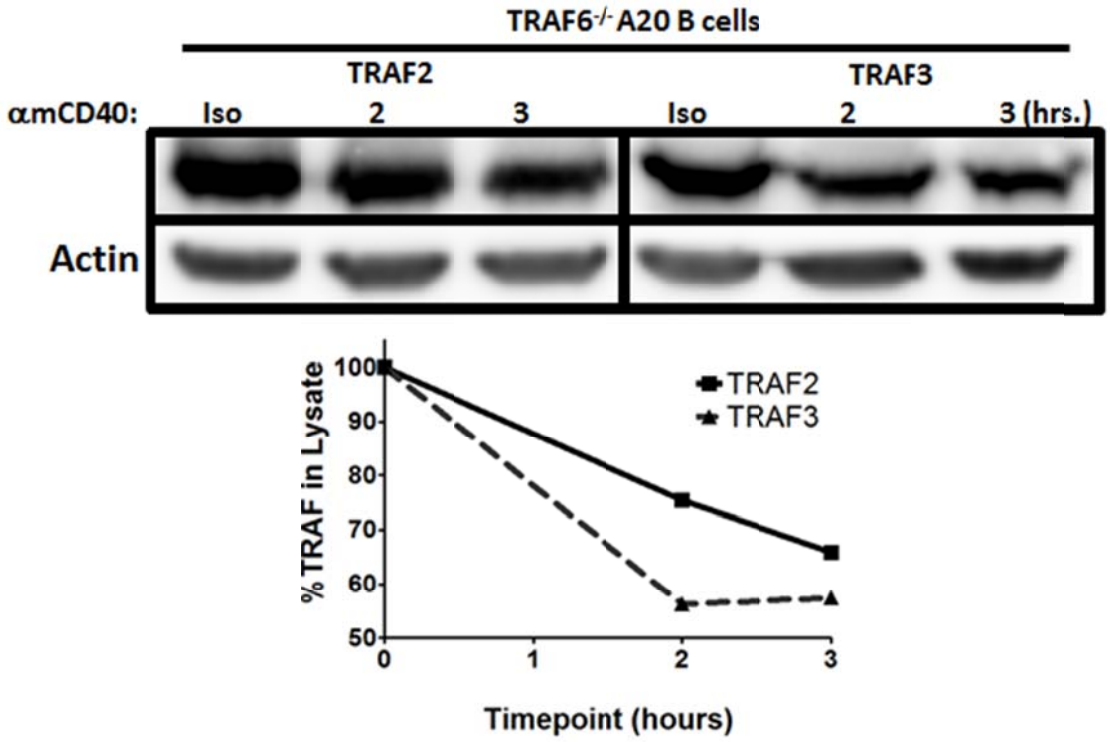


Figure 26. TRAF6 deficiency and CD40-mediated TRAF degradation. Whole cell lysates from TRAF6$^{-/-}$ A20 B cells were stimulated with isotype control Ab or anti-mCD40 Ab and analyzed for TRAF2 and TRAF3 degradation. Quantification of TRAFs was performed by measuring the intensities of TRAF2, TRAF3, and actin bands as described in Methods. The amount of each TRAF band was normalized to the corresponding actin band. These values were then normalized to the 0 time point, which was set as 100% of either TRAF2 or TRAF3. Black solid lines represent TRAF2 degradation whereas dashed grey lines represent TRAF3 degradation.

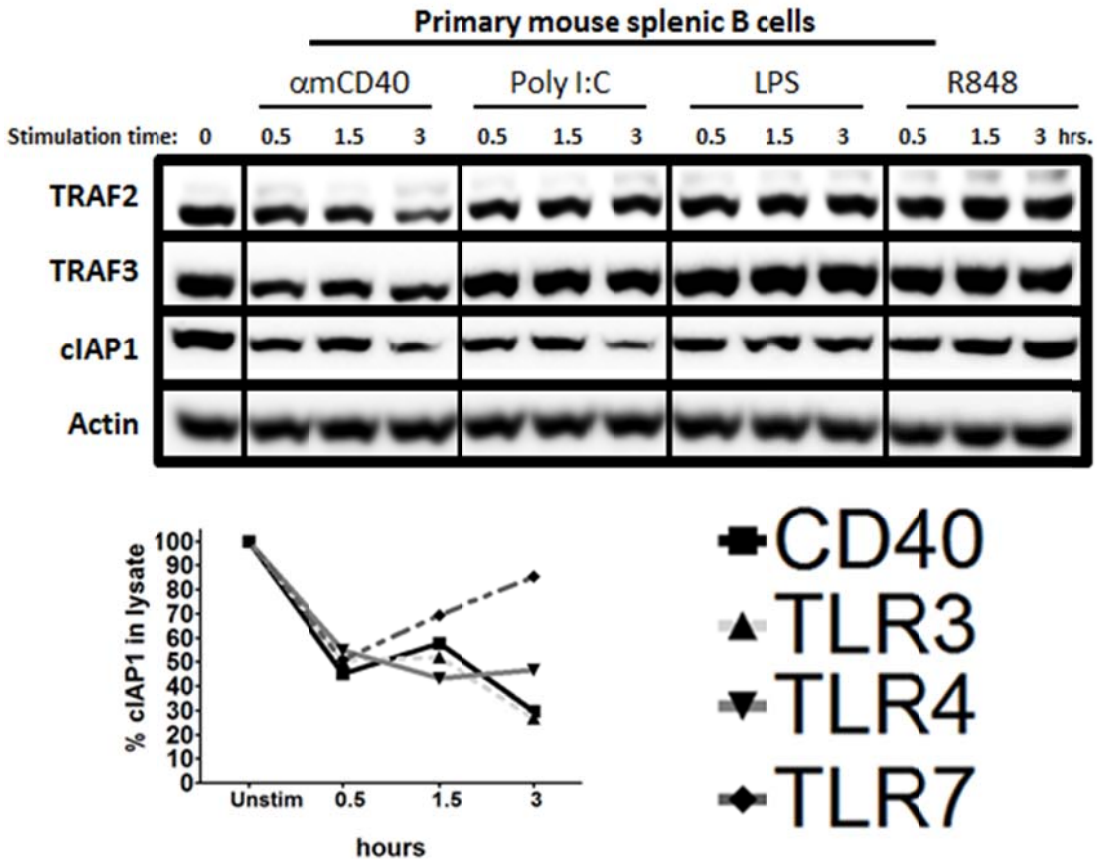


Figure 27. CD40 and TLR stimulation of mouse splenic B cells.
Whole cell lysates from mouse splenic B cells were stimulated with anti-mCD40 Ab, poly I:C, LPS, or R848 and analyzed for TRAF2, TRAF3, and cIAP1 degradation. Quantification of cIAP1 was performed by measuring the intensities of the cIAP1 and actin bands as described in Methods. The amount of cIAP was normalized to the corresponding actin band. These values were then normalized to the 0 hour time, which was set as 100%. Black solid lines represent CD40 stimulation, grey solid lines represent TLR4 stimulation, light grey dashed lines represent TLR3 stimulation, and dashed grey lines represent R848 stimulation.

CHAPTER VI

DISCUSSION

CD40-mediated TRAF degradation in immune regulation

The results of this study demonstrate that CD40-mediated TRAF degradation is a rapid and robust regulatory mechanism for both the CD40 signal itself and TRAF2/3-dependent signals delivered by other receptors on B lymphocytes and macrophages (Figures 4-11). TRAF2 degradation appears to regulate the CD40 signal in several ways. The initial phases of TRAF2 degradation following CD40 engagement promote the dissolution of the CD40 signaling complex ((145) and Figure 2). Blocking TRAF degradation by proteasome inhibition amplified the activation of MAPKs in as little as ten minutes of CD40 signaling, and sustained the signal to at least 30 minutes (Figure 2). This finding correlates with an increased CD40-TRAF2 association at 30 minutes post-signaling in proteasome-inhibited B cells (145). Additionally, in a proteasome-dependent manner, CD40 signaling blocked the ability of subsequent CD40LMP1-initiated pathways to activate JNK. Thus, CD40-mediated decreases in the total cellular pool of TRAF2 or TRAF3 dampened subsequent TRAF2- or TRAF3-dependent signaling (Figure 2-8).

CD40 signaling induces the degradation of its negative regulator, TRAF3 (98). In a recently proposed two-step model for CD40 signaling, a TRAF2-dependent, CD40-tethered multi-subunit complex of signaling factors forms upon CD40 engagement. According to this model, the TRAF2-dependent signaling

factor complex must be released from CD40 in order to gain access to, and activate, MAPK substrates in the cytosol. This model also predicted that TRAF3 prevents complex release from CD40, so for CD40 to mediate MAPK signaling TRAF3 must be degraded. Accordingly, TRAF3 degradation was assigned a role as a positive regulator of CD40 signals (145).

In the present study, we show that inhibiting TRAF degradation in mouse or human B cells and macrophages amplified, rather than abrogated, CD40-mediated MAPK activation (Figures 2 and 3). If a TRAF2-dependent multi-subunit complex of signaling factors must be released from CD40 in order to activate MAPKs, our data suggest that the proteasome-dependent degradation of TRAF2 or TRAF3 does not play a necessary role in this process. Our findings also indicate that sustained CD40-TRAF2 interactions (145) promote CD40-mediated MAPK activation (Figures 2 and 3). The differences between our results and the conclusions drawn in an earlier study (145) may be explained by the different cell types used in experiments. Primary splenic mouse B cells, used in the cited study, die quickly by apoptosis after isolation and removal from the presence of *in vivo* survival factors (192). Exposure of these sensitive cells to the stress-inducing compound MG132 may render them unresponsive to activation signals. Our results in mouse B cell lines were recapitulated in primary human macrophages (Figure 2A), which are less susceptible than mouse splenic B cells to early apoptosis in culture.

CD40 signaling was restrained in a proteasome-dependent manner, independently of the presence of or degradation of TRAF3 (Fig. 3). This

demonstrates that the proteasome-dependent mechanism mediating rapid negative regulation of CD40 signals was TRAF3 and TRAF3 degradation-independent. Furthermore, TRAF3 was not required for the ability of CD40 pre-stimulation to abrogate subsequent CD40 signals in mouse B cells (Figure 3B). Together, these results suggest that CD40-mediated degradation of TRAF3 may be more important to regulate the signaling of CD40-independent, TRAF3-dependent pathways in the CD40-expressing cell. Evidence supporting this concept is presented in Figs. 4-11.

Not only did CD40 signaling negatively regulate subsequent CD40LMP1-mediated JNK activation (Figures 4 and 5), it also affected downstream cytokine signaling, by both itself and other receptors. This CD40-mediated inhibition of LMP1 signaling suggests that CD40 agonists may be effective in the treatment of LMP1-driven, $CD40^{+}$ tumors (Figure 28).

The CD40 molecule is central in the transition from innate to adaptive immunity (19). It is well known that CD40 stimulation of an APC promotes the expression of immune promoting factors, such as chemokines, cytokines, and co-stimulatory molecules (22). This work adds a new dimension to CD40's function in immunity. Not only can CD40 signaling directly promote the expression of factors which shape an immune response, it can also work indirectly by modulating the responsiveness of an APC to host cytokines (Fig. 6) and pathogen-associated molecular patterns (PAMPs) (Fig. 8).

Here we show that CD40 can modulate the signaling of some, but not all TLR pathways (Fig.8). TLR3 signaling requires the signaling adaptor TIR-

domain-containing adaptor-inducing interferonβ (TRIF), whereas TLR7 requires the myeloid differentiation primary response gene 88 (MyD88) molecule (193). TLR4 utilizes both TRIF and MyD88 in the mediation of its signals (193). Our work shows that only TRIF-utilizing receptors were affected by CD40 pre-stimulation (Figure 8). The completely TRIF-dependent signals of TLR3 were abrogated by CD40 pre-stimulation whereas the signals of TLR4, which can employ either TRIF or MyD88 were lessened but not abrogated (Fig.8). This result suggests that in humans, the MyD88 pathway does not rely on TRAF2 or TRAF3 as positive mediators of its signals. Presumably, the MyD88 component of the TLR4 signal is compensating for the CD40-regulated signals of the TRIF pathway.

While it is known that TRAF3 mediates some aspects of the TLR signal (193), a role for TRAF2 in TLR signaling has yet to be described. TRAF2 may mediate some aspects of the TLR signal alone or in combinations with TRAF3 or TRAF6 or TRAF3 and TRAF6. Future work is needed to address these possibilities.

Both TLR3 and TLR7 recognize different forms of RNA, a PAMP present during some types of viral infections (20). TLR7 is a key mediator of the autoimmune disease systemic lupus erythematosus (SLE) (194), and CD40 ligand is required for TLR7-mediated lupus-like disease in mice (195). It is thus tempting to speculate that unlike TLR7, TLR3 signaling is not linked to SLE because of the restraint placed upon it by CD40-mediated TRAF degradation.

Overall, the findings presented here reveal a potentially important, powerful and newly appreciated function for CD40 in regulating immune responses. In addition to acting as a powerful stimulator of APC activation, CD40 signaling can also shape immune responses via the induction of TRAF degradation, which significantly modulated the signals of other TRAF2- or TRAF3-dependent immune receptors.

The role of the TRAF binding site in CD40 and LMP1 signaling

Although LMP1 is a functional mimic of CD40, these receptors use the TRAFs in distinct and sometimes contrasting ways (114, 134, 136). The present findings further indicate that physical association with LMP1 regulates the TRAF molecules differently than association with CD40. Here we demonstrate that the TBS of CD40 and hCD40LMP1 differentially regulated association with TRAFs and certain downstream functions.

Some studies suggest that CD40 and LMP1 signaling is regulated by cooperation of the TRAF2/3 binding site with the TRAF6 binding site, and cooperation between the CTAR1 and 2 regions, respectively (111, 112, 190). Investigations of CD40 and LMP1 molecules with mutated TBS clearly demonstrate the importance of the TBS in signaling (131, 140, 188). However, interpretation of these results is complex because mutations disrupting the integrity of the TBS may have either or both direct effects on proteins associating with the TBS, and indirect effects on signaling pathways which rely on cooperation between different structural regions. For CD40 signaling,

cooperation between the TRAF2/3 binding site and the TRAF6 binding site has been suggested to be important in regulating TNF-α secretion (190). Cooperation between the CTAR1 and 2 regions of LMP1 is important for TRAF3 binding and CD80 upregulation (97, 112). Here we show that hCD40 and hCD40LMP1-induced TNF-α secretion, CD80 upregulation, and TRAF3 binding were unaltered by TBS swapping. This indicates that any cooperative pathways emanating from hCD40 and hCD40LMP1 are intact in hCD40ADD and hCD40LMP1AEDL molecules. This work extends mutational analysis studies of the TBS in LMP1 and CD40 signaling by removing variables introduced by disruption of cooperative pathways.

Interestingly, the respective TBS of CD40 and LMP1 regulated the strength of TRAF2 but not TRAF3 binding (Fig. 12) (112, 185, 187). Our findings are consistent with earlier reports and further implicate regions outside of the TBS as important in TRAF3 binding (102, 188). This information is crucial for the design of potential therapeutics that might target LMP1-TRAF3 association. Most likely, a COOH-terminal portion of LMP1 called CTAR2 is influencing TRAF3 binding. Previous findings demonstrate cooperation between CTAR1 and 2 in LMP1 signaling and TRAF association (97, 111, 112). These studies show that mutation of either CTAR1 or 2 significantly alters TRAF association (97, 112). While the TBS in CTAR1 exhibits similar preference for TRAF1, 2, and 3, the CTAR2 region either directly influences preferential TRAF3 binding or interacts with additional factors which influence TRAF3 association (112). Further studies will be needed to examine these two possibilities.

The present report illustrates that a receptor's ability to recruit TRAF2 cannot fully explain whether TRAF2/3 degradation will occur upon signaling. Despite lowering the TRAF2 binding of hCD40ADD compared to hCD40, hCD40ADD retained its ability to induce TRAF2/3 degradation, although TRAF2 degradation was at a lower level than that induced by hCD40 at later times. This result, together with the finding that hCD40LMP1AEDL initiated TRAF2 degradation, suggests that the CD40 TBS is most important in enabling TRAF2 but not TRAF3 degradation. CD40 signaling-induced TRAF2/3 degradation is thought to be induced by TRAF2-dependent recruitment of ubiquitin ligases (145). Results presented here suggest that the TBS sequence of CD40 imparts to TRAF2 the ability to recruit a subset of these ubiquitin ligases, but other regions of CD40 may be required to recruit all of the participants necessary to promote maximal TRAF2 degradation and initiate TRAF3 degradation. The rate of TRAF3 degradation was improved by the replacement of the hCD40 TBS with that of hCD40LMP1. Perhaps factors which associate via the CD40 TBS compete for space with TRAF3 degradation factors that associate via other regions of CD40 within the signaling complex. By removing the CD40 TBS and the ability of TBS-associated factors to bind CD40, TBS-independent factors may be able to more efficiently bind CD40 and mediate TRAF3 degradation. It should prove interesting to determine what other regions and factors influence TRAF2 and 3 degradation. These results also illustrate differences in the way the TBS sequences of CD40 and LMP1 interact with and regulate TRAFs for signaling. While both CD40 and LMP1 bind TRAF2 via their respective TBS, the sequence

of the CD40 TBS induces TRAF2 to perform distinct functions compared to the TBS of LMP1.

The TBS of LMP1 clearly influences the exaggerated signaling nature of hCD40LMP1. The TBS of hCD40LMP1 imparted to hCD40ADD the ability to induce IL-6 in response to agonistic Ab, in contrast to hCD40 (98, 150). This ability was not merely the result of loss of a negative regulatory mechanism associated with the TBS of CD40, because hCD40LMP1AEDL retained the ability to induce IL-6 secretion in response to agonistic Ab.

In the case of signals activating the canonical NF-κB pathway, an interesting intermediate pattern of TBS influence emerged for both hCD40ADD and hCD40LMP1AEDL (Fig. 6). This suggests that, like IL-6 production, both TBS properties as well as additional factors regulate the ability to activate the canonical NF-κB pathway. Despite the effects of the TBS on activation of the canonical NF-κB pathway, the overall sum of both NF-κB activation pathways at later time points correlated with regions outside of the TBS in both hCD40 and hCD40LMP1 (Fig. 7). hCD40LMP1AEDL's maintenance of an hCD40LMP1-like NF-κB activation profile is most likely due to its ability to maintain IκBα degradation at later times, similar to hCD40LMP1. Further, regions outside of the TBS may be important in mediating non-canonical NF-κB activation. Inasmuch as these regions were not affected by switching of the TBS between hCD40 and hCD40LMP1, the mutant versions of these molecules would be able to activate the non-canonical pathway to a similar degree as their WT counterparts.

The activation of JNK showed a stronger dependence upon the distinct TBS, and this was also reflected in a downstream function of hCD40ADD, IL-6 production, heavily influenced by the JNK pathway (191). CD40LMP1AEDL signaling, however, maintained the hCD40LMP1-like ability of inducing IL-6 secretion in response to agonistic Ab despite its inability to activate the JNK pathway for prolonged periods of time. This suggests that other LMP1 TBS-independent pathways are capable of a compensatory contribution to this signaling outcome. Future studies comparing recruitment of factors important for JNK activation to hCD40ADD and hCD40LMP1AEDL in comparison to their parental counterparts should aid in determining whether differential association with positive or negative regulatory mechanisms, or both, contribute to differences in JNK activation.

LMP1 utilizes TRAF3 as a positive regulator of several of its signaling pathways, while TRAF3 is a negative regulator of CD40, either by direct signaling, or by competition for binding of TRAF2 (136). Although there is strong structural similarity in each receptor's proposed binding to TRAF3, several additional contacts are found for LMP1 (142). This indicates that there may be differences in the way TRAF3 associates with each TBS. TRAF2 appears to be functionally more critical for hCD40 than hCD40LMP1 signaling (112, 134), highlighting another difference in TRAF utilization by CD40 and its viral mimic. The present study provides additional insights into which aspects of TRAF2/3 association and hCD40LMP1 signals are regulated by the TBS, and to what extent. This information has strong potential value for designing small molecule

therapies that interrupt key pathogenic aspects of LMP1 signaling while avoiding disruption of CD40 signals, or if desired, therapies that would disrupt both signaling pathways.

The cIAPs and TRAF degradation

The TRAF binding site controls some but not all aspects of the CD40 or LMP1 signal (141). Interestingly the TBS, in a fashion redundant with other regions of CD40, can foster interaction with the E3 ubiquitin ligases, cIAP1 and cIAP2 (Fig. 15). Because the cIAPs are important positive regulators of CD40-mediated MAPK activation and negative regulators of NFκB activation (Figs. 15 and 16), the influence of the CD40 TBS on CD40 and LMP1AEDL signals is likely mediated in part by these factors.

Interestingly, CD40LMP1 uses the cIAPs in a manner different from that of CD40. CD40LMP1 does not recruit the cIAPs upon signaling whereas CD40 does (Fig. 15), which implies that CD40LMP1 utilizes the cIAPs in a cytosolic manner. Interestingly, LMP1AEDL, a molecule which behaves similarly to CD40 in some respects, directly recruits the cIAP molecules upon signaling. This suggests that CD40 uses the cIAPs at the receptor, as opposed to in the cytoplasm.

Contrary to a previous report, we found that the cIAPs were not absolutely required for CD40-mediated TRAF degradation, though they might be important for maximal TRAF degradation at early times (Fig. 18). In contrast to CD40, CD40LMP1AEDL requires the cIAPs for TRAF2 degradation (Fig.19). This

demonstrates that the ability to induce TRAF2 degradation can be mediated by the CD40 TBS, independent of other regions of the CD40 molecule. These results also imply that the CD40 TBS mediates TRAF degradation strictly through the cIAPs. Other regions outside of the CD40 TBS, absent in the CD40LMP1AEDL molecule but present in CD40ADD, must foster interactions with cIAP-redundant E3 ubiquitin ligases that can mediate both TRAF2 and 3 degradation. In Figure 29, a model illustrating these findings is given. Given that CD40 can mediate normal levels of TRAF degradation in the absence of TRAF6 (Figures 13 verses 22), TRAF6 most likely does not play a role in mediating interactions with these other redundant molecules

LMP1 mimics CD40 in many respects, but not in the ability to induce TRAF degradation, which is likely an important factor in the dysregulated nature of the LMP1 signal. CD40 and LMP1AEDL signaling both induce TRAF2 degradation and both have a greater TRAF2 binding ability than LMP1 (Fig. 12 and 13). To determine if a high TRAF2 binding capacity is sufficient for a receptor to induce TRAF2 degradation, we investigate the ability of CD40LMP1CTAR1 signaling to induce TRAF degradation. Like WT CD40LMP1 (Fig. 13), CD40LMP1CTAR1 was unable to induce TRAF degradation despite its greater TRAF2 binding potential (Fig. 20). Furthermore, the mitigation of any possible negative influences of TRAF1 or TRAF3 did not allow CD40LMP1CTAR1 to induce TRAF degradation (Fig. 24). These results suggest that merely having a high TRAF2 binding capacity is not sufficient for a receptor to induce TRAF degradation. Because LMP1AEDL can induce cIAP-dependent TRAF2

degradation (Fig. 22), we surmise that the unique sequence of the CD40 TBS imparts upon TRAF2 the ability to initiate TRAF degradation in a cIAP-dependent manner. LMP1 does not initiate this process because the sequence of its TBS does not foster TRAF2-cIAP interactions, and because LMP1 does not have, like CD40, regions outside of its TBS which can facilitate interactions with cIAP-redundant E3 ubiquitin ligases.

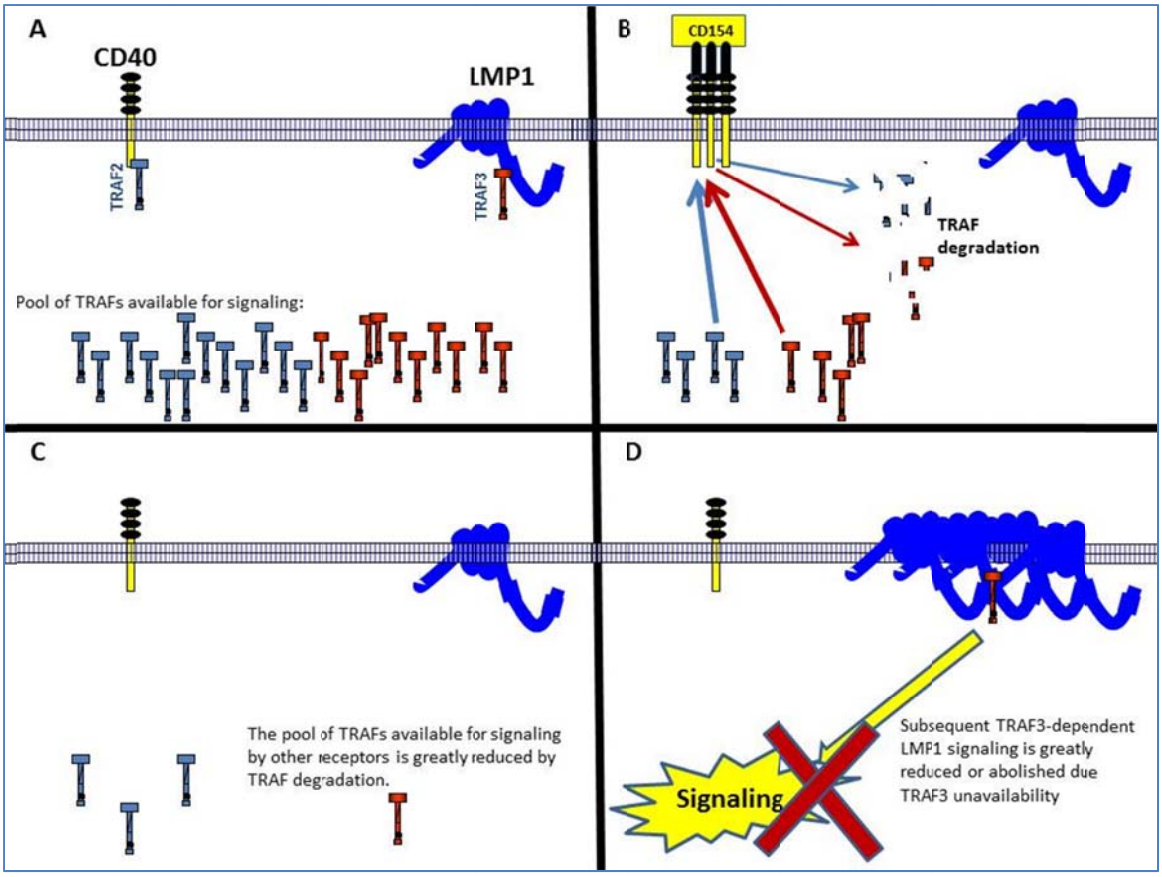


Figure 28. CD40 signaling-induced TRAF degradation can potentially limit other receptors signals.
In this example, prior CD40 signaling limits subsequent LMP1 signals through CD40's ability to induce TRAF degradation. **(A)** CD40 requires TRAF2 for signaling whereas LMP1 requires TRAF3. There is a pool of these TRAFs available for use in each molecule's signaling. **(B)** When CD40 is engaged by CD154, signaling induced TRAF2 and TRAF3 degradation occurs. **(C)** The ultimate effect of CD40 signaling-induced TRAF degradation is a greatly reduced pool of TRAFs available for subsequent signaling by receptors which utilize them. **(D)** Subsequent ligand-independent clustering of LMP1 initiates the process of LMP1 signaling. However, since the pool of necessary TRAF3 has been greatly diminished by prior CD40 signaling, the subsequent TRAF3-dependent LMP1 signal is greatly reduced or abolished.

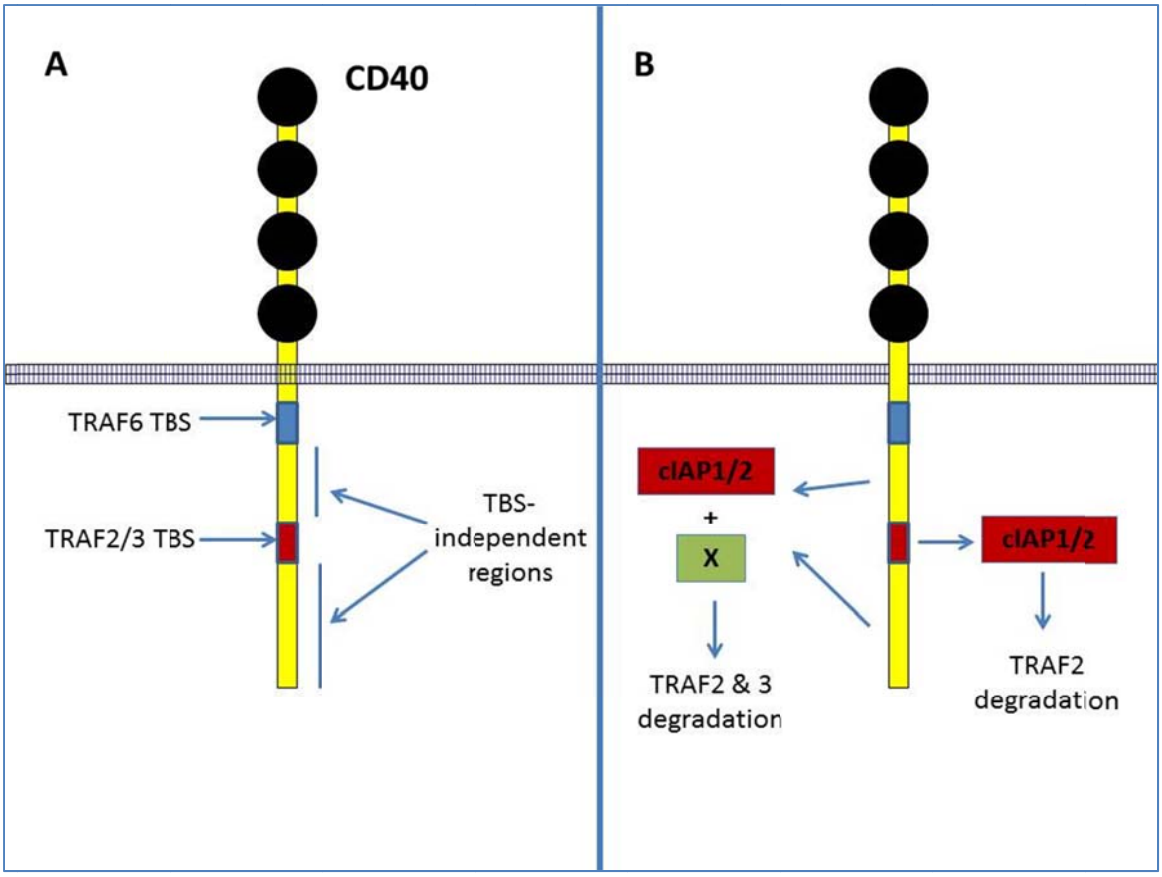


Figure 29. Model of CD40 signaling-induced TRAF degradation.
(A) In the CY tail of CD40, there are two TRAF binding sites. There is a membrane-proximal TRAF6 binding site and membrane-distal TRAF2/3 binding site. **(B)** TRAF2 degradation can be mediated by the cIAPs and cIAP-redundant E3 ubiquitin ligases ("x") via TBS-independent regions. Also, TRAF2 degradation can be mediated by the TRAF2/3 TBS in a cIAP-dependent manner. The degradation of TRAF3 is mediated by TBS-independent regions through cIAP-redundant E3 ubiquitin ligases ("x").

Future directions

CD40-mediated TRAF degradation in immune regulation

It is clear from this work that CD40 signaling-induced TRAF degradation is a potent regulatory mechanism capable of down-modulating TRAF2 and TRAF3-dependent pathways. However, this work did not address what the contributions of this mechanism are to immune responses *in vivo.* For instance, does CD40-mediatedTRAF degradation *in vivo* limit over exuberant immune responses, and/ or does it fine-tune and improve the quality of the immune response by modulation of immune promoting factors? To test these possibilities, future work should be directed at elucidation of the exact molecular mechanisms of CD40-mediated TRAF degradation. With this knowledge it might be possible to specifically interfere with CD40-mediated TRAF degradation while sparing all other CD40 signals and functions. With this capability one could test the contribution of TRAF degradation to immune responses by specifically removing the ability of CD40 to induce this event.

The role of the TBS in CD40 and LMP1 signaling

LMP1 requires TRAF3 for many facets of its signal (112). Hence, in LMP1-driven diseases specific disruption of the LMP1-TRAF3 association would be beneficial. Our knowledge of how to specifically disrupt this interaction is lacking. The TBS in CTAR1 exhibits similar preference for TRAF1, 2, and 3, the CTAR2 region either directly influences preferential TRAF3 binding or interacts with

additional factors which influence TRAF3 association (112, 141). Further studies addressing how CTAR2 mediates preferential LMP1 TBS-TRAF3 binding are therefore needed. If the CTAR2 region mediates the LMP1-TRAF3 association through uncharacterized CTAR2-binding proteins, than immunoprecipitation of CD40LMP1CTAR2 (112) and proteomic analysis of co-immunoprecipitated factors by mass spectrometry should yield the identity of these factors.

To further our understanding of the mechanism of TRAF degradation, it should prove interesting to determine what other TBS-independent regions of CD40 influence TRAF2 and 3 degradation. Mutagenesis of regions outside of the TBS of the CD40 cytoplasmic tail and subsequent assessment of the mutant's ability to induce TRAF degradation upon signaling may prove useful in identifying these regions.

LMP1-mediated JNK activation is integral to many aspects of LMP1's pathological nature. For instance, JNK activation is necessary for IL-6 secretion (150, 191), and IL-6 is an important factor in promoting some lymphomas (22). CD40 and LMP1-mediated JNK activation show a strong dependence upon each molecules distinct TBS (141). Therefore, future studies comparing the recruitment of factors important for CD40ADD and LMP1AEDL-mediated JNK activation in comparison to their parental counterparts should aid in determining whether differential association with positive or negative regulatory elements, or both, contribute to differences in JNK activation. This information is crucial in designing therapies which interfere with LMP1-mediated JNK activation but spare CD40's ability to activate JNK.

The cIAPs and TRAF degradation

Our work suggests that CD40 signaling mediates TRAF degradation in several ways. The ability of LMP1AEDL to induce TRAF2 degradation in a cIAP-dependent manner (Fig. 22) suggests that CD40 signaling can induce TRAF2 degradation through the cIAPs via unique interactions between its TBS and TRAF2. Additionally, regions outside the TBS may facilitate interactions with the cIAPs and other factors important for both TRAF2 and TRAF3 degradation. Interestingly, CD40ADD is able to mediate TRAF degradation and cIAP association upon signaling despite loss of the CD40 TBS (Figs. 13 and 18). Therefore, understanding the effects of cIAP depletion on the ability of CD40ADD to induce TRAF degradation is crucial for the development of future hypotheses. Because WT CD40 is able to mediate the degradation of *both* TRAF2 and TRAF3 in a cIAP-independent manner, we predict that this molecule would retain its ability to induce TRAF degradation in a cIAP depleted state.

CD40, LMP1, CD40ADD, and LMP1AEDL all have differential abilities to induce TRAF degradation upon signaling. Hence, immunoprecipitation and mass spectrometry studies comparing potential associations of binding partners of CD40, LMP1, CD40ADD, and LMP1AEDL will yield important information about the identity of these proteins. By comparing proteins that bind CD40 versus LMP1 (which doesn't induce TRAF degradation), important clues may be revealed about which are important for mediating TRAF degradation. Furthermore, comparisons of CD40ADD and LMP1AEDL will give clues as to how TBS-dependent and independent TRAF degradation are regulated.

CD40LMP1CTAR1 has a high TRAF2 binding ability, yet its signaling does not induce TRAF degradation (Figs. 23 and 24). LMP1AEDL signaling induces the degradation of TRAF2 but not TRAF3 (Figure 13). Hence, LMP1AEDL signaling may not induce TRAF3 degradation because it lacks the TBS-independent regions of CD40 necessary for TRAF3 degradation or because its CTAR2 region is suppressing this ability. To distinguish between these two possibilities it would be interesting to determine if a CD40LMP1CTAR1 molecule mutated to possess the TBS of CD40 (CD40LMP1AEDLCTAR1) is able to induce both TRAF2 and TRAF3 degradation upon signaling. If this molecule possessed the ability to induce both TRAF2 and TRAF3 degradation upon signaling than this would imply that one of the reasons LMP1 doesn't induce TRAF 3 degradation is because its CTAR2 region actively suppresses this process.

This work demonstrates that the mechanisms which drive CD40-mediated TRAF degradation are at least in part redundant, which suggests that CD40-mediated TRAF degradation plays an important role in regulation of immune responses. In support of this, CD40-mediated TRAF degradation affects the signals of a variety of other TRAF2- and TRAF3-dependent pathways. Hence, CD40 emerges as a global regulator of the immune response, both by direct promotion of response effector mechanisms, and by indirect modulation of the responsiveness of other TRAF2- and TRAF3-dependent pathways.

Clinical applications

Many EBV positive tumors express LMP1 (22), suggesting that LMP1 signaling may be vital to the tumor's survival. Therefore, a need exists to block LMP1 signaling within cancer cells. This work suggests that CD40 signaling-induced TRAF degradation may, in some tumors, be used to inhibit the pathogenic signals of LMP1. Future work needs to address this possibility by determining the effect of CD40 agonists on LMP1-driven CD40+ tumors.

Many dendritic cell or B lymphocyte-based cellular vaccines utilize CD40 agonists as components in cellular activation regimens. While CD40 signaling itself is able to activate antigen presenting cells, this work suggests that CD40 may modulate the responsiveness of the antigen presenting cell to additional agents used in cellular activation cocktails. Therefore, the sequence in which CD40 agonist and TRAF2- or TRAF3-dependent cellular activators are applied may positively or negatively affect the desired outcome.

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