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Title: **Caspase-dependent cleavage regulates protein levels of Epstein-Barr virus-derived latent membrane protein 1**

Running title: EBV-LMP1 is degraded by caspases

Authors: Sumihito Togi[a], Yosuke Hatano[a], Ryuta Muromoto[a], Eri Kawanishi[a], Osamu Ikeda[a], Koki Hirashima[a], Shigeyuki Kon[a], Yuichi Kitai[a], Teruhito Yasui[b], Kenji Oritani[c], and Tadashi Matsuda[a,¶]

[a]Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-12 Nishi-6, Kita-Ku, Sapporo, 060-0812, Japan

[b]Department of Molecular Immunology, Research Institute for Microbial Diseases, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan

[c]Department of Hematology and Oncology, Graduate School of Medicine, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan

[¶]Address correspondence to: Dr. Tadashi Matsuda, Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-Ku Kita 12 Nishi 6, Sapporo 060-0812, Japan Tel.: 81-11-706-3243, Fax: 81-11-706-4990, E-mail: tmatsuda@pharm.hokudai.ac.jp

Abstract

Epstein–Barr virus (EBV)-encoded latent membrane protein-1 (LMP1) plays pathogenetic roles in EBV-related diseases. Thus, host cells employ several mechanisms to regulate LMP1 functions, and we previously reported possible regulation by signal transducing adaptor protein-2 as well as BS69. Here, we found that caspase-3 mainly degraded LMP1 proteins in HeLa cells, leading to decreased NF-κB and STAT3 activation. Caspase-3 cleaved the consensus DNTD sequences in the CTAR3 region of LMP1. Of importance, LMP1 expression strongly enhanced caspase-3 activity. Taken together, the reduction of LMP1 protein levels by caspases is likely to be a newly identified host defense against EBV infection.

Abbreviations: EBV, Epstein–Barr virus; LMP, latent membrane protein; CTAR, C-terminal activation region; LUC, luciferase

Keywords: EBV, LMP1, caspase, degradation, NF-κB activation

1. Introduction

Epstein–Barr virus (EBV) is linked to the development of multiple malignancies, including post-transplant lymphoproliferative disorders, Hodgkin lymphomas, and nasopharyngeal carcinomas. EBV-encoded latent membrane protein 1 (LMP1) is expressed in many EBV-associated tumor cells and is responsible for the majority of the observed altered cellular growth properties. Structurally, LMP1 is an integral membrane protein composed of 386 amino acids (aa) with a short cytoplasmic N-terminal domain (aa 1-24), six transmembrane domains (aa 25-186), and a long cytoplasmic C-terminal tail (aa 187-386) [1, 2, 3]. The cytoplasmic C-terminal tail is composed of three C-terminal activation regions (CTAR1, CTAR2, and CTAR3, respectively), which are mainly involved in LMP1-mediated signaling events [1, 4, 5]. CTAR1 contains a consensus TRAF-binding motif (PXQXT), and can bind to TRAFs 1, 2, 3, and 5 [1,6-9]. CTAR2 interacts with TNFR-associated death domain protein (TRADD), receptor-interacting protein [1, 10, 11], and a multi-domain cellular protein BS69 [12]. Signals through the CTAR domains induce p100 and p105 NF-κB precursors and upregulate their processing into p52 and p50, respectively [13, 14], indicating that LMP1 is involved in both canonical and non-canonical activation of the NF-κB pathway.

Binding of LMP1 to TRAFs and TRADD also initiates the formation of a signaling complex, which leads to the activation of mitogen-activated protein kinase, p38 [15]. LMP1 also activates c-Jun N-terminal kinase (JNK) as well as PI3K/Akt and STAT3 signaling [12, 16-19]. We recently identified an adaptor protein, STAP-2, as a new binding partner of both CTAR1 and CTAR2, and demonstrated that STAP-2 negatively regulates LMP1-mediated NF-κB activation (20). Moreover, we reported that BS69 regulates LMP1-mediated NF-κB activation by influencing binding between LMP1 and TRADD [21, 22].

LMP1 is a short-lived protein, with a reported half-life ranging from 1.5 to 7 h, depending on the cell type [23-25]. Additionally, one report has found that LMP1 is in part degraded via the ubiquitin-mediated proteasome degradation pathway in COS7/5 fibroblasts [26]. Notably, LMP1 degradation was detected in lymphomas derived from the LMP1 transgenic mice expressing LMP1 in B lymphocytes [27]. However, very little information is available on the regulation of degradation rate and stability of LMP1 proteins in individual cells.

In the present study, we have found that LMP1 is mainly degraded by the caspase cascade in HeLa cells. In this situation, caspase-3 cleaves the CTAR3 region of LMP1 at consensus DNTD sequences and is, to the best of our knowledge, a previously

undescribed host mechanism to control LMP1 protein levels. Of importance, LMP1 expression strongly enhances caspase-3 activities. We also discuss possible meanings of LMP1 degradation by caspases during EBV infection.

2. Materials and Methods

2.1. Reagents and Antibodies

Recombinant human TNF-α, cycloheximide (CHX) and NH_4Cl were purchased from WAKO Chemicals (Osaka, Japan). MG132, Z-VAD, Z-IETD, Ac-DNLD, Ac-VEID, Ac-LEHD and Z-DEVD were purchased from Peptide Institute (Osaka, Japan). Expression vector for FLAG-tagged LMP1 was described previously [20], and its mutants were generated by PCR and cloned by using TaKaRa In-Fusion Kit (primer sequences are available upon request). The synthesized FLAG-tagged LMP1 4xANTD mutant was obtained from GenScript (Piscataway, NJ). STAT3-LUC and NF-κB-LUC were kindly provided by T. Hirano (Osaka University Medical School, Osaka, Japan) and T. Fujita (Kyoto University, Kyoto, Japan) [28, 29]. Anti-FLAG antibody was purchased from Sigma-Aldrich (St. Louis, MO). Anti–caspase-3, anti–caspase-7, and anti–caspase-8 antibodies were from Cell Signaling Technologies (Beverly, MA). Anti-actin antibody was from Millipore (Billerica, MA).

2.2. Cell Culture, Transfection, siRNA and luciferase assay

A human cervix carcinoma cell line, HeLa was maintained in DMEM containing 10 %

FCS. HeLa cells were transfected using jetPEI (Polyplus Transfection, Strasbourg, France) according to the manufacturer's instructions.. The siRNAs targeting human caspase-3, -7, or -8 used in this study were as follows: caspase-3 #1, 5'-GCGUGAUGUUUCUAAAGAAGA-3' (sense), 5'-UUCUUUAGAAACAUCACGCAU-3' (antisense) ; caspase-3 #2, 5'-CCCUGGACAACAGUUAUAATT-3' (sense), 5'-UUAUAACUGUUGUCCAGGGTT-3' (antisense) ; caspase-7 #1, 5'-GCAUCAUAAUAAACAACAAGA-3' (sense), 5'-UUGUUGUUUAUUAUGAUGCAU-3' (antisense) ; caspase-7 #2, 5'-GGAACUCUACUUCAGUCAAUA-3' (sense), 5'-UUGACUGAAGUAGAGUUCCUU-3' (antisense) ; caspase-8 #1, 5'-GAGUCUGUGCCCAAAUCAATT-3' (sense), 5'-UUGAUUUGGGCACAGACUCTT-3' (antisense) ; caspase-8 #2, 5'-CAGCCUACUUUCACACUAATT-3' (sense), 5'-UUAGUGUGAAAGUAGGCUGAG-3' (antisense) ; Control siRNA was obtained from Qiagen (nonsilencing; catalog No. 1022076). HeLa cells were plated on 24-well plates at $2x10^4$ cells/well and incubated with an siRNA-Lipofectamine 2000 (Invitrogen, Carlsbad, CA) mixture at 37 °C for 6 h followed by the addition of fresh medium

containing 10 % FCS [20]. Luciferase activities of HeLa cells transfected with NF-κB-LUC or STAT3-LUC as well as internal control, in which the α2-macroglobulin promoter drives expression of a luciferase reporter gene, were measured using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions.

2.3. Immunoblotting

Western blotting assays were performed, as described previously [30]. The cell lysates were resolved on SDS-PAGE and transferred to polyvinylidene difluoride transfer membrane (PerkinElmer, Boston, MA). The filters were then immunoblotted with each antibody. Immunoreactive proteins were visualized using an ECL detection system (Millipore).

2.4. RNA Isolation and Quantitative Real-time (qRT)-PCR

Cells were harvested and total RNAs were prepared by using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA). First-strand cDNA was synthesized from 1 μg of total RNA with ReverTra Ace (TOYOBO, Osaka, Japan). Quantitative real-time PCR analysis of mRNA transcripts was carried out using a combination of a KAPA

SYBR FAST qPCR master mix (KAPA Biosystems, Woburn, MA, USA) with an Mx3005P real-time PCR system (Stratagene, Santa Clara, CA, USA). The primers used for qPCR were: *IL-6*, 5'- AACTGGATATAATCAGGAAAT-3' (sense), 5'-CTAGGTTTGCCGAGTAGATCTC-3' (antisense); *ACTIN*, 5'-TGTTACCAACTGGGACGACA-3' (sense), 5'-GGGGTGTTGAAGGTCTCAAA-3' (antisense).

2.5. Analysis of caspase activity

Caspase-3 activity was measured using the Caspase-Glo 3/7 Assay kits essentially according to the manufacturer's instructions (Promega) [31].

3. Results

3.1. LMP1 is degraded in a caspase-dependent manner in HeLa cells

To confirm the degradation of LMP1 protein, we first assessed the stability of LMP1 proteins in LMP1-trasfected HeLa cells. HeLa cells ectopically expressing LMP1 were cultured in the presence of CHX to block protein synthesis. As shown in Fig. 1A and B, the treatment of LMP1-transfected HeLa cells with CHX induced a rapid reduction of full-length LMP1 (FL-LMP1) protein levels; in addition, multiple smaller LMP1-derived bands were clearly visible. This result likely indicates that LMP1 protein was comprehensively degraded in HeLa cells. We next examined effects of various protease inhibitors on LMP1 protein degradation using this experimental system. As shown in Fig. 1C and D, a pan-caspase inhibitor, Z-VAD, significantly blocked the degradation of LMP1 protein in HeLa cells while a proteasomal inhibitor, MG132, or a lysosomal inhibitor, NH_4Cl, did not. In the same situation, the treatment of HeLa cells with MG132 blocked TNF-α-induced IκBα degradation in HeLa cells (Fig.1S).

Therefore, in HeLa cells, ectopically expressed LMP1 is mostly degraded in a caspase-dependent manner.

3.2. Caspase-3 is responsible for LMP1 protein degradation in HeLa cells

To identify which caspase is responsible for LMP1 protein degradation in HeLa cells, LMP1-transfected HeLa cells were cultured in the presence of a caspase-3 specific inhibitor, Ac-DNLD, a caspase-3/7 specific inhibitor, Z-DEVD, a caspase-6 specific inhibitor, Ac-VEID, a caspase-8-specific inhibitor, IETD, a caspase-9 specific inhibitor, Ac-LEHD or a pan-caspase inhibitor, Z-VAD. As shown in Fig. 2A and B, the treatment with Z-DEVD, and Ac-VEID as well as Z-VAD significantly blocked LMP1 protein degradation in HeLa cells. Although these results indicate the involvement of caspases, the evaluation of each caspase in LMP1 degradation is difficult. This might due to the limited selectivity of the caspase inhibitors because they are designed based on the substrate specificity profiles and has similar constructs. To directly clarify this issue, we employed siRNAs techniques to specifically knockdown each caspase. We transfected specific siRNAs for caspase-3 (#1 and #2), caspase-7 (#1 and #2), or caspase-8 (#1 and #2) into HeLa cells expressing LMP1. The reduction of caspase-3, but not caspase-7 or caspase-8 expression, resulted in a significant increase of FL-LMP1 protein levels in LMP1-transfected HeLa cells (Fig. 2C and D). Therefore, caspase-3 is the main caspase responsible for degradation of LMP1 in HeLa cells. However, casapse-3 siRNA treatment of HeLa cells also increased accumulation of the

partially degraded fragment of LMP1 as shown in the case of the treatment with Ac-DNLD (Fig. 2A). Thus, we could not exclude the involvement of other caspases in LMP1 protein degradation in HeLa cells, although casapse-3 knockdown in HeLa cells by siRNA treatment was not complete.

3.3. LMP1 expression induces caspase-3 activation in HeLa cells

To investigate the relationship between LMP1 and caspase-3, we analyzed the caspase enzymatic activities of caspase-3 after LMP1-transfection into HeLa cells. As shown in Fig. 3A, the activities of these caspases markedly increased in correlation with LMP1 protein degradation in LMP1-transfected HeLa cells, whereas they were completely blocked by the addition of Z-VAD. This was also confirmed by western blot analyses (Fig. 3B) showing that the cleavage of caspase-3 and -8 was significantly increased in LMP1-transfected HeLa cells compared with vector-transfected HeLa cells. However, we could not detect the cleavage of caspase-7 in these cells (data not shown).

3.4. Caspase inhibition enhances LMP1-mediated signals in HeLa cells

To analyze the involvement of caspases on LMP1-mediated signals, we examined the effects of Z-VAD on LMP1-induced NF-κB or STAT3 activation using transient

reporter assays with NF-κB-LUC or STAT3-LUC. Both luciferase activities were markedly induced by the expression of LMP1 in HeLa cells (Fig. 4A and B). Importantly, LMP1-induced NF-κB-LUC or STAT3-LUC activities were significantly enhanced by Z-VAD treatment in parallel with the increase in LMP1 protein levels, suggesting that caspase activation negatively regulates LMP1-induced NF-κB and/or STAT3 activation.

NF-κB activation induces the expression of inflammatory cytokines, such as IL-6. To confirm the regulation of LMP1-induced NF-κB activation by caspases, LMP1-induced IL-6 mRNA expression was analyzed in the Z-VAD-treated or untreated cells. LMP1-induced IL-6 mRNA expression was markedly augmented by the treatment of LMP1-transfected HeLa cells with Z-VAD in (Fig. 4C). Interestingly, the treatment with Z-VAD had no effect on TNF-α-induced NF-κB activation (Fig. 4D).

Therefore, caspase activity negatively regulates NF-κB and/or STAT3 activation mediated by LMP1, but not TNF-α.

3.5. Caspase-3 regulates LMP1-mediated NF-κB activation in HeLa cells

To further explore whether casapase-3 regulates LMP1-induced NF-κB activation, we used siRNAs to reduce endogenous expression of caspase-3 in HeLa cells. HeLa

cells were transfected with specific siRNAs for caspase-3 (#1 and #2) or a control siRNA. We then determined the effects of caspase-3 siRNAs on LMP1-induced NF-κB activation in HeLa cells. As shown in Fig. 5A, siRNA-mediated knock down of caspase-3 resulted in a significant enhancement of LMP1-induced NF-κB-LUC activities in parallel with the increase in LMP1 protein levels. We also determined the effects of caspase-3 siRNAs on LMP1-induced IL-6 mRNA expression. LMP1-induced IL-6 mRNA expression was also significantly augmented by reduced expression of caspase-3 in LMP1-transfected HeLa cells (Fig. 5B).

Therefore, casapse-3 is responsible for LMP1 protein degradation and LMP1-induced NF-κB activation in HeLa cells.

3.6. Determination of caspase cleavage sites in LMP1 in HeLa cells

To determine which domains of LMP1 were involved in caspase-dependent degradation, HeLa cells were transfected with a series of FLAG-tagged LMP1 deletion mutants (Fig. 6A). The transfectants were treated with Z-VAD or left untreated, lysed, and immunoblotted with an anti-FLAG antibody. As shown in Fig. 6B, the protein levels of both FL-LMP1 and LMP1(1-349) were sensitive to Z-VAD treatment. However, no significant alteration of protein levels of both LMP1(1-192) and LMP1(1-231) was

observed in samples with Z-VAD. These results indicate that the caspase cleavage sites in LMP1 may exist in the CTAR3 region. We found four putative consensus casapase-3 cleavage sequences (DNTD) at the positions of aa 257–260, aa 268–271, aa 284–287, and aa 295–298 in that region (data not shown). We then examined these four repeated DNTD motifs using a Δ4xDNTD mutant with a deletion of aa 249–307 in LMP1(1-349) (Fig. 6C). As shown in Fig. 6D, LMP1(1-349/Δ4xDNTD) protein levels did not change before or after the Z-VAD treatment. In addition, the multiple degraded bands observed in control LMP1(1-349) samples were not seen in LMP1(1-349/Δ4xDNTD). Of note, LMP1(1-349/Δ4xDNTD) protein yielded two bands, which migrated a little faster, in the absence of Z-VAD, indicating that other cleavage sites may exist in LMP1(1-349/Δ4xDNTD). Upon investigation, another potential caspase cleavage site was identified as LMTD (aa338–341). To further evaluate the potential cleavage of LMP1 at this site, we generated a LMP1 mutant with substitution of glutamic acid for aspartic acid at residue 341 (D341E) in LMP1(1-349/Δ4xDNTD). The LMP1(1-349/Δ4xDNTD/D341E) protein showed a single protein band in the absence or presence of Z-VAD, indicating that the LMP1(1-349/Δ4xDNTD/D341E) protein gained complete resistance to caspase-dependent proteolysis. We also introduced these mutations into FL-LMP1 (FL-LMP1 (Δ4xDNTD/D341E)) (Fig. 6E).

As shown in Fig. 6F, the FL-LMP1 (Δ4xDNTD/D341E) protein was completely resistant to caspase-dependent proteolysis. We finally generated a LMP1 4xANTD mutant with substitution of alanine for aspartic acid at residues 257, 268, 284 and 295 in LMP1, and a LMP1 4xANTD/D341E mutant with substitution of glutamic acid for aspartic acid at residue 341 in LMP1 4xANTD. As shown in Fig. 6G, multiple smaller LMP1-derived degraded bands disappeared in the LMP1 4xANTD mutant, although LMP1 4xANTD protein yielded two bands as well as LMP1(1-349/Δ4xDNTD). More importantly, LMP1 4xANTD/D341E was completely resistant to degradation in HeLa cells (Fig. 6G). The treatment of Z-VAD tended to slightly increase the protein levels in LMP1 mutants, which do not contain the suggested caspase cleavage sites. However, there is no statistically significant difference, when we normalized the protein level LMP1 to the actin protein of the same sample (data not shown). Thus, we have identified four repeated DNTD motifs and a LMTD sequence in CTAR3 as consensus caspase cleavage sites responsible for degradation by caspase-3 and another caspase, respectively, although we could not exclude the possibility that the caspase-8 cleavage motif LMTD may be newly exposed by deletion of the four repeated DNTD motifs in the CTAR3 region.

4. Discussion

EBV, among other tumor-associated viruses, can affect host cell growth and survival, resulting in malignant transformation [32, 33]. Some virus proteins function to mediate escape from immune surveillance, while others directly regulate growth and/or apoptotic signals [34, 35]. In the case of EBV, a nuclear antigen, EBNA1, has anti-apoptotic effects, while EBNA3 binds to Rb and promotes cell-cycle progression in Burkitt lymphoma cells. The small nuclear RNA, EBER1, binds to and inhibits protein kinase RNA, which is an effector of the apoptotic signaling pathway. In addition, EBERs can induce resistance to apoptosis via increased expression of an anti-apoptotic factor BCL-2 [1, 33]. LMP1 also contributes to survival and/or tumor progression by providing CD40/CD40L-like signals or modifying the TNF receptor pathway [1, 2, 3, 33]. Here, we describe a new caspase cascade-dependent regulatory mechanism controlling LMP1. In HeLa cells, caspase-3 mainly degrades LMP1 proteins, resulting in decreased LMP1-induced NF-κB activation and IL-6 production. We have found that LMP1 expression induces caspase-3 activation, and activated caspase-3 in turn cleaves the CTAR3 region of LMP1. Therefore, caspases negatively regulate LMP1 functions by controlling protein levels.

Caspases are often targeted by a variety of viruses in infected cells because these host enzymes play a central role in controlling cell death machinery [33, 34]. Thus, many viruses can interfere with caspase activation or inhibit caspase activities. Indeed, caspase-8 activities are inhibited by various virus-encoded proteins, including the baculovirus p35 protein, the adenovirus E3 14.7 kDa protein, several poxvirus serpins and vFLIPs (viral FLIPs), the gammaherpesvirus vFLIPs, and the cytomegalovirus viral inhibitor of caspase 8 activation protein. In contrast, we have shown that caspase-3 activities are upregulated in response to LMP1 expression and that activated caspase-3 degrades LMP1 protein. As mentioned above, EBV-derived LMP1 provides strong signals for cell growth and survival. Thus, the relationship between caspase-3 and LMP1 suggests a likely target for the host defense to block EBV infection. However, our experimental model was restricted to LMP1, although EBV produces a number of viral proteins. Further analysis will clarify the extent to which caspases act to inhibit EBV infection in host cells. Of note, an interesting report indicates that the inhibition of caspase activities enhances virus replication in a HIV-infected T-cell line [36]. Virus replication was caused by sensitization of TNF-R1 towards endogenously produced TNF-α. The activation of Fas also triggered HIV replication, which was further enhanced in the presence of Z-VAD. The authors concluded that caspase inhibitors

sensitize both Fas and TNF-R1 to mediate the activation of HIV in latently infected cells. Therefore, caspases function to protect host cells from virus infection at multiple intracellular events, and the degradation of EBV LMP1 by caspase-3 is likely to be a potential mechanism of host defense against EBV.

With regard to LMP1 protein degradation, Aviel et al. reported that LMP1 protein was degraded by the ubiquitin-proteasome pathway in a fibroblast line COS7/5 [26]. LMP1 protein levels were significantly increased by treatment with a proteasome inhibitor MG132. As we show here, treatment of HeLa cells with Z-VAD, but not MG132, increased LMP1 protein levels. In addition, our preliminary experiments also indicated that LMP1 protein was degraded via both ubiquitin-proteasome and caspase pathways in B lymphocyte lines (data not shown). Therefore, at least two mechanisms exist for LMP1 protein degradation, and the use of these two pathways to degrade LMP1 protein is likely to be dependent on cell type.

The CTAR3 region of LMP1 contains 4xDNTD and LMTD, which are the consensus caspase cleavage sequences. CTAR3 has been shown to bind JAK3 via PXXPXP motifs (aa 275–280 and aa 302–307) that activate DNA binding of STAT1 [5]. CTAR3, but not CTAR1 or CTAR2, has been shown to interact with SUMO-conjugating enzyme Ubc9 [37]. Interactions between Ubc9 and CTAR3 mediate LMP1-induced protein

sumoylation, in turn influencing cellular migration. Furthermore, LMP1 expression induced sumoylation of IRF7 in a CTAR3-dependent manner, followed by decreasing its turnover, increasing nuclear retention, decreasing DNA binding, and limiting transcriptional activation [38]. Our results indicate that the cleavage of LMP1 protein at the CTAR3 region significantly reduces the ability to induce NF-κB activation and IL-6 production. Although the precise mechanisms are unknown, the CTAR3 region may be required for full LMP1 functions.

Here, we provide evidence that caspases can inhibit EBV LMP1-mediated NF-κB activation through protein degradation of LMP1. Our data suggest the possibility that controlling LMP1 protein levels may be a novel strategy for anti-viral therapy to regulate EBV LMP1-induced NF-κB activation.

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Disclosures

The authors have no financial conflicts of interest.

Author contributions

ST, YH, RM, EK, OI and KH performed experiments. SK and YK analyzed data. TY distributed materials. KO wrote paper and TM designed experiments, supervised the project and wrote the paper.

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Figure Legends

FIGURE 1. **LMP1 is degraded in a caspase-dependent manner in HeLa cells.** A, HeLa cells in a 24 well plate were transfected with FLAG-tagged LMP1 (500 ng). At 24 h after transfection, the cells were treated with cycloheximide (5 μg/ml) for the indicated periods. Cells were then lysed, and an aliquot of total cell lysate (TCL) (1%) was blotted with anti-FLAG or anti-actin antibody. B, Densitometric quantification of the above results was also shown. The relative intensity of FLAG-LMP1 was normalized to the actin protein of the same sample. Results are representative of three independent experiments, and the error bars represent the SD. *, $p<0.05$. **, $p<0.01$. C, HeLa cells in a 24 well plate were transfected with FLAG-tagged LMP1 (500 ng). At 12 h after transfection, the cells were treated with MG132 (10 μM), Z-VAD (10 μM) or NH_4Cl (10 μM) for an additional 12h, with or without cycloheximide (5 μg/ml). Cells were then lysed, and TCL (1%) was blotted with anti-FLAG or anti-actin antibody. D, Densitometric quantification of the above results was also shown. The relative intensity of FLAG-LMP1 was normalized to the actin protein of the same sample. Results are representative of three independent experiments, and the error bars represent the SD.**, $p<0.01$, ***, $p<0.001$.

FIGURE 2. **Caspase-3 is responsible for LMP1 protein degradation in HeLa cells.** A, HeLa cells in a 24 well plate were transfected with FLAG-tagged LMP1 (500 ng). At 12 h after transfection, the cells were treated with Ac-DNLD (10 μ M), Z-DEVD (10 μM), Ac-VEID (10 μ M), Z-IETD (10 μM), Ac-LEHD (10 μ M), or Z-VAD (10 μM) for an additional 12h. Cells were then lysed, and an aliquot of total cell lysate (TCL) (1%) was blotted with anti-FLAG or anti-actin antibody. B, Densitometric quantification of the above results was also shown. The relative intensity of FLAG-LMP1 was normalized to the actin protein of the same sample. Results are representative of three independent experiments, and the error bars represent the SD. *, $p<0.05$. **, $p<0.01$. C, HeLa cells in a 24-well plate were transfected with FLAG-tagged LMP1 (500 ng) together with control, caspase-3, -7, or -8 siRNA (20 pmol). At 36 h after transfection, the cells were lysed, and TCL (1%) was blotted with anti-FLAG or anti-actin antibody. D, Densitometric quantification of the above results was also shown. The relative intensity of FLAG-LMP1 was normalized to the actin protein of the same sample. Results are representative of three independent experiments, and the error bars represent the SD. *, $p<0.05$. **, $p<0.01$.

FIGURE 3. L**MP1 expression induces caspase-3/8 activation in HeLa cells.** A, HeLa

cells in a 24 well plate were transfected with FLAG-tagged LMP1 or empty vector (500 ng). At 12 h after transfection, the cells were treated with DMSO or Z-VAD (10 μM) for an additional 12h. Cells were then lysed and assessed for caspase-3/7 activities (Top panel). The results are indicated as -fold induction of caspase activity from triplicate experiments, and the error bars represent S.D. *, $p<0.05$. **, $p<0.01$. An aliquot of total cell lysate (TCL) (1%) was blotted with anti-FLAG or anti-actin antibody (Bottom panel). B, HeLa cells in a 24 well plate were transfected with FLAG-tagged LMP1 or empty vector (500 ng). At 24 h after transfection, the cells were lysed, and then TCL (1%) was blotted with anti- caspase-3, anti-caspase-8, anti-FLAG or anti-actin antibody.

FIGURE 4. **Caspase inhibition enhances LMP1-mediated signals in HeLa cells.** A, HeLa cells in a 24-well plate were transfected with NF-κB-LUC (250 ng), together with FLAG-tagged LMP1 or empty vector. At 12 h after transfection, the cells were treated with DMSO or Z-VAD (10 μM) for an additional 12 h. And then the cells were harvested and assayed for luciferase activity. The results are indicated as -fold induction of luciferase activity from triplicate experiments, and the error bars represent S.D. **, $p<0.01$. An aliquot of total cell lysate (TCL) (1%) was blotted with an anti-FLAG or anti-actin antibody (IB). B, HeLa cells in a 24-well plate were transfected with

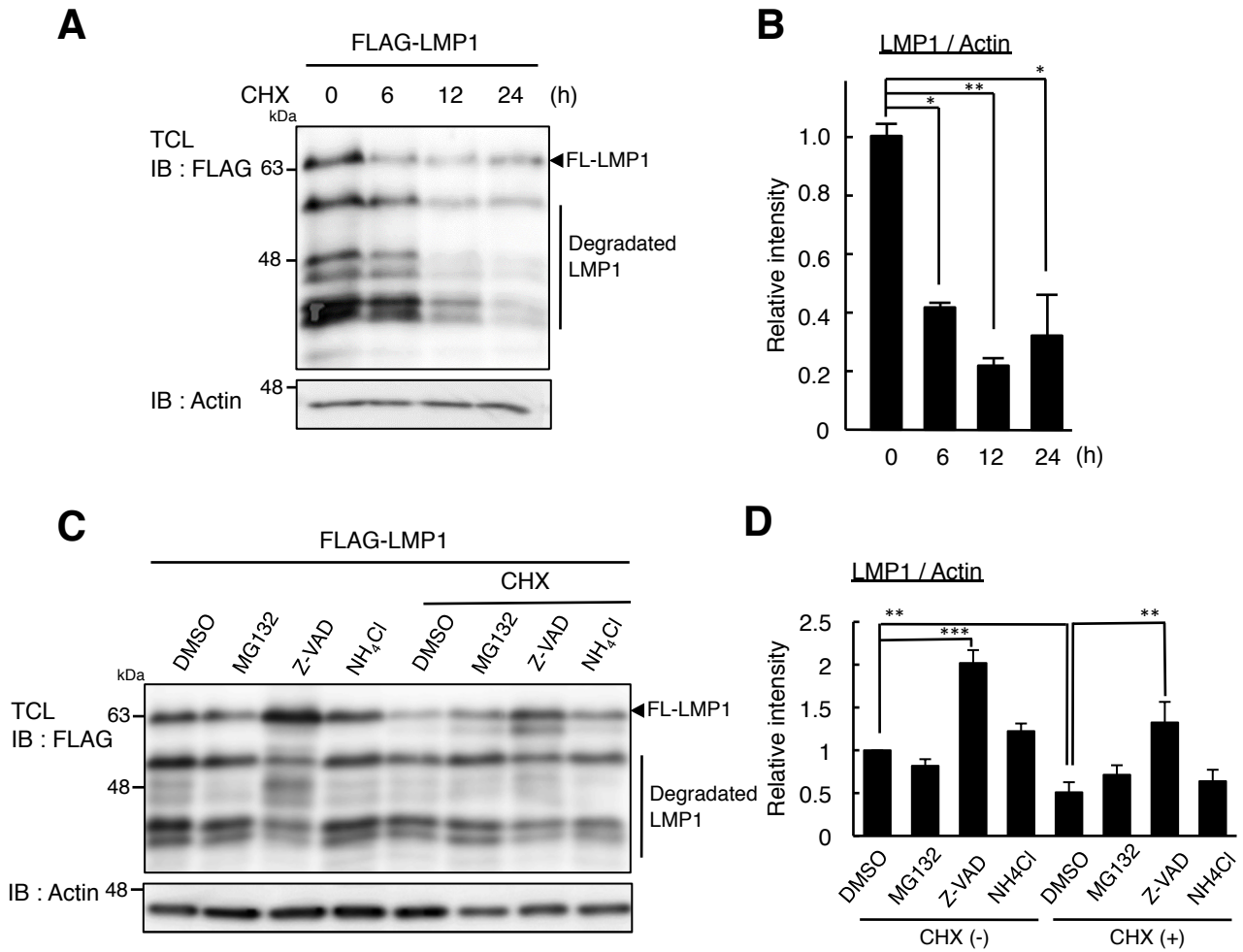
STAT3-LUC (250 ng), together with FLAG-tagged LMP1 or empty vector. At 12 h after transfection, the cells were treated with DMSO or Z-VAD (10 μM) for an additional 12 h. And then the cells were harvested and assayed for luciferase activity as described the above. **, $p<0.01$. TCL (1%) was blotted with an anti-FLAG or anti-actin antibody. C, HeLa cells in a 24-well plate were transfected with FLAG-tagged LMP1 or empty vector. At 12 h after transfection, the cells were treated with DMSO or Z-VAD (10 μM) for an additional 12 h. Total RNA samples isolated from these cells were also quantified by reverse transcription and quantitative real-time PCR analysis. Data represent the levels of *IL-6* mRNA normalized to that of a *ACTIN* internal control and are expressed relative to the value of control sample that was transfected with empty vector and treated with DMSO. The results are representative of three independent experiments, and the error bars represent the SD. **, $p<0.01$. D, HeLa cells in a 24-well plate were treated with DMSO or Z-VAD (10 μM) for 12 h, followed by TNF-α (10 ng/ml) stimulation for 30 min. Total RNA samples isolated from these cells were also quantified by reverse transcription and quantitative real-time PCR analysis as described the above.

FIGURE 5. **Caspase-3 regulates LMP1-mediated NF-κB activation in HeLa cells.** A,

HeLa cells in a 24 well plate were transfected with NF-κB-LUC (250 ng), together with FLAG-tagged LMP1 or empty vector. At 12 h after transfection, the cells were transfected with control or caspase-3 siRNA (20 pmol). 36 h later, the cells were harvested and assayed for luciferase activity as described the above. **, $p<0.01$. An aliquot of total cell lysate (TCL) (1%) was blotted with an anti-FLAG, anti-caspase-3 or anti-actin antibody. B, HeLa cells in a 24 well plate were transfected with FLAG-tagged LMP1 or empty vector. At 12 h after transfection, the cells were transfected with control or caspase-3 siRNA (20 pmol). 36 h later, the cells were harvested and total RNA samples isolated from these cells were also quantified by reverse transcription and quantitative real-time PCR analysis as described the above.

FIGURE 6. **Determination of caspase cleavage sites in LMP1 in HeLa cells.** A, C, E, Domain structure of LMP1 and its mutant fragments are shown schematically. B, D, F, G, HeLa cells in a 24 well plate were transfected with FLAG-tagged LMP1 or its mutants (500 ng). At 12 h after transfection, the cells were treated with DMSO or Z-VAD (10 μM) for an additional 12h. Cells were then lysed, and an aliquot of total cell lysate (TCL) (1%) was blotted with anti-FLAG or anti-actin antibody.

Figure 1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FLAG-LMP1
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kDa
TCL
IB : FLAG
63
48
FL-LMP1
Degradated LMP1
IB : Actin
48
B
LMP1 / Actin
Relative intensity
1.0
0.8
0.6
0.4
0.2
0
0 6 12 24 (h)
C
FLAG-LMP1
CHX
DMSO
MG132
Z-VAD
NH4Cl
DMSO
MG132
Z-VAD
NH4Cl
kDa
TCL
IB : FLAG
63
48
FL-LMP1
Degradated LMP1
IB : Actin
48
D
LMP1 / Actin
Relative intensity
2.5
2
1.5
1
0.5
0
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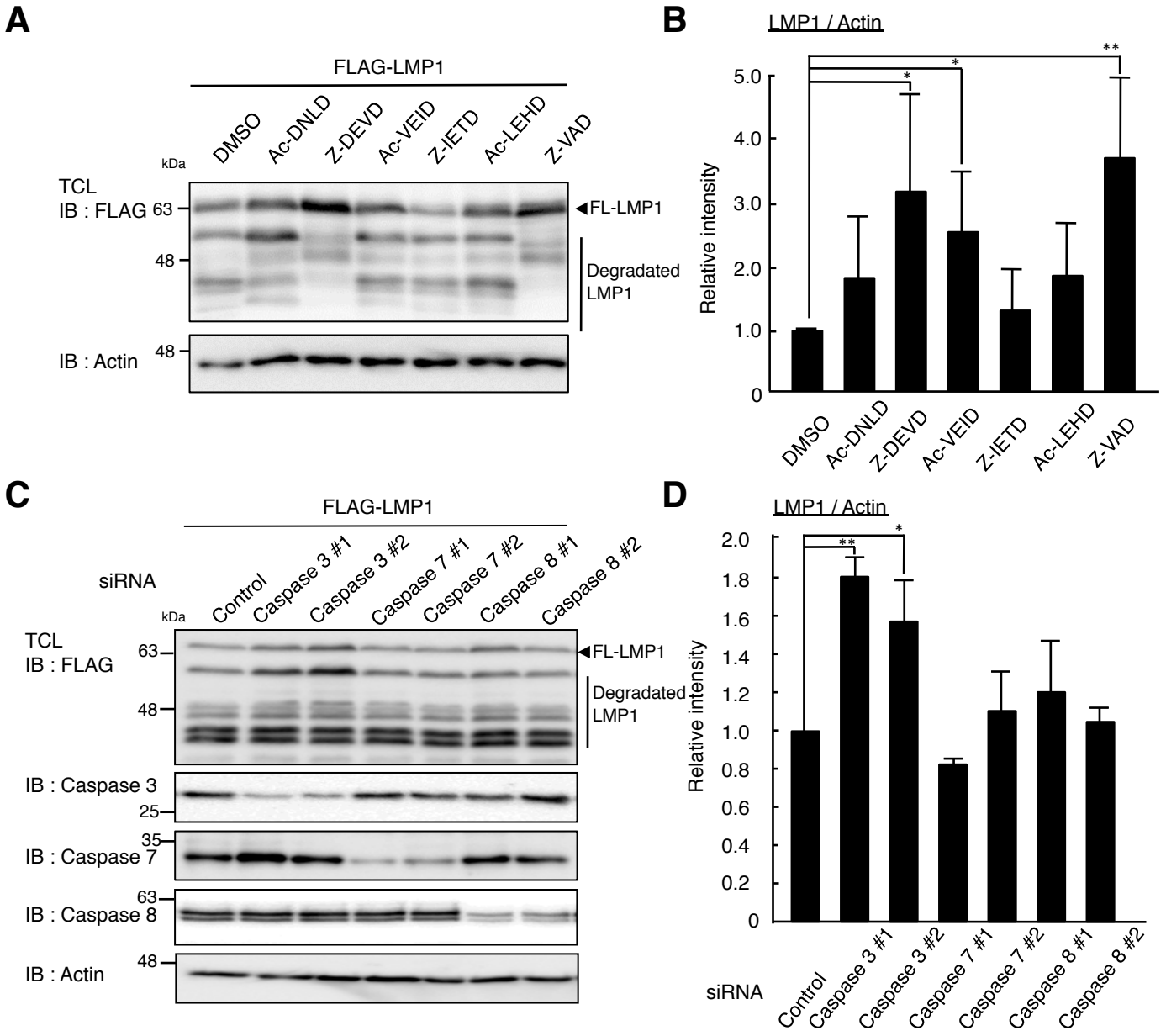
MG132
Z-VAD
NH4Cl
DMSO
MG132
Z-VAD
NH4Cl
CHX (-)
CHX (+)

Figure 2


A
FLAG-LMP1
DMSO
Ac-DNLD
Z-DEVD
Ac-VEID
Z-IETD
Ac-LEHD
Z-VAD
kDa
TCL
IB : FLAG
63
48
FL-LMP1
Degradated LMP1
IB : Actin
48
B
LMP1 / Actin
Relative intensity
5.0
4.0
3.0
2.0
1.0
0
*
*
**
DMSO
Ac-DNLD
Z-DEVD
Ac-VEID
Z-IETD
Ac-LEHD
Z-VAD
C
FLAG-LMP1
siRNA
Control
Caspase 3 #1
Caspase 3 #2
Caspase 7 #1
Caspase 7 #2
Caspase 8 #1
Caspase 8 #2
kDa
TCL
IB : FLAG
63
48
FL-LMP1
Degradated LMP1
IB : Caspase 3
25
IB : Caspase 7
35
IB : Caspase 8
63
IB : Actin
48
D
LMP1 / Actin
Relative intensity
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
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*
siRNA
Control
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Caspase 3 #2
Caspase 7 #1
Caspase 7 #2
Caspase 8 #1
Caspase 8 #2

Figure 3

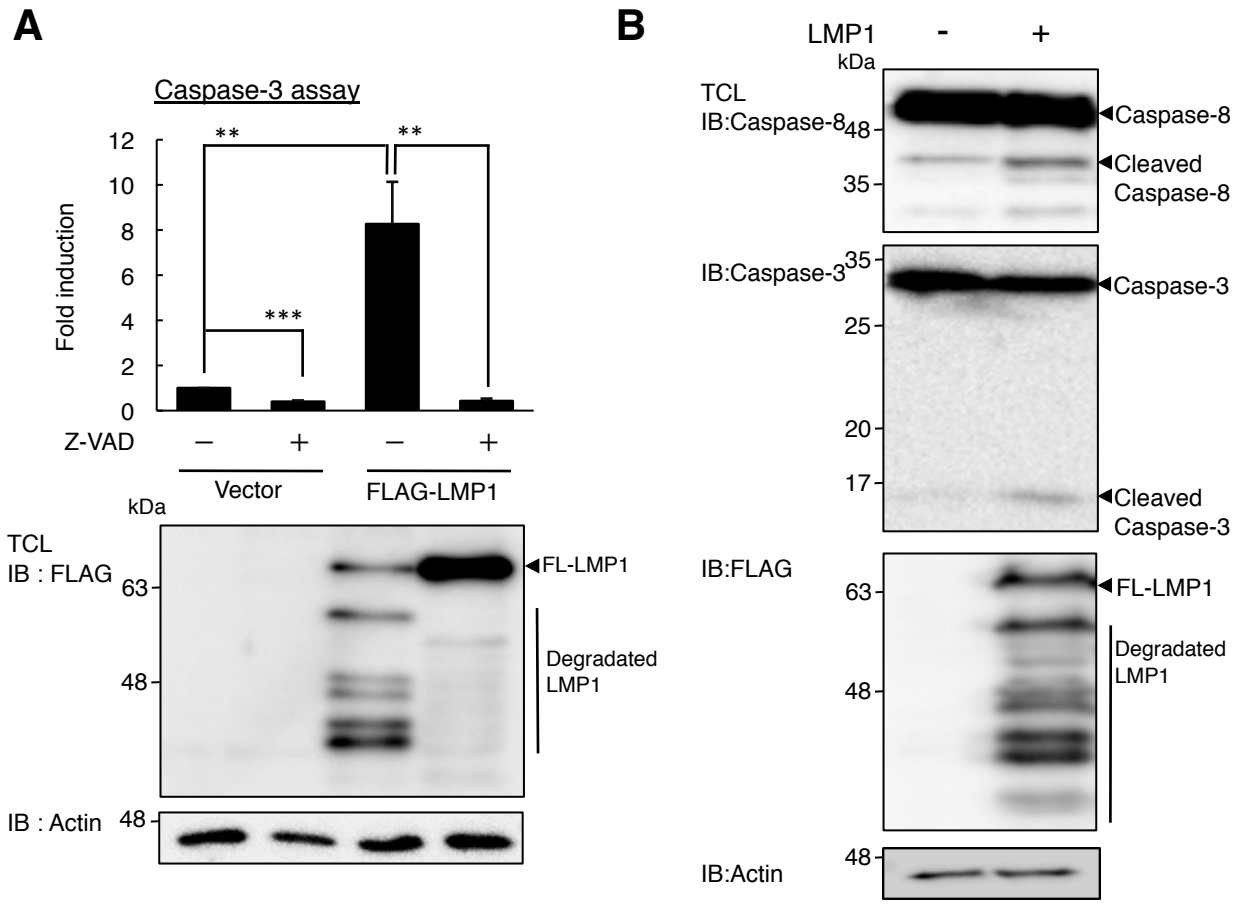

A
Caspase-3 assay
Fold induction
12
10
8
6
4
2
0
**
**
***
Z-VAD
–
+
–
+
Vector
FLAG-LMP1
kDa
TCL
IB : FLAG
63
48
FL-LMP1
Degradated
LMP1
IB : Actin
48
B
LMP1
-
+
kDa
TCL
IB:Caspase-8
48
35
Caspase-8
Cleaved
Caspase-8
IB:Caspase-3
35
25
20
17
Caspase-3
Cleaved
Caspase-3
IB:FLAG
63
48
FL-LMP1
Degradated
LMP1
IB:Actin
48

Figure 4

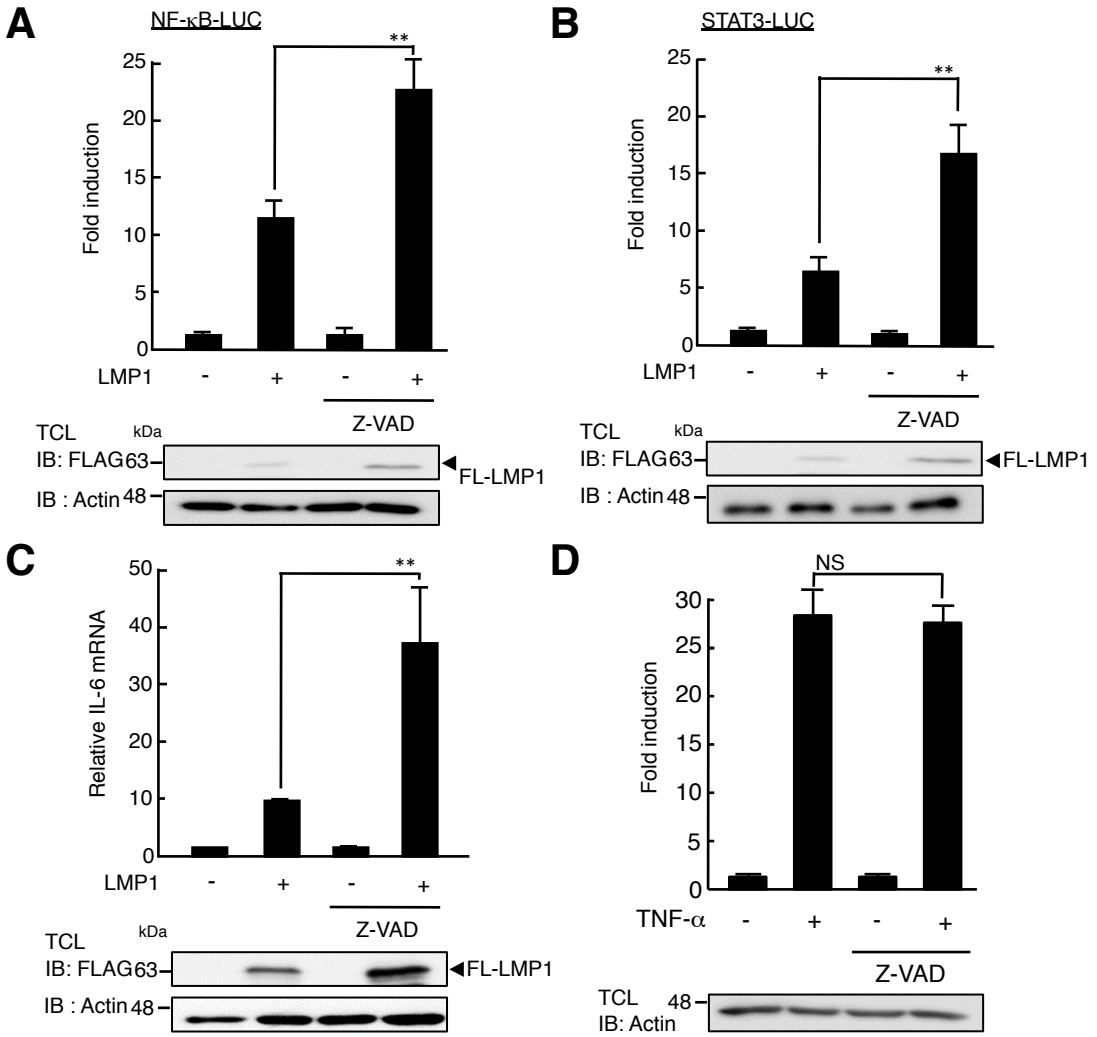

A
NF-κB-LUC
Fold induction
LMP1
Z-VAD
TCL
kDa
IB: FLAG
63
FL-LMP1
IB : Actin
48
B
STAT3-LUC
C
Relative IL-6 mRNA
D
NS
TNF-α
IB: Actin

Figure 5

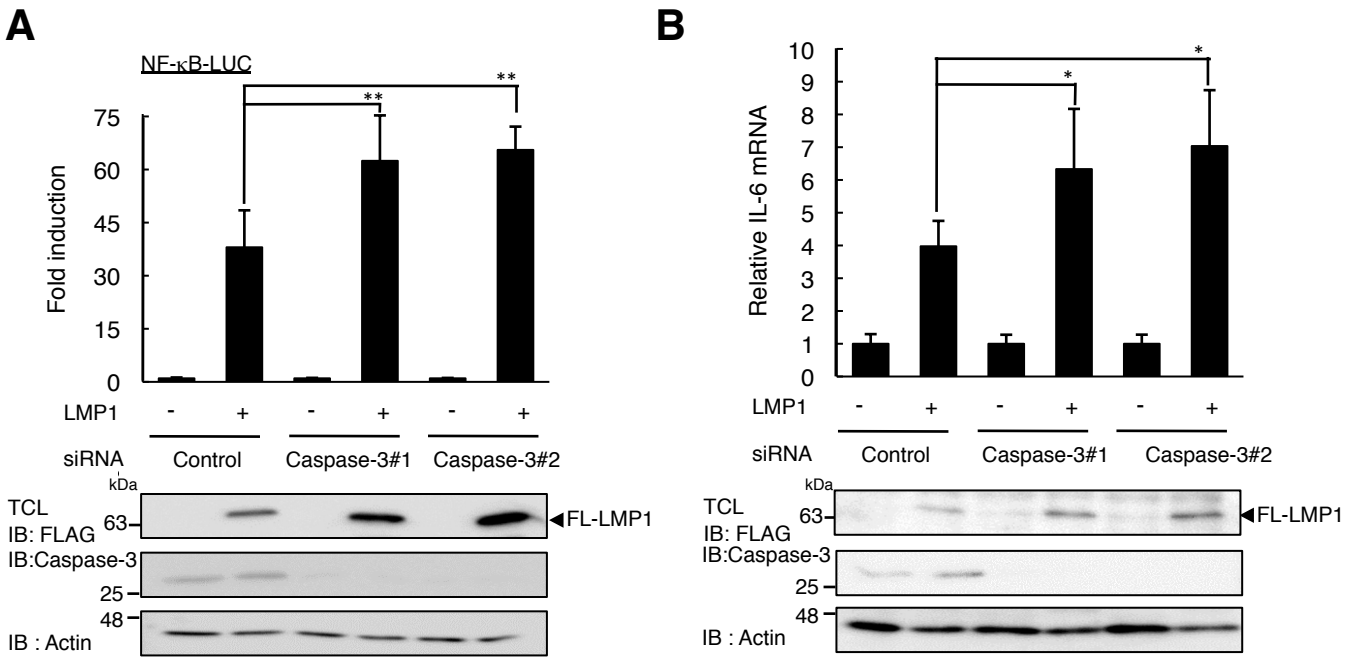
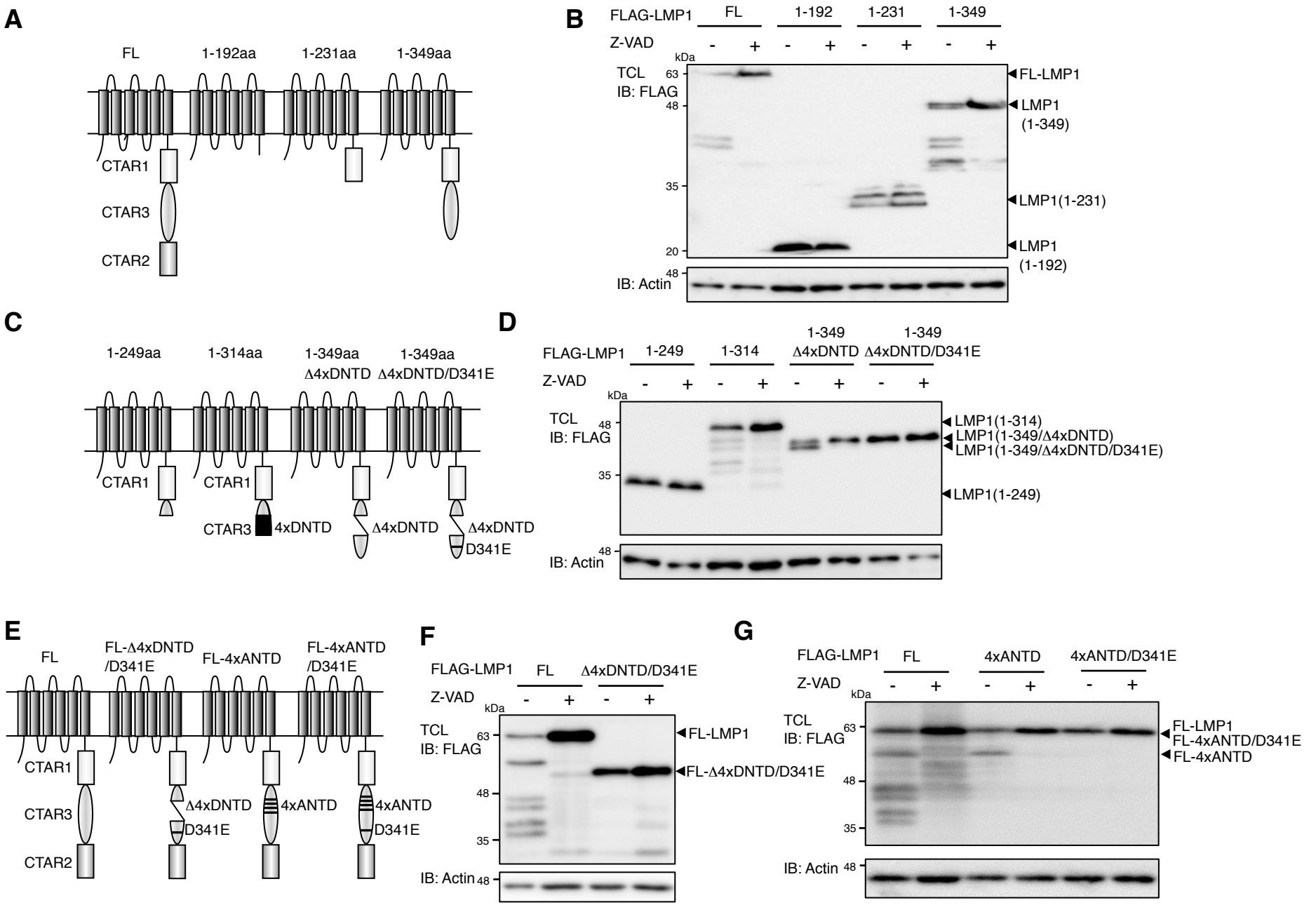

A
NF-κB-LUC
Fold induction
75
60
45
30
15
0
**
**
LMP1
-
+
-
+
-
+
siRNA
Control
Caspase-3#1
Caspase-3#2
kDa
TCL
IB: FLAG
63
FL-LMP1
IB:Caspase-3
25
48
IB : Actin
B
Relative IL-6 mRNA
10
9
8
7
6
5
4
3
2
1
0
*
*
LMP1
-
+
-
+
-
+
siRNA
Control
Caspase-3#1
Caspase-3#2
kDa
TCL
IB: FLAG
63
FL-LMP1
IB:Caspase-3
25
48
IB : Actin

Figure 6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FL
1-192aa
1-231aa
1-349aa
CTAR1
CTAR3
CTAR2
B
FLAG-LMP1
FL
1-192
1-231
1-349
Z-VAD
- + - + - + - +
kDa
TCL
IB: FLAG
63
48
35
20
FL-LMP1
LMP1 (1-349)
LMP1(1-231)
LMP1 (1-192)
IB: Actin
48
C
1-249aa
1-314aa
1-349aa Δ4xDNTD
1-349aa Δ4xDNTD/D341E
CTAR1
CTAR1
CTAR3
4xDNTD
Δ4xDNTD
Δ4xDNTD D341E
D
FLAG-LMP1
1-249
1-314
1-349 Δ4xDNTD
1-349 Δ4xDNTD/D341E
Z-VAD
- + - + - + - +
kDa
TCL
IB: FLAG
48
35
LMP1(1-314)
LMP1(1-349/Δ4xDNTD)
LMP1(1-349/Δ4xDNTD/D341E)
LMP1(1-249)
IB: Actin
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E
FL
FL-Δ4xDNTD /D341E
FL-4xANTD
FL-4xANTD /D341E
CTAR1
CTAR3
CTAR2
Δ4xDNTD
D341E
4xANTD
4xANTD
D341E
F
FLAG-LMP1
FL
Δ4xDNTD/D341E
Z-VAD
- + - +
kDa
TCL
IB: FLAG
63
48
35
FL-LMP1
FL-Δ4xDNTD/D341E
IB: Actin
48
G
FLAG-LMP1
FL
4xANTD
4xANTD/D341E
Z-VAD
- + - + - +
kDa
TCL
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63
48
35
FL-LMP1
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FL-4xANTD
IB: Actin
48