Molecular Mechanisms of TNFR-associated Factor 6 (TRAF6) Utilization by the Oncogenic Viral Mimic of CD40, Latent Membrane Protein 1 (LMP1)§

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Latent membrane protein 1 (LMP1), encoded by Epstein-Barr virus, is required for EBV-mediated B cell transformation and plays a significant role in the development of posttransplant B cell lymphomas. LMP1 has also been implicated in exacerbation of autoimmune diseases such as systemic lupus erythematosus. LMP1 is a constitutively active functional mimic of the tumor necrosis factor receptor superfamily member CD40, utilizing tumor necrosis factor receptor-associated factor (TRAF) adaptor proteins to induce signaling. However, LMP1-mediated B cell activation is amplified and sustained compared with CD40.

We have previously shown that LMP1 and CD40 use TRAFs 1, 2, 3, and 5 differently. TRAF6 is important for CD40 signaling, but the role of TRAF6 in LMP1 signaling in B cells is not clear. Although TRAF6 binds directly to CD40, TRAF6 interaction with LMP1 in B cells has not been characterized. Here we tested the hypothesis that TRAF6 is a critical regulator of LMP1 signaling in B cells, either as part of a receptor-associated complex and/or as a cytoplasmic adaptor protein. Using TRAF6-deficient B cells, we determined that TRAF6 was critical for LMP1-mediated B cell activation. Although CD40-mediated TRAF6-dependent signaling does not require the TRAF6 receptor-binding domain, we found that LMP1 signaling required the presence of this domain. Furthermore, TRAF6 was recruited to the LMP1 signaling complex via the TRAF1/2/3/5 binding site within the cytoplasmic domain of LMP1.

Epstein-Barr virus (EBV)³ latently infects >90% of humans (1) and is the causative agent of infectious mononucleosis (2, 3). Ablerrant EBV reactivation in the immunocompromised patient is strongly associated with Burkitt’s lymphoma, Hodgkin’s disease, nasopharyngeal carcinoma, and posttransplant lymphomas and lymphoproliferative disease (3–8). EBV reactivation is also seen in autoimmune disease, particularly systemic lupus erythematosus and rheumatoid arthritis (9–11). EBV preferentially establishes latency in memory B cells (2, 8, 12), but infected cells do not express LMP1 unless EBV partially emerges from latency, usually in the setting of immunosuppression or autoimmunity (2, 8, 12). LMP1 is expressed in the majority of EBV-associated malignancies and posttransplant lymphomas and lymphoproliferative disease and is required for EBV-mediated B cell transformation (3, 7, 12–15). Several studies also implicate LMP1 in the exacerbation of systemic lupus erythematosus (9, 16).

LMP1 is a transmembrane (TM) protein consisting of a short cytoplasmic (CY) N-terminal domain, six TM domains, and a long CY C-terminal domain (17, 18). The N-terminal domain anchors LMP1 to the plasma membrane and regulates LMP1 processing (17–19). The TM domains spontaneously self-aggregate and oligomerize within the plasma membrane, resulting in ligand-independent, constitutive activation of signaling terminated by rapid and constant processing of the protein into fragments (18–20). Two subdomains within the C-terminal domain, C-terminal activating region (CTR1) 1 and CTR2, are critical for LMP1 signaling (17, 19, 21). Our lab has shown in both isolated B cells and mice that the C-terminal domain is necessary and sufficient to mediate LMP1 functions in B cells (3, 22). Specifically, we have demonstrated that the LMP1 C-terminal CY domain mimics various aspects of LMP1 signaling, including early pathway activation and downstream biological functions of the B cell (3, 18, 20, 22–25).

LMP1 is a functional mimic of the tumor necrosis factor receptor superfamily member CD40, an activating receptor constitutively expressed on B cells, macrophages, and dendritic cells (20, 26). LMP1 and CD40 signaling result in kinase and NF-κB activation and the up-regulation of costimulatory and adhesion molecules (12, 18, 27). However, LMP1 signals to B cells are amplified and sustained compared with CD40 signals (18, 26). Both LMP1 and CD40 lack enzymatic activity (26) and thus use tumor necrosis factor receptor-associated factor (TRAF) adaptor proteins to mediate signaling, but they utilize TRAFs 1, 2, 3, and 5 in distinct and, in some cases, contrasting ways (18, 20, 28). TRAF3 negatively regulates CD40 signaling but serves as a positive regulator of LMP1 signaling, whereas TRAFs 1 and 2 promote CD40-mediated but not LMP1-mediated JNK and NF-κB activation (17, 20, 29–31). In the absence...

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§ The abbreviations used are: EBV, Epstein-Barr virus; TM, transmembrane; CY, cytoplasmic; CTR, C-terminal activating region; TRAF, tumor necrosis factor receptor-associated factor; Ab, antibody; Tg, transgenic; TBS, TRAF1/2/3/5 binding site; mCD40, mouse CD40.
of TRAF5, CD40-mediated surface molecule up-regulation and IgM production is reduced, but TRAF5 deficiency has no effect on CD40-mediated JNK and NF-κB activation (32, 33). However, TRAF5 was recently shown to be critical for LMP1-mediated activation of JNK and Akt and the production of IL-6, TNF-α, and IL-17 (24).

The role of TRAF6 in LMP1 signaling in B cells is a significant knowledge gap in the field and is the focus of this study. TRAF6 plays a critical role in CD40 signaling and directly binds to CD40 (34), but the binding site is distinct from that shared by TRAFs 1, 2, 3, and 5 (28, 32, 35, 36). Although TRAF6 has been implicated in LMP1-mediated NF-κB and p38 activation, the role of TRAF6 in LMP1-mediated JNK activation is controversial (19, 37, 38). It is important to note that all of the studies reported to date investigating TRAF6 in LMP1 signaling were performed in mouse embryonic fibroblasts or a human kidney adenovirus-transformed adenocarcinoma cell line, with exogenously overexpressed proteins (19, 37, 38). One caveat of experiments performed using overexpression systems is that nonphysiologic levels of signaling proteins can lead to aberrant pathway activation, complicating the interpretation of results. Thus, studying signal transduction with proteins expressed at endogenous levels in relevant cell types is crucial. Results of a study of TRAF6−/− mouse embryonic fibroblasts showed that the absence of TRAF6 does not abolish LMP1-mediated p38 activation (19). However, prior studies indicate that TRAF functions can vary depending upon receptor and cell type (39). Thus, we used B cells, the primary target of EBV, in which proteins are expressed at endogenous levels. Additionally, although TRAF6 has been shown to directly associate with CD40, TRAF6 interaction with LMP1 has not been determined (28, 32, 35, 36). Studies have suggested that TRAF6 may associate with the CTAR2 subdomain of LMP1, but there is no direct evidence to support this (19, 37, 40). It has also been proposed, on the basis of overexpression studies, that the adapter protein TRADD interacts with CTAR2 and TRAF6, linking TRAF6 to CTAR2 (41, 42). However, this finding has not been reproduced at normal protein levels in B cells (18, 43). Interestingly, not all receptors that employ TRAF6 to promote signaling must directly bind TRAF6. It was recently shown that CD40 can utilize a strictly CY mutant of TRAF6 to mediate a subset of B cell activation signals (29). Similarly, the innate immune Toll-like receptors have been demonstrated to use TRAF6 strictly as a CY adaptor, without direct binding (44).

To understand how LMP1 might employ TRAF6 to promote its effects on B cells, we used TRAF6-deficient mouse B cells expressing a hybrid receptor, in which the LMP1 extracellular and TM domains have been replaced with those of human CD40 (hCD40), to allow control of early LMP1 signaling (18, 25, 28). This approach allowed us to determine for which LMP1 functions and early signaling pathways TRAF6 may be required. This hybrid molecule has been shown in mouse and human B cell lines, freshly isolated B cells, and mice to accurately represent LMP1-mediated signals to B cells (18, 25, 28). To address TRAF6 association with LMP1, we used mouse B cells sufficient or deficient in TRAF6, expressing either hCD40LMP1 or variants in which either CTAR1 or CTAR2 was deleted from the LMP1 molecule. Results presented here reveal for the first time that TRAF6 was required for LMP1-mediated B cell activation and associates with the CTAR1 subdomain of LMP1.

EXPERIMENTAL PROCEDURES

Cell Lines—The mouse B cell lines A20.2J and CH12.LX (45, 46) have been described previously. This study also used A20.2J cells deficient in TRAF6 (29), TRAF2 (30), or TRAF3 (20), CH12.LX cells deficient in TRAF1 (31), TRAF2 (31), TRAF1 and 2 (31), and TRAF3 (31), A20.2J TRAF6−/− cells stably transfected to express endogenous levels of WT TRAF6 or a TRAF6 molecule lacking the receptor-binding TRAF domain (TRAF6ΔTRAF) (29), A20.2J and CH12.LX cells stably expressing hCD40LMP1 (18, 22, 28), and the CD40 CY domain truncation mutant hCD40ΔS5 (47), hCD40CTAR1 (18, 43), hCD40CTAR2 (18, 43), or hCD40LMP1PQAA1 (43). These cell lines were maintained in RPMI 1640 (Invitrogen) with 10 μM 2-mercaptoethanol (Invitrogen), 10% heat-inactivated FCS (Atlanta Biologicals, Atlanta, GA), and antibiotics (Invitrogen) (this medium is referred to as BCM-10). The cell lines expressing hCD40LMP1, hCD40ΔS5, hCD40CTAR1, hCD40CTAR2, or hCD40LMP1PQAA1 alone were maintained in 400 μg/ml G418 disulfate (Research Products International, Mt. Prospect, IL). The TRAF6-deficient A20.2J cell lines expressing TRAF6 or TRAF6ΔTRAF were maintained in 400 μg/ml G418 disulfate, 200 μg/ml hygromycin (Invitrogen), and 400 μg/ml zeocin (Research Products International).

Reagents—Sorbitol was purchased from Fisher. Disuccinimidyl suberate (DSS) was purchased from Thermo Scientific (Rockford, IL). LPS (Escherichia coli strain 0111:B4) was purchased from Sigma. Rabbit anti-phospho-JNK antibody (Ab), rabbit anti-phospho-p38 Ab, rabbit anti-pTAK1 Ab, rabbit anti-plkBα Ab, and rabbit anti-total IkBα Ab were purchased from Cell Signaling Technology (Danvers, MA). Rabbit anti-total JNK Ab, rabbit anti-TRAF5 Ab, rabbit anti-TRAF1 Ab, and rabbit anti-TRAF3 Ab were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-actin Ab was purchased from Millipore (Billerica, MA). Rabbit anti-TRAF2 Ab and chicken anti-TRAF6 Ab were purchased from Medical and Biological Laboratories (Japan). S12 (mouse anti-LMP1 IgG Ab) was a gift from Dr. Fred Wang (Harvard University, Cambridge, MA). Goat anti-mouse IgG, goat anti-rabbit IgG, goat anti-chicken IgG, and donkey anti-sheep IgG secondary Abs and goat anti-rat IgG were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). MOPC-21 Ab (mouse IgG1), FITC-labeled hamster anti-mouse CD80 Ab, and FITC-labeled Armenian hamster IgG Ab were purchased from BioLegend (San Diego, CA). HM40.3 (hamster anti-mCD40 Ab), rat anti-mouse CD16/32 Ab (to block FcR binding), FITC-labeled mouse anti-hCD40 Ab, and FITC-labeled mouse IgG2a were purchased from ebioscience (San Diego, CA). The G28.5 hybridoma producing mouse IgG1 anti-hCD40, was purchased from the ATCC. The 1C10 hybridoma producing rat IgG2a anti-mCD40, was a gift from Dr. Frances Lund (University of Rochester, Rochester, NY). The sheep anti-mCD40, was a gift from Dr. Frances Lund (University of Rochester, Rochester, NY). The 1C10 hybridoma producing rat IgG2a anti-mCD40, was a gift from Dr. Frances Lund (University of Rochester, Rochester, NY). The sheep anti-mCD40 Ab (48) was generated in our laboratory.
and rested for 45 min at 37 °C. Cells were then stimulated for 10, 15, 30, or 60 min with anti-hCD40 Ab (G28.5, 10 μg/ml), 6 M sorbitol (100 μl/ml), or anti-mCD40 Ab (HM40.3, 10 μg/ml). Whole cell lysates were prepared by pelleting the cells, removing the supernatant, and adding 200 μl of 2× SDS-PAGE loading buffer to the cell pellet. Lysates were sonicated using a Branson Sonifier 250 (VWR International) with 20 pulses at 90% duty cycle, output 1.5. Samples were denatured for 10 min at 95 °C. NF-κB1 activation was determined by measuring protein levels of phosphorylated and total IκBα (see below), as IκBα is phosphorylated and then degraded upon activation of the NF-κB1 pathway.

Western Blots—Up to 10 μl of sample were resolved on 10% SDS-PAGE. Proteins were transferred to Immobilon-P PVDF membranes (Millipore). Membranes were blocked with 10% dry milk in TBST for 1 h, washed in TBST (NaCl, Tris, Tween 20, and H2O), and incubated overnight at 4 °C with one of the above Abs. Blots were washed in TBST and incubated with secondary Abs for 1 h or overnight and developed using Supersignal West Pico (Pierce). Western blot chemiluminescence was read with an LAS–4000 low-light camera and analyzed with Multi Gauge software (Fujifilm Life Science, Edison, NJ).

**CD80 Up-regulation and Flow Cytometry—**2.5 × 10^5^ cells were stimulated in a 24-well plate in 1 ml BCM-10 for 72 h with 2 μg/ml of either MOPC-21 isotype mAb or G28.5 (anti-hCD40 mAb), or 20 μg/ml of LPS. Anti-mouse CD16/32 Ab (0.5 μg/ml) was added 10 min prior to staining with FITC-labeled anti-mouse CD80 Ab or FITC-labeled Armenian hamster IgG to block nonspecific binding to B cell Fcγ receptors. Flow cytometry was performed on a FACScalibur (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

**Immunoprecipitation—**Dynal protein G magnetic beads (Invitrogen) were coated with G28.5 or MOPC-21 Ab (10 μg/10 μl beads) or goat anti-rat IgG followed by anti-mCD40 Ab (1C10) as described previously (29). Abs were conjugated to the beads using disuccimidyl suberate according to the manufacturer’s protocols. Cells (2.0 × 10^7^ for cell lines, 1.5 × 10^7^ for primary cells) were incubated in 1 ml of BCM-10 with Ab-coated beads for either 30 min at 37 °C or 45 min at room temperature. Beads and cells were then pelleted and lysed as described previously (29). Bead-bound proteins were resuspended in 2× SDS-PAGE loading buffer and boiled for 10 min at 95 °C.

**Mouse Splenic B Cells—**The mCD40^+/−^ and mCD40LMP1 transgenic (Tg) mice were described previously (25, 49). The mCD40LMP1PQAA1 mice were generated as described for the mCD40LMP1 mice, and the mCD40LMP1PQAA1 construct was created using site-directed mutagenesis as described previously (43). Mice of all transgenic strains used develop and induce hCD40LMP1 signaling for 0–60 min. Stimulation with 10 μl/ml of either MOPC-21 isotype mAb or G28.5 (anti-hCD40 mAb) will induce up-regulation of costimulatory and adhesion molecules (3). To understand how LMP1 uses TRAF6 to promote its effects on B cells, we first examined TRAF6-sufficient (TRAF6^+/−^) and TRAF6-deficient (TRAF6^−/−^) mouse B cells, which were created via gene targeting by homologous recombination as described previously (29). As described under “Experimental Procedures,” these cell lines were both transfected to stably express hCD40LMP1, which was measured by flow cytometry to pair subclones based on their similar levels of hCD40LMP1 expression. Anti-hCD40 Ab was used to stimulate these cells and induce hCD40LMP1 signaling for 0–60 min. Stimulation via sorbitol, a sugar that induces osmotic stress (22), was included as a TRAF6-independent control stimulus. Fig. 1, B and C, shows that the absence of TRAF6 abolished the ability of hCD40LMP1 to activate JNK, p38, TAK1, and NF-κB1. Impor-
up-regulated by both CD40 and LMP1 (3, 22). Previous studies revealed that CD40 does not require the TRAF6 TRAF-C domain to induce CD80 expression in B cells (29). To examine this requirement for LMP1-mediated CD80 up-regulation, TRAF6−/− cells or TRAF6−/− cells transfected to express WT TRAF6 or TRAF6ΔTRAF were cultured in the absence of stimulation or in the presence of an isotype control Ab or anti-hCD40 Ab to induce hCD40LMP1 activation. LPS (a TLR4 agonist) served as a positive control. The absence of TRAF6 abrogated the ability of hCD40LMP1 to induce CD80 expression (Fig. 3). In TRAF6−/− cells, exogenous expression of WT TRAF6, but not similar levels of TRAF6ΔTRAF, induced normal up-regulation of CD80. Although there appears to be a slight shift in CD80 expression when TRAF6−/− cells expressing TRAF6ΔTRAF were stimulated via hCD40LMP1, this change was very modest. Thus, TRAF6ΔTRAF was unable to effectively restore LMP1-mediated CD80 up-regulation. This LMP1-mediated event was revealed to require the TRAF6 TRAF-C domain, suggesting that direct TRAF6 association was needed.

TRAF6 Association with LMP1—Although no TRAF6 binding motif has been described in the LMP1 CY domain to date, the results presented in Figs. 2 and 3 indicated that TRAF6 associates with the CY domain of LMP1. This domain contains two important subdomains, CTAR1 (amino acids 187–241) and CTAR2 (amino acids 242–386), which mediate the binding of signaling molecules (17–19, 21). Previous studies have shown that CTAR1 and CTAR2 cooperate to mediate LMP1 functions, including activation of early signaling pathways (53). To determine TRAF6 association with LMP1, we used mouse B cell lines stably expressing hCD40LMP1 or mutant hCD40LMP1 molecules described earlier (18, 43), expressing only LMP1 CTAR1 (hCD40CTAR1) or only CTAR2 (hCD40CTAR2) (Fig. 4A). An hCD40 molecule lacking nearly all of the CY domain (hCD40Δ55) and shown not to deliver any signals to B cells (47) was included as a control for nonspecific binding. The results presented in Fig. 4B demonstrate that TRAF6 associated with hCD40LMP1 and the CTAR1, but not the CTAR2, subdomain of hCD40LMP1 in B cells.

TRAF6 Association with the TRAF1/2/3/5 Binding Site—The major TRAF1/2/3/5 binding site (TBS) is located within the CTAR1 subdomain of LMP1 (43). Our laboratory previously demonstrated that TRAFs 1, 2, 3, and 5 associate with CTAR1 but not CTAR2 in B cells (18). Although the TRAF6 binding site in CD40 is distinct from the TBS shared by the other TRAFs (32, 35, 36), no TRAF6 consensus binding site has been described in LMP1. To test whether TRAF6 was recruited to LMP1 via the TBS, we stimulated mouse B cells stably expressing hCD40LMP1 or an hCD40LMP1 receptor with the TBS mutated to disrupt the binding of TRAFs 1, 2, 3, and 5 (hCD40LMP1QAA1) (43). Disruption of the TBS abolished the ability of hCD40LMP1 to recruit TRAF6 (Fig. 5A). To confirm this important finding in freshly isolated B cells, we took advantage of a new mCD40LMP1 Tg mouse. Our lab previously produced mice that express the mCD40LMP1 Tg on a CD40−/− background so that the endogenous ligand for CD40, CD154, induces signaling through LMP1 only (25). Mice that expressed mCD40LMP1 with a mutated TBS
(mCD40LMP1PQAA1 Tg) were developed in the same manner, as described under “Experimental Procedures.” Using purified splenic B cells from mCD40−/− mice or mCD40−/− mice expressing mCD40LMP1 or mCD40LMP1PQAA1, we determined TRAF6 association with LMP1 in freshly isolated mouse B cells, an important confirmation of the results obtained in mouse B cell lines.

**DISCUSSION**

LMP1 is a functional mimic of CD40, yet these receptors use TRAFs 1, 2, 3, and 5 differently (18, 20, 28). CD40-mediated JNK activation and CD80 up-regulation in B cells are TRAF6-dependent but TRAF-C domain-independent (29), whereas in the present study we demonstrate that LMP1-mediated JNK, p38, NF-κB1 activation, and CD80 up-regulation required the presence of this TRAF6 domain. Interestingly, previous work showed a decrease in LMP1-mediated CD80 up-regulation in the absence of TRAF3, although there was still residual CD80 up-regulation in TRAF3−/− B cells compared with TRAF6−/− B cells (20). Data in this study show that in the absence of TRAF6 there was no CD80 up-regulation, thus none of the other TRAFs have a redundant role in this process. However, it is possible that TRAF3 and TRAF6 function together to promote CD80 expression, as both TRAFs play a role in this LMP1-mediated function. Furthermore, whereas TRAF6 binds to a site on CD40 distinct from the major TBS (28, 32, 35, 36), our results revealed that TRAF6 association required the TBS on LMP1, further highlighting differences between the manner in which LMP1 and CD40 utilize TRAF6.
The TRAF-C domain mediates receptor binding to TNFR superfamily members (50, 51), and the absence of this domain abolishes TRAF6 binding to members of this superfamily (29, 54, 55). Interestingly, the TRAF6 TRAF-C domain is not required for several important CD40-mediated TRAF6-dependent events, indicating that CD40 may use TRAF6 as a strictly CY adaptor protein in certain situations (29), similar to TRAF6 use by the Toll-like receptors (44). In LMP1 signaling, the absence of the TRAF6 TRAF-C domain resulted in the abrogation of all tested LMP1-mediated signaling events. Thus, LMP1 likely requires receptor-associated TRAF6 to mediate the effects tested. However, it is possible that the TRAF6 TRAF-C domain is dispensable for some as yet unknown LMP1 functions. The present studies used A20 B cells, selected because they are responsive to CD40 and LMP1-mediated activation signals (20, 29). Importantly, subclones completely and specifically deficient in TRAF6 were available (29).However, A20 cells cannot be induced to secrete Ig, and their production of a variety of cytokines and chemokines is not reliably detectable (data not shown). B cell-specific TRAF6/H11002 mice are not commercially available, and they do not express LMP1 (EBV does not infect mouse B cells). Thus, we cannot exclude the possibility that LMP1 mediates some effects without requiring the TRAF6 TRAF-C domain, but it is clear that LMP1 is much more dependent upon this domain for signaling to B cells than is CD40.

The results presented here showed that TRAF6 associated with the CY domain of hCD40LMP1, specifically the TBS, following stimulation. We cannot simply directly compare the in vivo functions of LMP1 in mCD40LMP1Tg versus mCD40LMP1PQAA1Tg mice because the expression levels of the mCD40LMP1PQAA1 Tg molecule in founder lines are only approximately half the level of expression of mCD40LMP1 WT on B cells of this transgenic mouse (data not shown). This does not affect immunoprecipitation experiments because we adjusted conditions in vitro so that mCD40LMP1 levels were the same for each sample, but this discrepancy in surface levels could affect the interpretation of in vivo characteristics of the mCD40LMP1PQAA1 mouse. Although the mCD40LMP1PQAA1 Tg mice have similar levels of total B cells and B cell subsets compared with the mCD40LMP1 Tg mice, mCD40LMP1PQAA1 mice fail to form spontaneous germinal centers (in contrast to mCD40LMP1 mice), and B cells from mCD40LMP1PQAA1 mice fail to switch from IgM to IgG1 in vitro.4

4 K. Arcipowski and G. Bishop, unpublished data.
**TRA6 in LMP1 Signaling**

There are several ways in which TRAF6-LMP1 interaction may occur. One possibility is that TRAF6 is recruited to LMP1 via other TRAFs. This mechanism of association has been shown for CD40, as TRAF1 shows very minimal direct binding to CD40 but is largely recruited to the CD40 CY domain by association with TRAF2 (28). It has been demonstrated previously that TRAFs 1 and 2 are not required for LMP1-mediated B cell activation (31), and we confirmed that TRAF1 and TRAF2 were not responsible for mediating recruitment of TRAF6 to LMP1 (supplemental Figs. 1 and 3). TRAFs 1, 2, 3, and 5 were not required for TRAF6 interaction with LMP1. Interestingly, we found that more TRAF6 was recruited to LMP1 in the absence of either TRAF1 or TRAF2, or in the absence of both TRAF1 and TRAF2 (supplemental Figs. 1 and 3). We also found that the absence of TRAF3 had little effect on TRAF6-LMP1 interaction (supplemental Figs. 1 and 3). TRAF5 has been recently reported to be an essential component of LMP1 signaling to B cells both in vitro and in vivo (24). Interestingly, TRAF5 required the presence of TRAF3 to associate with LMP1 (supplemental Fig. 2), indicating that TRAF5 cannot be required for TRAF6 recruitment, as the latter occurred independently of TRAF3 (supplemental Figs. 1 and 3).

TRA1 showed increased association with LMP1 in the absence of TRAF2 (supplemental Fig. 3), whereas TRAF2 depended on TRAFs 1 and 3 for optimal association with LMP1 (supplemental Fig. 3). Thus, it appears that various TRAFs can compete or cooperate for binding to LMP1, as has been shown for CD40 (31). The enhanced binding of TRAF6 to LMP1 in the absence of TRAFs 1 and/or 2 is consistent with direct binding of TRAF6 to LMP1, although unidentified non-TRAF proteins may also participate. Whereas the structural motif in CD40 responsible for TRAF6 (QPQEQINF) (56) is not present in LMP1, TRAF6 could directly interact with LMP1 via a novel TRAF6 binding site, namely the TBS. This idea has precedent, as it was demonstrated that TRAF2 associates with CD40 via the originally described TBS (PXQXT) (57) and a second binding site (SXXE) identified later (56). Future studies involving comprehensive structure-function analysis could test whether TRAF6 directly binds to LMP1. However, given the cooperative and competitive interactions seen for LMP1-TRAF binding, such analyses would need to include additional TRAFs in physiologically relevant proportions, as these can clearly influence TRAF6 binding. Simple *in vitro* assays of interactions between single purified proteins would not accurately model the *in vivo* situation.

Another possibility is that one or more non-TRAF proteins recruit TRAF6 to LMP1. Future studies could test the possible role of proteins shown to associate with TRAF6, CATAR1, or CD40, including A20, BS69, NEMO, Act1, Malt1, and HOIP (58–63). A detailed proteomics study could identify any novel proteins involved in TRAF6 recruitment to LMP1.

Finally, TRAF6 could directly bind to LMP1, but additional proteins could enhance the avidity or stability of this association. Examination of these possibilities will be the subject of future studies.

Understanding the exact mechanisms by which LMP1 employs TRAF6 is important for the development of therapies that target the pathogenic effects of LMP1. As LMP1 is rapidly and constantly processed from the cell surface and lacks a typical extracellular domain (18–20, 53), LMP1 itself may not be an ideal target for therapies. Thus, it would be beneficial to disrupt downstream LMP1 signaling, possibly by targeting TRAF6. As TRAF6 associates with LMP1, it could be feasible to target this interaction while not disrupting key CD40-mediated signals that do not require direct TRAF6 association. As LMP1 does not possess a canonical TRAF6 binding site, it could also be possible to disrupt TRAF6-LMP1 association while leaving its binding to CD40 intact. Although TRAF6 is used by multiple receptors, it is clearly used in different ways. Identifying the unique requirements of TRAF6 in LMP1 signaling adds important new information to our understanding of how the CD40 mimic LMP1 drives B cell pathogenesis.

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