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Mini-Review

TRAF6 as a critical regulator of B lymphocyte functions

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ABSTRACT

The adaptor protein tumor necrosis factor receptor-associated factor 6 (TRAF6) plays a central role in B cell activation pathways initiated by a wide variety of receptors, including members of the tumor necrosis factor receptor (TNFR) superfamily, Toll-like receptors (TLRs), cytokine receptors, and the Epstein-Barr virus (EBV)-encoded latent membrane protein 1 (LMP1). TRAF6 has the most divergent receptor-binding TRAF-C domain among the TRAFs, and interacts with receptors in a manner distinct from that of other TRAFs. TRAF6 plays an important role in regulating a diverse range of processes in many cell types, including various important roles in the function of immune cells. This review focuses upon the molecular mechanisms of TRAF6 function in B lymphocyte activation, and the importance of TRAF6 in normal and pathologic B cell functions.

KEYWORDS: B lymphocyte, TRAF, signal transduction, TNFR superfamily, EBV (Epstein-Barr virus)

INTRODUCTION

TRAFs are adaptor molecules that directly bind to the cytoplasmic domains of many different cell surface receptors, particularly members of the TNFR superfamily and the EBV-encoded oncoprotein LMP1 [1]. TRAF molecules are genetically conserved in mammals as well as other multi-cellular organisms, including *Drosophila* and *Caenorhabditis elegans* [2]. All TRAF molecules consist of a coiled-coil (TRAF-N) domain, which mediates TRAF oligomerization, and a TRAF-C domain, which is required for receptor binding [1, 3]. With the exception of TRAF1, TRAFs also contain an N-terminal really interesting new gene (RING) domain, which promotes protein-protein interactions. Removal of this RING domain produces a non-functional molecule that retains receptor binding, resulting in a dominant-negative (DN) TRAF mutant [1, 3].

TRAF6 was initially identified via both an yeast two-hybrid screen for CD40-associated proteins [4], and an expressed sequence tag screen to discover new TRAF molecules [5]. TRAF6 contains a TRAF-C domain, a coiled-coil domain, a RING domain, and five zinc fingers [4, 5]. The TRAF-C domain of TRAF6 is most homologous to that of TRAF2, with 36% sequence identity [4], and the overall sequence identity between TRAF6 and other TRAF molecules is ~30% [5]. However, the TRAF-C domain sequence identities among the other TRAFs range from 42% to 66%, and thus, TRAF6 contains the most divergent TRAF-C domain [4]. Immunoprecipitation and yeast two-hybrid studies show that TRAF6 forms trimeric complexes with itself, TRAF2, and TRAF3 [5].

TRAF6 is ubiquitously expressed [4-6]. The early lethality of TRAF6-deficient mice is largely explained by the critical role of TRAF6 in signaling by the TNFR superfamily member, receptor activator of NF-κB (RANK) [7-11].

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TRAF6-deficient mice exhibit a variety of gross abnormalities, including reduced body mass, abnormal bone formation, lack of sweat glands and lymph nodes, failure of tooth eruption, splenomegaly, and disrupted splenic architecture, demonstrating the importance of TRAF6 signaling via many different receptors expressed on a wide variety of cell types [9, 12-14]. Here, we restrict our comments to the roles of TRAF6 in signaling pathways of receptors expressed by B cells.

BAFFR

Studies by our laboratory demonstrated that in mouse and human B cell lines, as well as primary B cells, TRAF6 associates with BAFFR (receptor for B cell activating factor belonging to the TNF superfamily) [15], in a manner independent of TRAF2 and TRAF3 binding to BAFFR. Reciprocally, binding of TRAFs 2 and 3 to BAFFR does not depend upon TRAF6 [15]. Stimulation of TRAF6-deficient B cells through endogenous BAFFR or a CD40BAFFR chimera (extracellular domain of human CD40 and the transmembrane and cytoplasmic domains of BAFFR) leads to decreased activation of the canonical NF-κB1 pathway, but normal activation of non-canonical NF-κB2 signaling [15, 16]. In the absence of TRAF6, BAFFR-mediated rescue of B cells from CD95 (Fas)-induced apoptosis is impaired [15]. Thus, TRAF6 is important for a subset of BAFFR-mediated B cell functions. TRAF6 also binds to TACI (transmembrane activator and calcium modulator and cyclophilin ligand interactor), another TNFR superfamily member expressed on B cells that induces signals in response to stimulation of cells with BAFF [17]. However, the precise requirements for TRAF6 in TACI-mediated signaling pathways have not been characterized.

TLRs

Several groups have demonstrated an important role for TRAF6 in TLR-mediated signaling pathways in B cells. Proliferation in response to stimulation with either TLR4 or TLR9 agonists is impaired in TRAF6-deficient B cells [9, 16]. TRAF6 is also critical for both TLR4 and TLR9-mediated activation of NF-κB, and the kinases JNK (c-jun N-terminal kinase), p38, ERK (extracellular signal-regulated kinase), and Akt, as well as production of the proinflammatory cytokine interleukin-6 (IL-6) [16, 18]. Although TRAF6 does not directly bind to TLRs, LPS-induced JNK and NF-κB activation depends upon the TRAF6 TRAF-C receptor-binding domain [18]. Thus, the TRAF-C domain likely allows TRAF6 interaction with other adaptor proteins important in TLR signaling cascades.

CD40

TRAF6 binds *in vitro* to amino acids 231-238 in the cytoplasmic domain of human CD40 [4, 19, 20]. This binding site (QEPQEINF) is N-terminal to the PxQxT binding motif shared by other TRAFs (amino acids 250-254) [4, 19, 20]. TRAF6 was initially identified as a CD40-binding protein [4], and numerous studies have investigated the role of TRAF6 in CD40-mediated B cell functions. TRAF6 plays a critical role in CD40 signaling pathways in B cells, including activation of the kinases JNK, p38, ERK, Akt, and TAK1, as well as the NF-κB1 and NF-κB2 pathways [16, 18, 21, 22]. In response to CD40 stimulation, B cell proliferation, upregulation of the co-stimulatory molecule CD80, and protection from CD95 (Fas)-induced apoptosis are also diminished in the absence of TRAF6 [9, 16, 18, 21]. Furthermore, a mutant dominant-negative TRAF6 molecule blocks CD40-mediated B cell production of IL-6 and IgM [23]. CD40 activation does not influence basal protein levels of TRAF6, and CD40 stimulation does not result in TRAF6 degradation [6, 24]. Previous studies using a hybrid TRAF molecule composed of the Zn-binding domains of TRAF6 and the TRAF domains of TRAF2 revealed that the TRAF6 Zn-binding domains do not contribute to CD40-mediated TRAF2 or 3 degradation, and this TRAF6-TRAF2 hybrid molecule is not degraded following CD40 signals [24]. Additionally, B cell-specific TRAF6-deficient mice display normal germinal center (GC) formation in response to immunization, a function that is CD40-dependent [16, 25]. TRAF6 deficiency in B cells does not affect the production of antigen-specific IgM or antibody (Ab) affinity maturation in response to the T cell-dependent (TD) antigen NP-KLH, but results in reduced NP-specific IgG1 and IgG2b levels, as well as decreased plasma cell

numbers [16]. B cell-specific TRAF6-deficient mice exhibit impaired TNP-specific Ab responses to immunization with the T cell-independent (TI) antigens TNP-Ficoll or TNP-LPS, as well as reduced basal levels of serum IgM and IgG2b [16]. These results correlate with studies in B cell lines demonstrating a role for TRAF6 in CD40-mediated Ig isotype switching [26]. Taken together, these studies show that TRAF6 is important for both early signaling pathway activation by CD40, and a subset of CD40-mediated immune functions of B cells *in vivo*.

Expression in TRAF6-deficient B cells of a TRAF6 molecule lacking the receptor-binding TRAF-C domain restores defects in CD40-induced JNK activation and CD80 upregulation, but not CD40-mediated rescue from CD95-induced apoptosis [18, 21]. Additional studies involved disruption of the CD40 TRAF6 binding site to explore the requirements of TRAF6-CD40 interaction in CD40 signaling pathways. To prevent TRAF6 association with CD40, one group of investigators mutated a proline and a glutamine residue in the TRAF6 binding site [18], while another group mutated two glutamine residues [23]. These subtle differences in mutations could explain the differential results obtained from these studies. Both found that the CD40 TRAF6 binding site is not important for JNK or NF-κB activation in response to CD40 stimulation [18, 23]. One study further showed that disruption of the CD40 TRAF6 binding site does not affect CD40-mediated upregulation of CD11α, CD54, or CD95, but does lead to decreased IL-6 and IgM production following CD40 activation [23]. However, reports diverged as to whether disruption of the CD40 TRAF6 binding site affects CD40-mediated upregulation of CD80 [18, 23]. Collectively, these results clearly show that direct TRAF6 interaction with CD40 is not needed for all CD40-mediated TRAF6-dependent signaling events.

The structural requirements of TRAF6 binding in CD40 functions were further explored *in vivo*, using $CD40^{-/-}$ mouse models expressing WT or mutant CD40 transgenic molecules [27, 28]. In two studies, the CD40 TRAF6 binding site was mutated, and mutant CD40 was expressed in $CD40^{-/-}$ mice [27, 28]. However, in one study the CD40 transgene was driven by the I-Eα promoter [27], whereas the other study used the B cell-specific promoter EμVh [28]. Importantly, CD40 molecules expressed via the I-Eα promoter are expressed on B cells, macrophages, and dendritic cells, all of which also normally express CD40 [29]. These antigen presenting cells upregulate co-stimulatory molecules and produce cytokines and chemokines that can affect immune cell function, as CD40 plays important roles in these functions in both B and myeloid cells [30]. Thus, in addition to affecting CD40-mediated functions in B cells, the TRAF6 binding site mutant could also influence macrophage and dendritic cell functions. Studies using the EμVh promoter to drive expression of CD40 molecules resulted in effects attributable only to B cell CD40 [28]. However, these B cell-specific transgenic mice were created on a $CD40^{-/-}$ background, and thus all non-B cells normally expressing CD40 also lack CD40 in this model [28]. The absence of CD40 on macrophages and dendritic cells could impact the influence of these cell types on B cell functions during immune responses. Another significant difference between these two studies is that the CD40 molecules expressed via the I-Eα promoter contained the extracellular domain of human instead of mouse CD40 [27], which could affect CD40 signals *in vivo*, as mCD154 activates mCD40 more effectively than hCD40 [31]. Thus, differences in the design of these two CD40 transgenic mouse models may explain differences in the results obtained. Disruption of TRAF6-CD40 interaction in mice in which CD40 expression is driven by the I-Eα promoter reduces antigen-specific serum IgG1 levels following TD immunization [27], but when CD40 expression is restored only in B cells, disruption of TRAF6 binding enhances antigen-specific IgG1 levels [28]. Disruption of TRAF6 binding to CD40, regardless of its transgenic promoter, does not affect CD40-mediated activation of JNK, p38, or NF-κB *in vitro* [27, 28], indicating that these early CD40 signaling pathways do not fully predict antigen-specific immune responses *in vivo*. Finally, a third study used $CD40^{-/-}$ mice expressing an EμVh promoter-driven mutant hCD40 molecule containing the TRAF6 binding site, but lacking the distal portion of the cytoplasmic domain (including the TRAF1/2/3/5 binding site) [32]. The TRAF6 binding site is sufficient for CD40-mediated B cell

proliferation, production of IgM and IgG1, and upregulation of the co-stimulatory/adhesion molecules CD23, CD54, CD80, CD86, and CD95 *in vitro* [32]. The CD40 TRAF6 binding site also partially rescues TD antigen-specific Ab isotype switching, but does not rescue Ab affinity maturation or germinal center formation *in vivo* [32]. Thus, TRAF6 binding to CD40 is required for some, but not all, CD40-mediated functions in mice *in vivo*.

LMP1

The Epstein-Barr virus (EBV)-encoded latent membrane protein 1 (LMP1) makes important contributions to EBV-mediated B cell transformation and EBV-associated malignancies [33-35], as well as exacerbation of autoimmunity [36, 37]. LMP1 is an integral membrane protein that contains a short cytoplasmic N-terminal domain, six transmembrane domains, and a long cytoplasmic C-terminal domain [38]. The N-terminal domain anchors LMP1 to the plasma membrane and controls LMP1 processing [39, 40]. The transmembrane domains spontaneously self-aggregate, oligomerizing within the plasma membrane to promote ligand-independent, constitutive signals [39, 41]. LMP1 is a functional mimic of CD40 in B cells; signaling through both molecules leads to MAPK and NF-κB activation, followed by co-stimulatory and adhesion molecule upregulation and cytokine production [30]. However, LMP1 induces amplified and sustained signals to B cells compared to CD40 [30]. Both CD40 and LMP1 depend upon TRAFs to mediate signaling, but they use TRAFs differently [18, 42-47].

LMP1 delivers signals in a constitutive manner, which complicates the study of early LMP1 signaling events [42]. In order to control the initiation of early LMP1 signaling, our laboratory created hybrid receptors containing the extracellular and transmembrane domains of mouse or human CD40, and the cytoplasmic domain of LMP1 [48]. The C-terminal domain of LMP1 is necessary and sufficient to induce most LMP1 functions in both mouse and human B cells, as well as in mice *in vivo*, including activation of early signaling pathways and downstream B cell effector functions [29, 43, 46, 48-51].

In the absence of TRAF6, the LMP1 signaling domain is unable to induce activation of JNK, p38, TAK1, or NF-κB1 [42]. LMP1-mediated upregulation of the co-stimulatory molecule CD80 is also abolished in TRAF6-deficient B cells [42]. Furthermore, decreased expression of TRAF6 induced via shRNA leads to reduced IRF7 ubiquitination and transcriptional activity in EBV-transformed human Raji B cells, which express high levels of LMP1 [52]. In contrast to CD40, LMP1 signaling requires the TRAF6 TRAF-C domain to activate JNK, p38, and NF-κB1, as well as upregulate CD80 [18, 42].

The C-terminal cytoplasmic domain of LMP1 lacks the consensus TRAF6 binding site found in CD40 [30]. Both CD40 and LMP1 contain the PxQxT TRAF1/2/3/5 core binding motif, but the variable residues within and around the core motif differ between the two molecules [20, 30, 53, 54]. TRAF6 associates with the LMP1 TRAF1/2/3/5 binding site in both mouse B cell lines and primary mouse B cells, which is in contrast to CD40, where TRAF6 binds at a site distinct from that shared by the other TRAFs [18, 42]. Furthermore, TRAF6 recruitment to LMP1 does not depend upon the presence of other TRAFs [42]. However, TRAF6 association with LMP1 is enhanced in the absence of TRAF1 and/or TRAF2 [42].

B cell development

Given the important role of TRAF6 in signaling to B cells by numerous receptors, the requirement of TRAF6 in B cell survival and development was examined using B cell-specific TRAF6-deficient mice [16]. The absence of TRAF6 does not affect the percentage of apoptotic splenic B cells after 14 h in culture, in the presence or absence of serum and/or stimulation [16]. Pre-B, pro-B, and immature B ($B220^{lo}$, IgM^{+}, IgD^{-}) cell subsets in the bone marrow of B cell TRAF6-deficient mice are comparable to those of control mice [16]. However, the number of mature recirculating B cells ($B220^{hi}$, IgM^{+}, IgD^{+}) and percentage of splenic B cells, particularly mature B cells, are reduced in mice whose B cells lack TRAF6 [16]. Furthermore, B cell TRAF6-deficient mice exhibit decreased numbers of B-1a cells in the peritoneal cavity [16]. Collectively, these findings suggest a possible role for TRAF6 in the regulation of mature B cell development. Alternatively, the

reduced B cell numbers could reflect the role of TRAF6 in B cell proliferation in response to TLR or CD40 signals [9, 16].

TRAF6 and B cell malignancies

Multiple myeloma (MM) is a malignancy of bone marrow plasma B cells with high morbidity and mortality rates [55]. In many MM cells, the NF-κB pathway is constitutively activated, promoting survival and maintenance of tumor cells [55]. As summarized above, TRAF6 plays a critical role in NF-κB activation in B cells, mediated by multiple receptors. Treatment of certain MM cell lines with TRAF6 siRNA significantly decreases cell proliferation and increases apoptosis and susceptibility to chemotherapeutic drugs *in vitro* [55, 56]. MM cells expressing TRAF6 siRNA also exhibit impaired growth *in vivo* following transplantation into the bone marrow of irradiated recipient mice [55, 56]. MM leads to bone disease in the majority of patients, largely due to abnormal bone remodeling and enhanced osteoclast activation [55, 57]. Signaling via the TNFR superfamily member RANK leads to osteoclast differentiation and activation [8, 55], and in combination with M-CSF, stimulation of bone marrow cells or human PBMCs with RANKL induces osteoclast differentiation [57]. TRAF6 is important for RANK-mediated signaling, particularly activation of the NF-κB pathway [7, 10, 11, 55], and osteoclast function, as TRAF6-deficient mice exhibit osteopetrosis [9]. Treatment of bone marrow macrophages with a TRAF6 inhibitory decoy peptide impairs osteoclast differentiation and bone resorption in response to RANKL [55, 58]. The proteasome inhibitor bortezomib is commonly used to treat MM patients [55]. Treatment of MM cells with bortezomib leads to decreased TRAF6 protein and mRNA levels, resulting in a reduction in cell proliferation and RANKL-induced osteoclast differentiation [57]. Overexpression of TRAF6 blocks these bortezomib-mediated effects [57]. Thus, TRAF6 likely plays a role in both signaling directly to MM cells, as well as the enhanced osteoclast activation characteristic of MM.

LMP1 contributes to numerous EBV-associated B cell malignancies, including Burkitt's lymphoma, Hodgkin's disease, and post-transplant lymphomas [30]. The role of LMP1 in cancer was recently reviewed [30]. However, given the critical role of TRAF6 in LMP1-mediated signaling pathways, and the differential association of TRAF6 with CD40 versus LMP1, TRAF6-mediated signaling pathways may be possible therapeutic targets in LMP1-associated malignancies [42].

TRAF6 in autoimmune and inflammatory diseases

LMP1 has been implicated in the exacerbation of autoimmune diseases, particularly systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [36, 59, 60]. Recently, several groups identified polymorphisms in TRAF6 that are associated with increased risk of SLE [61] and RA [62, 63]. However, the role of these different disease-associated TRAF6 polymorphisms in LMP1-mediated signaling has not been investigated. TRAF6 is critical for LMP1 signaling pathways [42], and it is possible that one or more of these TRAF6 proteins could lead to enhanced LMP1-mediated signaling, thus contributing to disease. Alternatively, these polymorphisms could affect signaling by other TRAF6-dependent receptors, and enhanced signaling by these receptors in combination with LMP1 signals drives pathogenesis.

Disruption of CD40 signaling in *Apoe*$^{-/-}$ mice reduces the development of inflammation-linked atherosclerosis in this model [64-67]. MHC class II promoter-driven expression of a CD40 transgene with a disrupted TRAF6 binding site in CD40$^{-/-}$ *Apoe*$^{-/-}$ mice reduces the development of atherosclerosis compared to mice expressing WT CD40 [68]. This abolished disease phenotype is not observed in mice expressing a CD40 transgene in which the canonical TRAF binding site was mutated [68], suggesting that TRAF6-dependent CD40 signaling uniquely contributes to atherosclerosis.

CONCLUSION

TRAF6 is a critical regulator of many signaling pathways in B lymphocytes, induced by a wide variety of receptors. Elucidating the role of TRAF6 in B cells, and the molecular mechanisms by which it exerts its impact, is important to our understanding of normal immune functions, as well as our understanding of mechanisms that contribute to diseases involving B cell dysfunction.

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