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EBV Can Protect Latently Infected B Cell Lymphomas from Death Receptor-Induced Apoptosis[1]

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The relationship between EBV infection and sensitivity to death receptor (DR)-induced apoptosis is poorly understood. Using EBV$^-$ and EBV$^+$ BJAB cells, we provide the first evidence that EBV can protect latently infected B cell lymphomas from apoptosis triggered through Fas or TRAIL receptors. Caspase 8 activation was impaired and cellular FLIP recruitment was enriched in death-inducing signaling complexes formed in EBV-infected BJAB cells relative to parent BJAB cells. Furthermore, latent membrane protein 1 expression alone could reduce caspase activation and confer partial resistance to DR apoptosis in BJAB cells. This protective effect was dependent on C-terminal activating region 2-driven NF-κB activation, which in turn up-regulated cellular FLIP expression in latent membrane protein 1$^+$ BJAB cells. Thus, the ability of latent EBV to block DR apoptosis may help to ensure the survival of host cells during B cell differentiation, and contribute to the development of B cell lymphomas, especially in immunocompromised individuals. *The Journal of Immunology,* **2006, 177: 3283–3293.**

Epstein-Barr virus is a B-lymphotropic γ-herpesvirus that benignly infects >90% of the human population (1). Following lytic infection, EBV expresses several latent genes that provide growth and survival signals to expand the pool of infected naive, tonsillar B cell hosts and ensure that a fraction of these cells survive through maturation (2–4). The virus persists for the lifetime of the host in a small pool of long-lived, resting memory B cells, where latent proteins are no longer expressed to avoid recognition by CTL. In immunocompromised individuals, however, latent gene expression can transform the infected B cell in the absence of CTL immunosurveillance, generating lymphomas associated with posttransplant lymphoproliferative disease (PTLD)[4] and AIDS.

Much work on the transforming capability of EBV has focused on latent membrane protein 1 (LMP1), an oncogene that is indispensable for EBV-mediated transformation of B cells (5). LMP1 is constitutively active and functionally mimics the CD40 molecule, providing survival and differentiation signals to B cells (6). Indeed, LMP1 is perhaps best known for its ability to up-regulate the expression of several antiapoptotic genes, primarily through induction of NF-κB activity.

Apoptosis, the best-characterized form of programmed cell death, can occur through two main pathways: intrinsic or extrinsic. Whereas the intrinsic pathway of apoptosis is activated by various stress-induced stimuli that destabilize mitochondria, the extrinsic pathway is triggered through dedicated death receptors (DRs) comprising a subset of the TNFR superfamily (7–9). Ligand-induced oligomerization of DRs stimulates the formation of death-inducing signaling complexes (DISCs), which serve as platforms for the cleavage and activation of apical caspases (caspases 8 and 10). Released, active caspase 8 can cleave downstream effector caspases such as caspase 3, which in turn cleave multiple death substrates leading to DNA fragmentation and eventual apoptosis. Fas/APO1, the best-characterized DR, is critical for normal lymphocyte homeostasis; mutations in the death domain of Fas result in uncontrolled lymphoproliferation and autoimmunity in both mice and humans (10, 11). The more recently characterized TRAIL/Apo2L can stimulate apoptosis through two functional DRs (DR4 and DR5), and may play a role in T cell homeostasis and immunosurveillance against tumors (12–14). Previous work has demonstrated that soluble TRAIL preferentially kills tumor cells while sparing primary cells, making it an attractive candidate for use in cancer therapy (8, 15).

EBV has evolved mechanisms for combating apoptosis following infection. The lytic cycle gene *BHRF1* encodes a homologue of Bcl-2 capable of inhibiting apoptosis induced by both intrinsic and extrinsic stimuli (16). Earlier studies established that EBV latent genes, LMP1 in particular, can protect B cells from apoptosis in vitro following serum withdrawal (17, 18). The relationship between EBV latency and DR-mediated apoptosis, however, remains undefined. Previous work from our laboratory and others suggested that EBV-infected lymphoblastoid cell lines (LCL) have differential sensitivity to Fas-induced apoptosis (19–21). We have noted that EBV$^+$ LCL derived from PTLD patients also demonstrate universal resistance to TRAIL-induced apoptosis (22).

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[4] Abbreviations used in this paper: PTLD, posttransplant lymphoproliferative disease; LMP, latent membrane protein; BL, Burkitt's lymphoma; cFLIP, cellular FLIP; $cFLIP_L$, long isoform of cFLIP; $cFLIP_S$, short isoform of cFLIP; CTAR, C-terminal activating region; DISC, death-inducing signaling complex; DR, death receptor; EBNA, EBV nuclear Ag; EGFP, enhanced GFP; FADD, Fas-associated death domain protein; FasL, Fas ligand; IP, immunoprecipitation; NGFR, nerve growth factor receptor; PI, propidium iodide; TRAF, TNFR-associated factor; WB, Western blot; WT, wild type.

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In this study, we investigated whether latent EBV infection can directly modulate apoptosis signaled through DRs, by using the Fas ligand (FasL)/TRAIL-sensitive B cell lymphoma line BJAB and its B95.8 EBV-infected counterpart, BJAB_B95. We demonstrate that BJAB_B95 cells are completely resistant to apoptosis triggered through Fas and DR4/DR5. Furthermore, we show that DR signaling is blocked at the DISC in EBV-infected BJAB cells, where increased cellular FLIP (cFLIP) may act to prevent sufficient activation of caspase 8. Finally, we show that LMP1 expression alone can confer partial protection against DR apoptosis in BJAB transfectants through the activation of NF-κB, which drives increased expression of cFLIP. These results provide direct evidence that EBV itself can protect latently infected B cell lymphomas from DR-dependent apoptosis, mediated in part by the anti-apoptotic function of LMP1.

Materials and Methods

Cells, plasmids, and reagents

The B cell lymphoma BJAB and its EBV-infected counterpart BJAB_B95 were provided by E. Kieff and E. Cahir-McFarland (Harvard Medical School, Boston, MA). Jurkat (E6.1) and SKW6.4 cells were acquired from American Type Culture Collection. All human cell lines and derived clones were maintained in RPMI 1640 + 10% heat-inactivated FBS (Mediatech) + 50 U/ml penicillin-streptomycin (Invitrogen Life Technologies). Fresh tumor tissue was obtained from the National Disease Research Interchange and Stanford University School of Medicine. For fresh tumor analysis, cells were isolated from biopsied tissue by Ficoll gradient centrifugation. The following pSG5-Flag-LMP1 expression plasmids were provided by K. Izumi (University of Texas Health Science Center, San Antonio, TX): Flag-LMP1 (wild type (WT)), Flag-LMP1 (1-231), Flag-LMP1 (1-231 PQ-AA), Flag-LMP1 (Δ187-351), and Flag-LMP1 (Δ187-351 ID). pSG5-EBV nuclear Ag (EBNA)2 was a gift from B. Zhao (Harvard Medical School, Boston, MA). Empty pSuper vector (a gift from R. Agami, Netherlands Cancer Institute, Amsterdam, The Netherlands) was modified with an expression cassette containing the SV40 promoter coupled to enhanced GFP (EGFP) and used as a reporter plasmid in LMP1 cotransfections. The nerve growth factor receptor (NGFR).LMP1 expression vector was provided by W. Hammerschmidt (GSF-National Research Center for Environment and Health, Munich, Germany). The NGFR.LMP1 chimera was modified to delete aa 272–276 of NGFR (FKRWN) and the 3-aa linker region (RGI), and add aa 185–189 of LMP1 (HGQRH). The modified construct was then subcloned into pcDNA3 using *Hin*dIII and *Xba*I. Pharmacological inhibitors of JNK (JNK inhibitor I), p38 MAPK (SB203580), ERK (PD98059), PI3K (LY294002), and NF-κB (BAY11-7082) were purchased from Calbiochem. Unless specified, all other reagents were obtained from Sigma-Aldrich.

Abs and Western blotting (WB)

The following primary Abs were used for WB: anti-Fas-associated death domain protein (FADD) (Zymed Laboratories); anti-caspase 8, anti-caspase 3, and anti-IκB (BD Pharmingen); anti-FLIP (NF6), anti-DR4, and anti-DR5 (Alexis); anti-Fas and anti-TNFR-associated factor (TRAF)1 (Santa Cruz Biotechnology); anti-LMP1 and anti-EBNA2 (DakoCytomation); and anti-β-actin (AC15) and anti-Flag (M2, M5). For secondary reagents we used the following: HRP-conjugated donkey anti-mouse or goat anti-rabbit Ig (Jackson ImmunoResearch Laboratories), and HRP-conjugated goat anti-mouse IgG1 (x56; BD Pharmingen). Stimulated cells were lysed in 50–100 μl of lysis buffer (50 mM Tris (pH 7.4), 1% Nonidet P-40, 0.5% deoxycholate, 150 mM NaCl, 0.5 mM EDTA, 2 mM PMSF, 5 μg/ml each of aprotinin and leupeptin, and 0.5 μg/ml pepstatin A) on ice for 30 min. Insoluble material was removed by centrifugation, and lysate protein concentration was determined using the D_C protein assay (Bio-Rad). Lysates (30–40 μg) were separated by 12% SDS-PAGE and transferred to nitrocellulose. After blocking in 5% milk in TBS/0.1% Tween 20 (TBST), blots were probed for 2 h with primary Abs diluted in 3% BSA/TBST, washed three times for 5 min in TBST, and then incubated for 1 h with diluted HRP-conjugated secondary Ab. Blots were washed again and developed with ECL (Amersham Biosciences).

Apoptosis assays

Where indicated, cells (5×10^5 cells/ml) were cultured for 18–24 h at 37°C in the presence of the following reagents: 100 ng/ml soluble, homotrimeric rTRAIL (a gift from A. Ashkenazi, Genentech, South San Francisco, CA), 200 ng/ml mouse anti-human Fas (clone CH-11; Coulter-Immunotech), 50 μM etoposide, or 2 μM staurosporine. Percentage of apoptotic cells with hypodiploid content was determined by cell cycle analysis using PI staining and flow cytometry, as previously described (21). Alternatively, annexin V-EGFP or annexin V-PE Apoptosis Detection Kits (BioVision Research Products) were used, according to the manufacturer's protocol. Flow cytometry was performed on a FACScan flow cytometer using CellQuest software (BD Biosciences). Caspase enzymatic activity was quantitated in stimulated lysates using caspase 8 or caspase 3 Colorimetric Assay Kits (BioVision Research Products), according to the manufacturer's protocol.

Flow cytometry

For Fas cell surface staining, cells (1×10^6) were washed once in cold FACS buffer (1× PBS, 1% FBS, 0.1% sodium azide) and incubated for 30 min on ice with 10 μg/ml PE-conjugated mAb to human Fas (BD Pharmingen), or a PE-conjugated isotype-matched control Ab in 100 μl of FACS buffer. To determine TRAIL receptor expression, cells were incubated as above with 6 μg/ml anti-DR4, anti-DR5, anti-DcR1, or anti-DcR2 mAbs (provided by Genentech), washed twice in FACS buffer, then incubated with 1/50 dilution of PE-conjugated goat anti-mouse $F(ab')_2$ secondary reagent (DakoCytomation). Fresh lymphoma cells were stained with FITC-conjugated anti-CD19 (Caltag Laboratories) or PE-conjugated anti-CD3 (BD Pharmingen). NGFR-LMP1 was detected on BJAB clones using 2 μl of biotinylated anti-NGFR (Chromaprobe) and 1 μl of streptavidin-PE (BD Pharmingen). For intracellular detection of LMP1, cells were fixed 10 min in CytoFix solution (BD Pharmingen), permeabilized 15 min in FACS buffer + 1% saponin, and stained with 20 μl of PE-conjugated anti-LMP1 mAb (S12; BD Pharmingen) or isotype-matched control Ab. In each case, stained cells were washed twice in FACS buffer and analyzed by flow cytometry.

Immunoprecipitation (IP) and DISC analysis

Cells (10^7/IP) were stimulated for 15 min with 1 μg of Flag-TRAIL (Alexis) + 2 μg of M2, 3 μg of anti-Fas mAb APO1-3 (Kamiya Biomedical), or left untreated. For Fas DISC IPs, 1.2×10^7 BJAB_B95 cells were used to ensure precipitation of equal amounts of Fas protein vs BJAB. To analyze the long isoform of cFLIP ($cFLIP_L$) recruitment to the TRAIL-DISC, we used 5 μg of biotinylated soluble TRAIL instead. TRAIL was biotinylated using an EZ-link Sulfo-NHS-LC-Biotinylation Kit (Pierce). After quenching in cold PBS, cells were lysed for 15 min on ice in 1 ml of DISC lysis buffer (30 mM Tris (pH 7.4), 1% Triton X-100, 10% glycerol, 150 mM NaCl, 1 mM PMSF, 5 μg/ml each of aprotinin and leupeptin, and 0.5 μg/ml pepstatin A). Insoluble material was removed by centrifugation, and DISCs were precipitated using 15 μl of GammaBind Sepharose or streptavidin Sepharose beads (Amersham Biosciences) for 4–16 h at 4°C. Beads were washed four times in lysis buffer and resuspended in 30 μl of 2× reducing SDS-sample buffer. Samples were boiled for 3 min, separated by 12% SDS-PAGE, and subjected to WB analysis, as described above. For quantitation of DISC-associated caspase 8 activity, cells were stimulated for designated times and IPs were performed as above. Washed beads were transferred to white-walled 96-well plates in 100 μl of PBS and mixed with 100 μl of Caspase 8-Glo substrate (Promega) for 30 min at room temperature. Luminescence was detected using a SpectraMax Gemini EM microplate reader (Molecular Devices).

Immunofluorescence

For intracellular detection of EBNA2 or LMP1, cells (1×10^6) were washed in PBS and adhered to poly(L-lysine)-coated coverslips. Cells were then fixed in 3.7% formaldehyde for 10 min and permeabilized in 0.1% Triton X-100 for 5 min, washing three times in 1 ml of PBS after each incubation. After blocking in 1% BSA/PBS to eliminate nonspecific binding, cells were incubated with mAbs specific for EBNA2, LMP1, FLAG, or an IgG1 isotype-matched control Ab for 30 min at 37°C. Following three washes in PBS, cells were blocked as above and stained with a PE-conjugated rabbit anti-mouse $F(ab')_2$ secondary reagent (DakoCytomation) for 30 min at 37°C. Cells were washed six times in PBS and visualized by fluorescent microscopy. To visualize nuclei, stimulated cells were stained in 4 μM Hoechst 33342 or 4′,6′-diamidino-2-phenylindole (Molecular Probes), washed three times in PBS, and visualized as above.

Transfection of LMP1 constructs

For transient expression, 5×10^6 BJAB cells were transfected by electroporation using a Bio-Rad GenePulser at 210 V, 960 μF at room temperature in 0.4 ml of RPMI 1640 + 10% FBS. In addition to LMP1 or EBNA2 expression constructs (25 μg per transfection), all cells were cotransfected

with 10 μg of pSuper.EGFP, which served as an EGFP reporter plasmid. Transfection efficiency ranged from 30 to 60%. Cells were allowed to recover in complete medium for 48 h before analysis. In some cases, $EGFP^{high}$ cells were sorted (>95% purity) at the Stanford Shared FACS Facility. For generation of stable BJAB-NGFR.LMP1 clones, cells were electroporated as above with 15 μg of pCDNA3.NGFR.LMP1. After 48 h, cells were plated in 96-well plates (2.5×10^3/well) in complete medium + 3 mg/ml G418 (Invitrogen Life Technologies). Selected clones were expanded and screened for NGFR.LMP1 expression by flow cytometry.

Results

EBV-infected BJAB cells are resistant to FasL/TRAIL-induced apoptosis

To investigate how EBV infection might directly modulate DR apoptosis in B cell lymphomas, we used the EBV-negative B cell lymphoma line BJAB and its EBV-infected counterpart, BJAB_B95. BJAB cells are known to be highly sensitive to apoptosis induced through both Fas and the TRAIL receptors DR4 and DR5 (23); however, the susceptibility of BJAB_B95 cells to DR-induced apoptosis has not been reported. BJAB_B95 cells demonstrated an EBV infection status typical of type III latency, as evidenced by simultaneous expression of LMP1 and EBNA2, comparable to other type III EBV-infected cell lines (Fig. 1, *A* and *B*). Hence, the type III viral latency status of EBV in BJAB_B95 cells is representative of that frequently observed in EBV^+ non-Hodgkin's lymphomas, particularly PTLD.

Treatment with either soluble, homotrimeric rTRAIL, or the agonistic anti-Fas mAb CH-11 efficiently induced apoptosis in BJAB cells, indicated by increased annexin V binding (Fig. 1*C*). In contrast, BJAB_B95 cells were almost completely annexin V^- upon stimulation with TRAIL or anti-Fas, suggesting these cells are resistant to DR stimuli. To confirm this difference in DR-mediated apoptosis sensitivity between BJAB and BJAB_B95, we performed cell cycle analysis using propidium iodide (PI) to distinguish apoptotic cells with hypodiploid DNA content. Whereas both TRAIL and anti-Fas induced a substantial increase in the

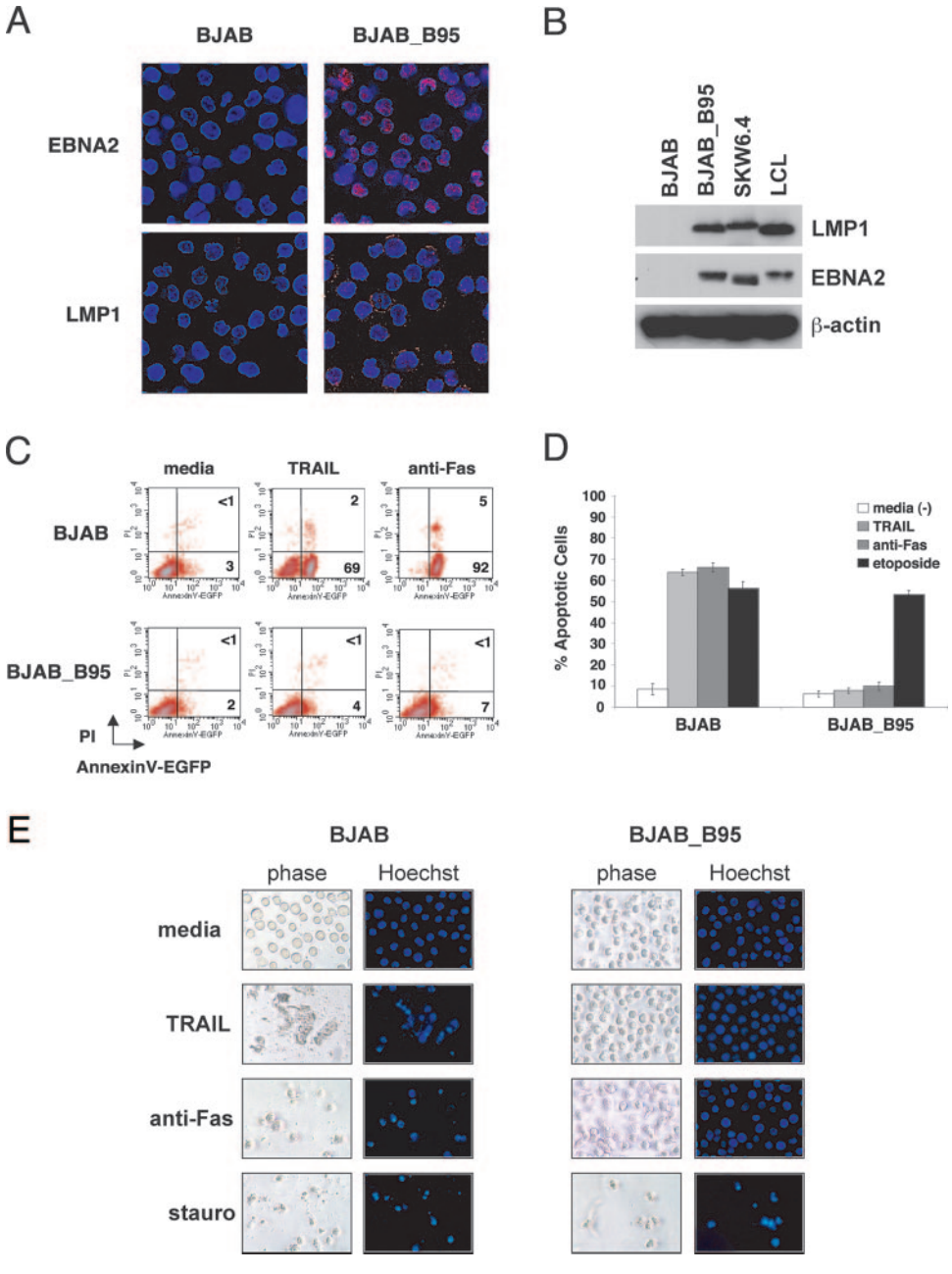

FIGURE 1. EBV-infected BJAB cells are resistant to DR apoptosis. *A*, BJAB_B95 cells exhibit type III EBV latency by expressing LMP1 and EBNA2. Normal EBV^- BJAB cells, or BJAB cells infected with the B95.8 prototype strain of EBV (BJAB_B95) stained intracellularly using mAbs against EBNA2 or LMP1 plus 4′,6′-diamidino-2-phenylindole and analyzed by fluorescent microscopy. Isotype-matched control Ab staining was negative (data not shown). *B*, WB analysis of LMP1 and EBNA2 expression in BJAB, BJAB_B95, SKW6.4, and an LCL generated by in vitro infection with the B95.8 strain of EBV. Proteins are indicated at *right*; β-actin served as a loading control. *C*, Cells were treated for 18 h with medium alone, soluble rTRAIL (100 ng/ml), anti-Fas mAb CH-11 (200 ng/ml), or 50 μM etoposide. Apoptosis was quantified using annexin V-EGFP + PI. The percentage of annexin V^+ apoptotic cells is indicated for the *lower* and *upper right quadrants*. Data are representative of three independent experiments. *D*, Cells were treated as above in the presence of medium alone (□), TRAIL (light gray), anti-Fas mAb CH-11 (dark gray), or etoposide (■). Following treatment, cells were stained with PI and analyzed by flow cytometry; apoptosis was measured as the percentage of cells with hypodiploid content. Data represent mean ± SEM of three separate experiments. *E*, To visualize nuclei, treated cells were incubated in 4 μM Hoechst 33342, washed, and analyzed by fluorescent microscopy.

percentage of apoptotic BJAB cells, very few BJAB_B95 cells underwent apoptosis following treatment (Fig. 1*D*). However, both BJAB and BJAB_B95 cells showed similar sensitivity to apoptosis stimulated by etoposide, indicating there is no global apoptosis defect in BJAB_B95. Finally, Hoechst nuclear staining revealed that unlike BJAB, BJAB_B95 cells stimulated with TRAIL or anti-Fas did not show significant chromatin condensation or blebbing characteristic of apoptotic cell death (Fig. 1*E*). Hence, we demonstrate using multiple methods for detection of apoptosis that BJAB cells harboring a latent EBV infection show marked resistance to DR stimuli.

Impaired caspase activation in EBV-infected BJAB cells

To investigate the nature of the biochemical defect in DR signaling in BJAB_B95 cells, we first assessed caspase activation following ligation of Fas or DR4/DR5. Whole cell lysates prepared from cells treated with soluble TRAIL or anti-Fas for 0, 2, or 4 h were analyzed by WB with Abs capable of recognizing both the zymogen and cleaved forms of caspase 8 and caspase 3. Jurkat and SKW6.4 cells were used as positive controls for TRAIL- and anti-Fas-mediated caspase activation, respectively. Cleavage products of both caspase 8 (p43/41, p26/23) and caspase 3 (p19/17/15) were readily detected in BJAB cells at 2 h and increased by 4 h when stimulated with either TRAIL or anti-Fas (Fig. 2*A*). In comparison, virtually no caspase cleavage could be seen in BJAB_B95 cells treated with TRAIL and anti-Fas, suggesting DR signaling is blocked in these cells before apical caspase activation. WB profiling of downstream Bcl-2 family proteins (i.e., Bcl-2, Mcl-1, Bax, and Bim) in BJAB vs BJAB_B95 revealed no obvious correlation between expression pattern and apoptosis sensitivity (data not shown).

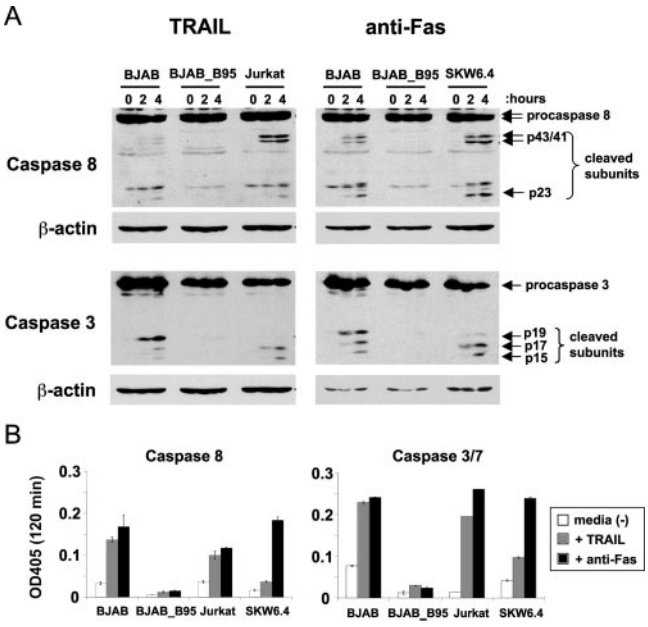

FIGURE 2. Caspase activation is impaired in EBV-infected BJAB cells. *A*, Cells were stimulated for 0, 2, or 4 h with either 100 ng/ml TRAIL or 200 ng/ml anti-Fas mAb CH-11 and lysed. WB analysis of caspase 8 and caspase 3 cleavage was conducted. Proforms and cleavage products of each caspase are indicated by arrows. Blots shown are representative of three separate experiments. *B*, Lysates (0- or 4-h stimulation) were incubated with IETD-pNA (caspase 8 substrate) or DEVD-pNA (caspase 3/7 substrate), and colorimetric change was recorded over 2 h as a measure of enzymatic activity. Data are presented as the difference of readings taken at 120 min minus background readings taken at 5 min in duplicate.

Because the extent of caspase cleavage does not always reflect the amount of enzymatic activity, we quantitated caspase activity using colorimetric assays. Cells were treated as described for 0 or 4 h, and their lysates were incubated with tetrapeptide substrates specific for caspase 8 or caspases 3 and 7. As shown in Fig. 2*B*, caspase enzymatic activity was severely impaired in both TRAIL- and anti-Fas-treated BJAB_B95 cells in comparison with BJAB cells. In summation, both TRAIL-R and Fas signaling are obstructed in EBV-infected BJAB cells before caspase 8 activation, thereby precluding downstream caspase activation and eventual apoptosis.

Defective caspase 8 activation is localized to the DISC in EBV-infected BJAB cells

Next, we assessed membrane expression of Fas and TRAIL receptors by flow cytometry. The DR expression profile was similar between BJAB and BJAB_B95 cells (Fig. 3*A*), ruling out the possibility that EBV-infected BJAB cells were resistant to FasL/TRAIL-mediated apoptosis due to differences in DR expression on the cell surface.

Given that caspase 8 activation could not be detected in BJAB_B95 cells after DR engagement, we hypothesized that DISC formation might be defective in these cells compared with the parent BJAB cell line. To test this, we examined recruitment of FADD and caspase 8 to DISCs in BJAB and BJAB_B95 cells following DR stimulation and IP. Interestingly, DISC formation appeared to be comparable in BJAB and BJAB_B95 cells, with only slightly less FADD and procaspase 8 (p55/53) present in Fas or TRAIL receptor-associated DISCs isolated from BJAB_B95 (Fig. 3*B*). Despite relatively intact DISC assembly, caspase 8 cleavage was somewhat reduced within the DISCs of BJAB_B95 cells. This was particularly evident in the Fas-DISC, where the second cleavage event represented by p26/23 subunit generation was not detected. To directly test the possibility that DISCs are less functional in BJAB_B95 cells, we quantitated specific DISC-associated caspase 8 activity (24). DISC IPs from BJAB and BJAB_B95 cells were incubated with a synthetic caspase 8-specific substrate (z-LETD-aminoluciferin), whose cleavage was detected by an increase in luminescence. Caspase 8 activity was easily detected in DISCs derived from BJAB cells, peaking at 10–30 min of DR stimulation time (Fig. 3*C*). However, DISC-associated caspase 8 activity was reduced in BJAB_B95 cells at every time point, suggesting caspase 8 turnover is decreased in both Fas and TRAIL-R DISCs derived from stimulated BJAB_B95 cells. Taken together, these data pinpoint DR-mediated apoptosis resistance in EBV-infected BJAB cells to a defect in DISC-associated caspase 8 activation, explaining the absence of active caspase 8 in the cytoplasm of these cells following DR stimulation.

Based on these results, we explored the involvement of cFLIP, a catalytically inactive homologue of caspase 8 capable of binding to the DISC and inhibiting caspase 8 activation (24). Indeed, endogenous levels of both $cFLIP_L$ and short isoform of cFLIP ($cFLIP_S$) were slightly elevated (~1.5-fold) in BJAB_B95 cells compared with parent BJAB cells (data not shown). Strikingly, recruitment of cFLIP was increased 2- to 3-fold in both Fas- and TRAIL-R-associated DISC IPs from BJAB_B95 cells vs BJAB cells (Fig. 3*D*). Like caspase 8, we detected cleavage of $cFLIP_L$ within the DISC to a 43-kDa product, consistent with previous reports (25). Furthermore, $cFLIP_S$ was only detectable in TRAIL-R-induced DISCs from BJAB_B95 cells. These data suggest that recruitment of more cFLIP to the BJAB_B95 DISC may act to impede caspase 8 activation. We are careful to note that the modest increase in

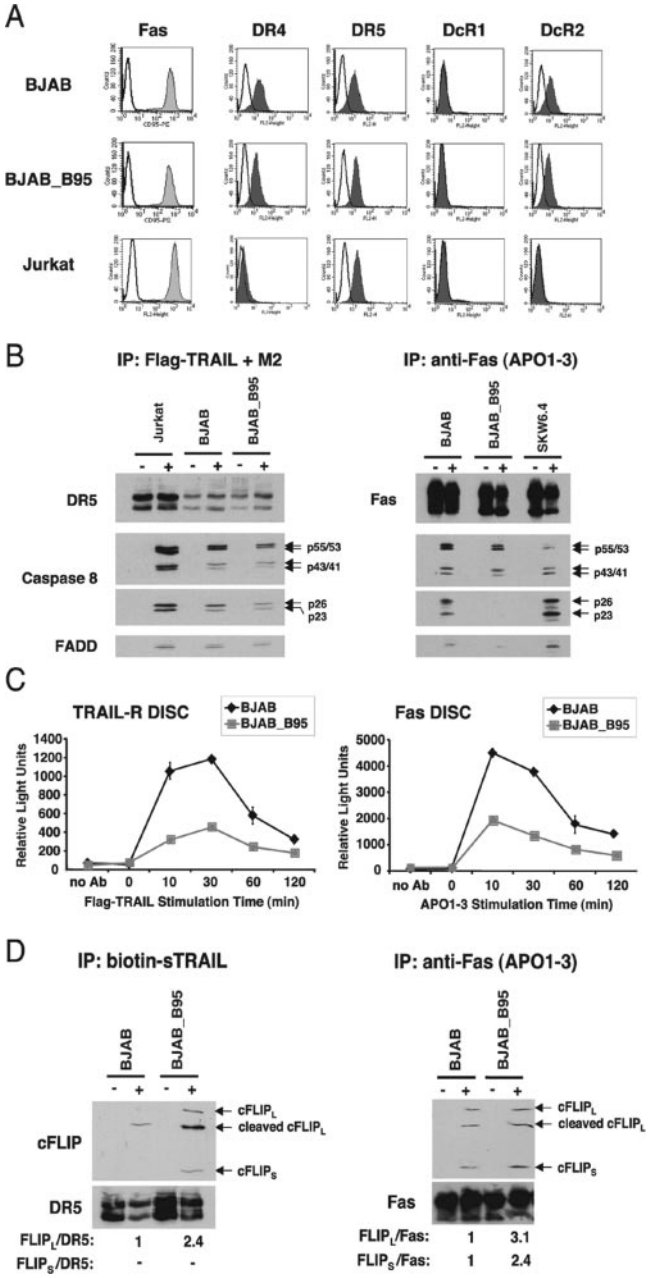

FIGURE 3. Caspase 8 activity is reduced and FLIP recruitment is enhanced in the DISC of EBV-infected BJAB cells. *A*, Surface expression of DRs. Cells were stained with PE-conjugated anti-Fas mAb, or anti-TRAIL-R mAbs followed by PE-conjugated secondary Ab, and analyzed by flow cytometry. Specific DR staining (filled histograms) is compared with staining with appropriate isotype-matched control Abs (open histograms). *B*, IP of Fas and TRAIL-R DISC. DR stimuli (indicated at the *top*) were added either after lysis (−) or 20 min before cell lysis (+). DISC-associated proteins were visualized by WB. Arrows at *right* indicate proforms (p55/53) and cleavage products (p43/41, p26/23) of caspase 8. Jurkat and SKW6.4 cells served as positive controls for TRAIL-DISC or Fas-DISC formation, respectively. *C*, Measurement of DISC-associated caspase 8 activity. Cells were stimulated as above in duplicate for indicated times and subjected to DISC IP. Noneluted beads were incubated with the caspase 8-specific luminescent substrate z-LETD-aminoluciferin. Data represent mean ± SEM of duplicate IPs for each time point. *D*, DISC IPs were performed as above and analyzed by WB. To avoid cross-reaction of anti-Flag IgH and $cFLIP_L$, TRAIL-DISC formation was instead stimulated with biotinylated TRAIL. Arrows at *right* mark the position of both isoforms of cFLIP, including the cleaved portion of $cFLIP_L$. Spot densitometry analysis of the ratio of cFLIP isoforms to DR protein (normalized to BJAB) is indicated at the *bottom* of designated lanes.

total cFLIP expression in BJAB_B95 cannot completely account for the enhanced recruitment of cFLIP to DISCs in these cells, suggesting additional mechanisms for directing FLIP to DRs may be at work. Nevertheless, our data are consistent with a role for cFLIP in EBV-mediated protection from FasL- and TRAIL-driven apoptosis in latently infected BJAB cells.

LMP1 expression alone partially protects BJAB cells from DR apoptosis

Next, we sought to determine whether LMP1 expression is responsible for resistance to DR-mediated apoptosis in infected B cells. Previous studies have shown that LMP1 signaling can up-regulate several antiapoptotic proteins (Bcl-2, Mcl-1, Bfl-1, A20, and cellular inhibitor of apoptosis protein 2) via NF-κB activation, and subsequently protect transfected cells from stress-related apoptotic stimuli (18, 26–30). To examine whether this protective function could extend to DR-specific apoptosis, we cotransfected BJAB cells with WT LMP1 and EGFP to specifically track DR apoptosis sensitivity in $EGFP^+$ cells. At 48 h posttransfection with pSG5-LMP1, immunofluorescence analysis identified high expression of LMP1 in $EGFP^+$ cells vs cells transfected with empty pSG5 (Fig. 4*A*). LMP1 could also be detected in BJAB transfectants by intracellular flow cytometry using a mAb recognizing the LMP1 cytoplasmic tail (Fig. 4*B*). The amount of LMP1 expression correlated linearly with the amount of EGFP found in the same cells (Fig. 4*B*, *lower left panel*), allowing us to use EGFP as a surrogate marker for the level of LMP1 expression. Robust expression of LMP1 could also be detected by WB in lysates made from these transfectants (Fig. 4*C*). Moreover, a marked increase in TRAF1, known to be directly up-regulated by LMP1 signaling, confirmed that LMP1 was functional in transfected BJAB cells.

To test the effect of LMP1 expression on DR apoptosis sensitivity, we treated BJAB transfectants with TRAIL or anti-Fas and assayed for apoptosis using annexin V-PE staining. In each case, we noted a small population of $EGFP^{high}$ cells that remained annexin V^-, suggesting high LMP1 expression could mediate apoptosis resistance in these cells (data not shown). To focus on this cell population specifically, we sorted $EGFP^{high}$ cells by FACS and conducted the same apoptosis assay described above. Higher levels of background cell death were evident in pSG5-LMP1 transfectants over empty vector controls (26 vs 9% annexin V^+), consistent with previous reports that ectopic LMP1 expression can be somewhat toxic to host cells at high levels (31, 32). Nevertheless, DR-induced apoptosis was reproducibly diminished in cells expressing LMP1, indicated by ~20% fewer annexin V^+ cells (Fig. 4*D*). Compiled data representing several experiments demonstrated that the percentage of annexin V^+ apoptotic cells (minus background in medium controls) was significantly reduced in TRAIL- and anti-Fas-treated $LMP1^+$ BJAB transfectants compared with cells receiving empty vector (Fig. 4*E*). Surprisingly, high LMP1 expression also reduced apoptosis in cells exposed to etoposide, implying LMP1 could also confer protection against intrinsic apoptosis pathways in this situation. Nonetheless, these data show that LMP1 expression alone can partially protect BJAB cells from DR-mediated apoptosis.

The C-terminal activating region (CTAR)2 signaling domain is primarily responsible for LMP1-mediated DR apoptosis resistance

The cytoplasmic tail of LMP1 contains two main signaling domains known as CTAR1 and CTAR2 (or transformation effector sites 1 and 2) that mediate signaling through interactions with TRAFs and other adapter molecules. To determine which signaling domains of the LMP1 cytoplasmic tail are critical for DR apoptosis

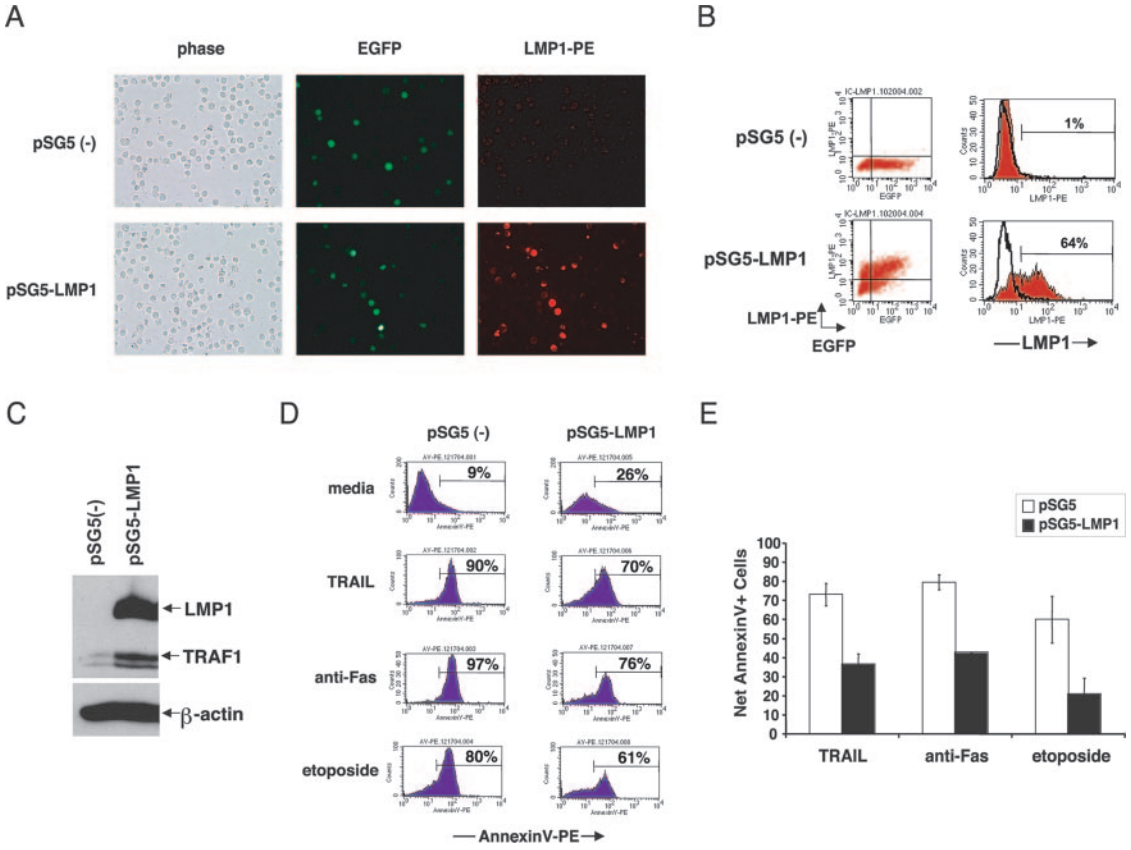

FIGURE 4. LMP1 partially protects BJAB transfectants from apoptosis. *A*, BJAB cells were cotransfected with pSuper.EGFP as a reporter plasmid plus pSG5 or pSG5-LMP1, as indicated at *left*. Forty-eight hours posttransfection, cells were stained intracellularly using PE-conjugated anti-LMP1 mAb and analyzed by fluorescent microscopy. EGFP was detected in ~70% of LMP1$^+$ cells. *B*, Cells were transfected as above and analyzed 48 h later by intracellular flow cytometry, staining with a PE-conjugated anti-LMP1 mAb (filled histogram) or an isotype-matched control Ab (open histogram). EGFP fluorescence intensity corresponds to the amount of LMP1 detected in LMP1$^+$ cells (*bottom left panel*). *C*, WB of lysates made from pSG5 or pSG5-LMP1 transfectants 48 h posttransfection. *D*, Cells were transfected as above, and GFP^{high} cells were sorted by FACS. Sorted GFP^{high} cells were stimulated, and apoptosis was quantified 24 h later using annexin V-PE. The percentage of annexin V-PE$^+$ cells is noted within each histogram. *E*, Compiled apoptosis data from experiments described in part (*D*), subtracting background apoptosis (percentage of annexin V-PE$^+$ cells in medium controls). Data represent the mean ± SEM of three separate transfection experiments.

resistance, we used a panel of previously described Flag-tagged LMP1 mutant constructs (Fig. 5*A*) (33, 34). EGFP cotransfection experiments were performed in BJAB cells using these different LMP1 constructs. Similar transfection efficiency and levels of LMP1 mutant expression were noted in EGFP$^+$ cells by immunofluorescence (Fig. 5*B*). Treatment of the various transfectants with TRAIL or anti-Fas revealed differential protective effects against these apoptotic stimuli (Fig. 5*C*). Cells expressing LMP1 mutant constructs that were incapable of signaling due to mutation/deletion of both CTARs (M5, M7) were as sensitive to TRAIL and anti-Fas as empty vector controls, indicating signaling is absolutely required for its protection. Whereas deletion of CTAR2 (M4) partially reversed the protective effect rendered by WT LMP1 in BJAB transfectants, deletion of CTAR1 (M6) conferred the same level of protection against DR apoptosis as WT LMP1. These data suggest that CTAR2 is more critical for LMP1-mediated resistance to TRAIL- and anti-Fas-induced apoptosis than CTAR1. A similar pattern of protection was also observed in testing LMP1 mutants against etoposide-mediated apoptosis (data not shown). However, deletion of CTAR2 alone did not completely abolish apoptosis protection, implying that signals originating from both CTAR1 and CTAR2 must cooperate to mediate the antiapoptotic effect.

Confirmation of LMP1-mediated DR apoptosis resistance using an inducible LMP1 chimeric protein

Because the partial protective effect of WT LMP1 against TRAIL/anti-Fas-mediated apoptosis could only be observed in a subpopulation of EGFP^{high} cotransfectants, we explored further methods for confirming this finding. Given the toxicity associated with long-term, high expression of LMP1 in BJAB cells, we turned to a modified construct for inducible LMP1 signaling originally developed by Hammerschmidt and colleagues (35). This chimeric protein is comprised of the extracellular portion of the low affinity NGFR fused to the cytoplasmic tail of LMP1. Without the LMP1 transmembrane domains, LMP1 signaling can only be induced by cross-linking with anti-NGFR plus anti-mouse secondary Ab, providing specific activation of LMP1 signaling as needed in BJAB cells, which do not express NGFR endogenously.

We generated two stable BJAB clones (18 and 20) expressing different levels of NGFR-LMP1 at the cell surface (Fig. 6*A*). Clones were treated with secondary Ab alone (0 h) or with anti-NGFR plus secondary Ab to cross-link NGFR-LMP1 and activate LMP1 signaling for 8 h before the addition of apoptotic stimuli. Prior activation of LMP1 signaling led to a substantial reduction in annexin V$^+$ cells following overnight exposure to TRAIL or anti-Fas (Fig. 6*B*). PI cell cycle analysis revealed the same trend of

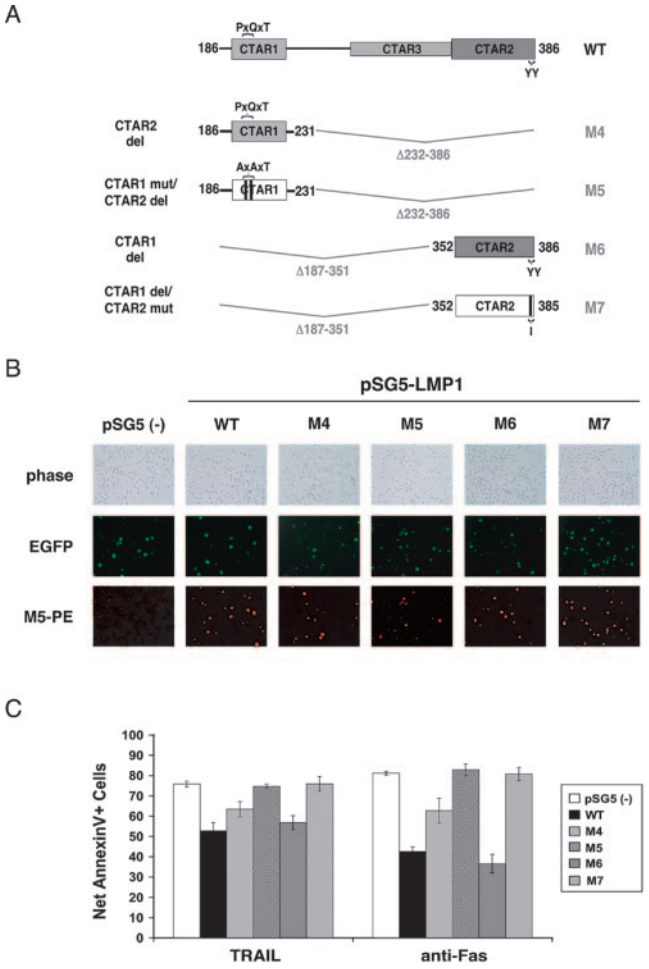

FIGURE 5. LMP1 confers protection against DR apoptosis primarily through CTAR2 signaling. *A*, Schematic diagram of LMP1 cytoplasmic tail mutants used. Labels on the *left* specify which domains are mutated/deleted; labels on the *right* indicate shorthand references for each construct. Point mutations in motifs critical for CTAR1 and CTAR2 signaling are indicated at the *top* of each domain. Deleted residues are noted in gray; □, indicate nonsignaling domains. *B*, BJAB cells were cotransfected with EGFP and Flag-LMP1 mutant constructs. Forty-eight hours posttransfection, cells were stained with anti-Flag M5 mAb plus PE-conjugated secondary Ab and analyzed by fluorescence microscopy. *C*, Cells were transfected as above and treated with TRAIL or anti-Fas 48 h later. Following 24-h incubation, apoptosis in GFP^{high}-gated cells was measured by annexin V-PE staining (subtracting medium background). Data represent mean ± SEM of at least three separate experiments.

partial protection against DR-mediated apoptosis following 8 h of cross-linking of NGFR-LMP1 in both clones (Fig. 6*C*). No significant reduction in etoposide-induced apoptosis was recorded upon NGFR-LMP1 signaling. In comparison with transiently transfected BJAB expressing WT LMP1 described above, this difference may be attributed to a difference in signal strength, the absence of WT LMP1 transmembrane domains in the NGFR-LMP1 molecule that may influence signal transduction, or the relative expression level. Nevertheless, these data demonstrate that inducible LMP1 signaling also confers partial protection against TRAIL- and anti-Fas-driven apoptosis in BJAB cells.

Stable BJAB NGFR-LMP1 clones also allowed us to examine DR signaling biochemically. Following apoptotic stimuli, both caspase 8 and caspase 3/7 activity were reduced in the presence of LMP1 signaling compared with controls (Fig. 6*D*), suggesting that LMP1 signaling can perturb caspase activation following DR engagement. These results show that the block in caspase 8 activation observed in EBV-infected BJAB cells may be explained in part by the action of LMP1.

NF-κB activation drives LMP1-mediated resistance to DR apoptosis

Because the CTAR2 of LMP1 appeared to be important for LMP1-mediated DR apoptosis resistance, and because CTAR2 drives ~80% of LMP1-induced NF-κB activity (36, 37), we asked whether NF-κB activation was required for this protective effect. To address this question, we used the NF-κB inhibitor BAY11-7082 (BAY11), which specifically inhibits the p65/p50 complex of NF-κB (38). When BAY11 was added concomitantly with NGFR cross-linking reagents, NGFR-LMP1-induced resistance to TRAIL- and anti-Fas-mediated apoptosis was reversed (Fig. 7*A*). The addition of pharmacological inhibitors specific for other LMP1-driven signaling pathways (p38 MAPK, JNK, ERK, PI3K) did not affect LMP1-mediated apoptosis resistance (data not shown), suggesting NF-κB activation is the primary signal driving resistance to DR stimuli.

As expected, NGFR-LMP1 signaling induced both IκB degradation and TRAF1 up-regulation in BJAB clones, consistent with NF-κB activation, and these effects were reversed by concomitant BAY11 treatment (Fig. 7*B*). More importantly, a substantial increase in the expression of both $cFLIP_L$ and $cFLIP_S$ was observed with 8-h NGFR-LMP1 signaling, and was markedly inhibited by BAY11. Taken together, these results present strong evidence for the involvement of NF-κB activity and subsequent cFLIP up-regulation in LMP1-driven protection against Fas/TRAIL-R-mediated apoptosis.

Finally, we tested whether NF-κB inhibition could also sensitize BJAB_B95 cells to DR apoptosis. BJAB_B95 cells were pretreated with BAY11 and then stimulated with TRAIL, anti-Fas, or etoposide. Although BAY11 alone induced considerable spontaneous apoptosis, it also conferred a synergistic effect with TRAIL and anti-Fas, leading to substantial increases in apoptosis (Fig. 7*C*). Moreover, BAY11 also reduced endogenous levels of cFLIP protein in BJAB_B95 cells (Fig. 7*D*). In total, these findings demonstrate that latent EBV infection can protect BJAB cells from Fas/TRAIL-R-mediated apoptosis, explained in part by the ability of LMP1 to activate NF-κB and up-regulate cFLIP, which in turn blocks sufficient caspase 8 activation in the DISC.

Discussion

Herpesviruses have evolved multiple strategies of immune evasion to promote persistence in host cells. Two dedicated DR pathways used by CTL and NK cells to eliminate virally infected cells are Fas/FasL and TRAIL/DR. In this study, we provide evidence that EBV itself can protect latently infected BJAB cells from DR-induced apoptosis. Thus, we describe a novel mechanism by which EBV may subvert important immune effector pathways of cell death. Specifically, our data demonstrate that DISC-associated caspase 8 turnover is disrupted in type III EBV-infected BJAB cells, resulting in impaired downstream caspase activation and inhibition of Fas- or TRAIL-R-triggered apoptosis. Our results also show that LMP1 contributes to DR apoptosis resistance through NF-κB activation. This feature of LMP1 signaling is consistent with other studies showing that LMP1 can protect cells from intrinsic apoptotic stimuli (16). It is also worth noting that fresh tumor cells collected from two patients recently diagnosed with EBV^+ B cell lymphoma were also resistant to TRAIL- or anti-Fas-induced death (data not shown), consistent with our findings in EBV-infected BJAB cells.

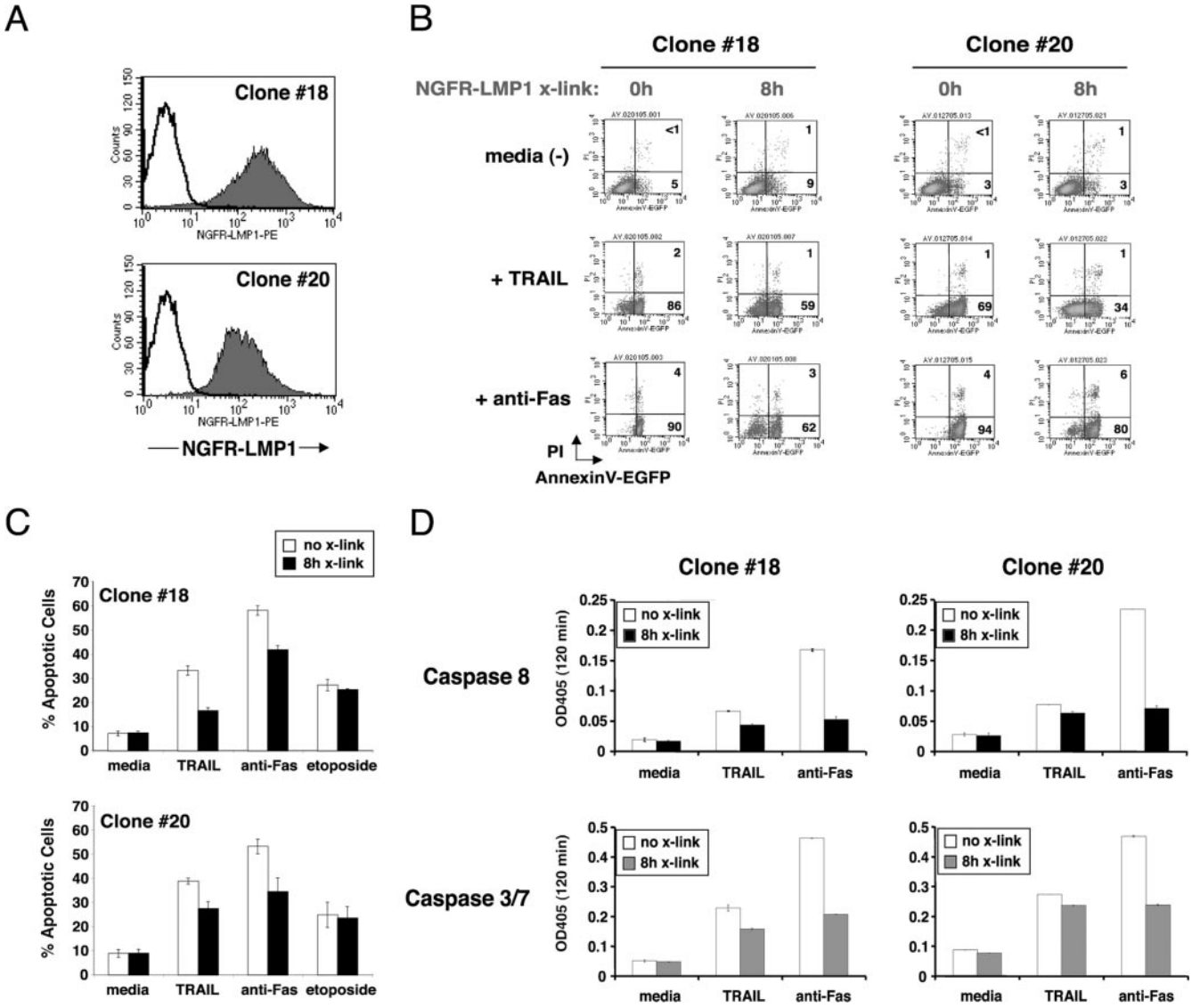

FIGURE 6. Inducible LMP1 signaling partially protects BJAB clones from DR apoptosis. *A*, Stable BJAB clones expressing NGFR-LMP1 chimeric protein were generated. Surface expression of NGFR-LMP1 was detected on clones 18 and 20 (filled histograms) by flow cytometry using biotinylated anti-NGFR mAb plus streptavidin-PE. Open histograms represent NGFR staining on parent BJAB cells. *B*, Clones were cross-linked with anti-NGFR mAb plus goat anti-mouse secondary Ab for 0 or 8 h to induce LMP1 signaling, and then stimulated with TRAIL or anti-Fas for 18–24 h. Apoptosis was measured by staining with annexin V-EGFP + PI. The percentage of annexin V^+ apoptotic cells is noted within the *lower* and *upper right quadrants*. *C*, Clones were cross-linked as above for 0 (□) or 8 h (■) before adding apoptosis stimuli. Apoptosis was analyzed by PI staining. Data represent mean ± SEM of five independent experiments. *D*, Cells were cross-linked as above; stimulated for 4 h with medium alone, TRAIL, or anti-Fas; and lysed. Enzymatic activity of specified caspases in each lysate was quantitated using the colorimetric assay described in Fig. 2*B*.

For the most part, the relationship between EBV infection and FasL- or TRAIL-mediated apoptosis has been only indirectly addressed, and no conclusive findings have emerged. For instance, one study reported an apparent correlation between EBV infection and TRAIL resistance in Burkitt's lymphoma (BL) lines (39). Although the authors dismiss the significance of this association by documenting TRAIL resistance in both EBV^- and EBV^+ Akata BL clones, their experiments do not address how EBV infection modulates apoptosis in a TRAIL-sensitive cell line such as BJAB. More recently, Tafuku et al. (40) described NF-κB-dependent TRAIL resistance in EBV^+ BL lines, independent of Fas sensitivity. Inasmuch as some EBV^+ LCL display differential sensitivity to DR apoptosis, particularly to Fas (i.e., SKW6.4), variability among EBV^+ LCL may be reflective of differential latent gene expression, natural sequence variants with differing intrinsic signaling properties (e.g., LMP1), or perhaps most importantly, derivation from myriad sources of host B cells. Indeed, early work on Fas sensitivity in LCL and BL lines by Falk et al. (41) emphasized this point. One recent report demonstrated that overexpression of LMP1 sensitized the LCL PRI to Fas-mediated apoptosis, ostensibly via increased Fas expression (42).

Unlike the aforementioned studies, our study directly explored the ability of EBV (and LMP1 alone) to modulate DR apoptosis in a controlled system, using highly sensitive BJAB cells, amenable to biochemical analysis of DR signaling. In this study, we show through two different approaches that LMP1 signaling in BJAB provides partial protection against DR killing through NF-κB. Moreover, we found that inhibition of protein synthesis with cycloheximide sensitized BJAB_B95 cells to DR-induced apoptosis (data not shown), presumably through the loss of short-lived proteins such as FLIP. Altogether, our results are consistent with the notion that NF-κB is commonly associated with up-regulation of antiapoptotic proteins, including FLIP.

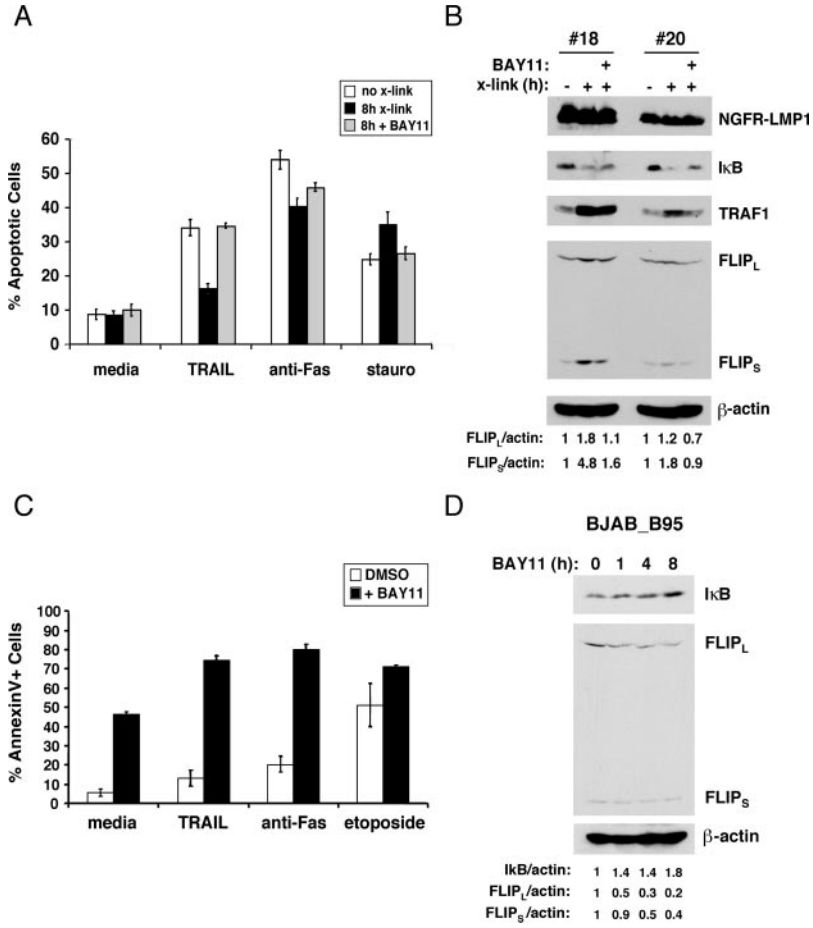

FIGURE 7. NF-κB activity is responsible for cFLIP up-regulation and DR apoptosis resistance in NGFR-LMP1 BJAB clones and EBV-infected BJAB cells. *A*, BJAB NGFR-LMP1 clones were cross-linked with anti-NGFR mAb + anti-mouse secondary Ab for 0 (□) or 8 h in the absence (■) or presence (▨) of BAY11 before the addition of apoptosis stimuli. Apoptosis was measured 24 h later by PI staining. Data represent mean ± SEM of three separate experiments. *B*, Clones were cross-linked as above for 0 h, or 8 h in the absence or presence of BAY11. Lysates prepared from each were subjected to WB analysis. Spot densitometry analysis of the ratio of cFLIP isoforms to β-actin (normalized to uncross-linked cells) is indicated at the *bottom* of each lane. *C*, BJAB_B95 cells were pretreated with 1 μM BAY11 for 1 h before stimulation with apoptosis reagents indicated on the *bottom*. Apoptosis was quantified by annexin V-EGFP staining, and data represent mean ± SEM of three separate experiments. *D*, Lysates derived from BJAB_B95 cells treated with 1 μM BAY11 for designated times were subjected to WB analysis of IκB degradation and cFLIP expression. Spot densitometry analysis of the ratio of cFLIP isoforms to β-actin (normalized to untreated cells) is indicated at the *bottom* of each lane.

The partial protective effect exhibited by LMP1 suggests other latent genes may participate in inhibiting DR signaling in EBV-infected BJAB cells. We found that EBNA2 did not confer additional protection against TRAIL or anti-Fas stimuli, whether or not LMP1 was expressed concomitantly (data not shown). LMP2A is another candidate viral protein that could affect apoptosis sensitivity in EBV$^+$ B cell lymphomas. LMP2A inhibits BCR signaling while simultaneously delivering certain tonic signals required for B cell survival (43). Although we could not detect LMP2A expression in EBV-infected BJAB cells (data not shown), LMP2A may still augment the protective effect of LMP1.

Given that our data localize the defect in DR signaling to impaired caspase 8 activation in EBV-infected and LMP1-transfected BJAB cells, cFLIP is the most likely candidate for inhibiting DISC function. Our results confirm a recent report identifying *cFLIP* as an NF-κB-responsive gene up-regulated by LMP1 signaling (38). The role of cFLIP in modulating DR sensitivity has been controversial. Whereas cFLIP$_S$ is accepted as a true inhibitor of FasL- and TRAIL-mediated apoptosis, various studies have shown cFLIP$_L$ can inhibit or promote apical caspase activation (44–46). More recent reports using RNA interference have conclusively shown cFLIP$_L$ is inhibitory to both FasL- and TRAIL-stimulated apoptosis (24, 47, 48). Our results are consistent with these latter studies, in which both isoforms of cFLIP are enriched and caspase 8 activity is diminished in DR DISCs. In fact, IP analysis revealed only partial processing of caspase 8 in the Fas-DISC of BJAB_B95 cells, an effect attributed to cFLIP$_L$ function (49). For unknown reasons, our attempts to silence cFLIP and other genes failed to significantly knock down target gene expression in BJAB_B95 cells. Interestingly, ~80% knockdown of cFLIP$_L$ expression in SKW6.4 cells succeeded in sensitizing these cells to TRAIL-induced apoptosis (data not shown). Because SKW6.4 cells do not express cFLIP$_S$ and are Fas sensitive, this could indicate that cFLIP$_S$ may be more critical for blocking Fas-mediated apoptosis in EBV$^+$ B cell lymphomas. Taken together, our data complement a larger model by which LMP1 signaling can inhibit apoptosis induced by DRs as well as certain stress-related stimuli, such as growth factor withdrawal.

Our results have pertinent implications for the genesis and persistence of EBV-infected B cell lymphomas. By targeting DR signaling in host B cells, latent infection neutralizes potential weapons (FasL/TRAIL) used by CTL to destroy EBV-infected targets. For instance, the finding that some PTLD lymphomas persist or relapse in immunocompetent patients may be due in part to DR apoptosis resistance, particularly if other cytotoxic effector molecules (perforin, granzymes) are also rendered ineffective. Our findings also prompt careful evaluation of TRAIL as a potential cancer therapeutic for EBV-associated B cell lymphomas. As demonstrated for other types of tumors, soluble TRAIL may need to be used in conjunction with other drugs to effectively kill lymphoma cells (15, 50).

Furthermore, LMP1-directed DR apoptosis resistance may play an important role in normal EBV biology. During initial infection, LMP1 expression during type III latency may aid in the expansion and survival of infected B cells in extrafollicular areas, delivering CD40-like costimulatory signals required for survival and differentiation. We propose that DR apoptosis resistance may be an important component of this signal. In addition, the protective effect of LMP1 may be particularly relevant to the rescue of type II (EBNA1$^+$, LMP1$^+$, LMP2A$^+$) latently infected germinal center B

cells that have failed somatic hypermutation, thought to be the precursors of EBV$^+$ Hodgkin lymphoma (51). In this scenario, LMP1 and LMP2A may function to both drive proliferation and prohibit apoptosis of crippled germinal center B cells lacking a functional BCR, known to give rise to some EBV$^+$ Hodgkin and PTLD lymphomas (52, 53). Although the role of TRAIL in B cell development is unknown, Fas-mediated apoptosis has been implicated in B cell selection and suppression of lymphomagenesis (54). Regardless of where LMP1 is expressed during B cell maturation, our findings suggest LMP1 effects may be dependent on signal strength or the expression of other viral genes, which potentially provides a mechanism for regulating its effect. If this signal is not regulated properly, lymphomas can develop, as demonstrated in LMP1 transgenic mice (55).

In summation, the antiapoptotic signal provided by LMP1 extends to DR apoptosis, which may influence the survival of normal and transformed EBV-infected B cells. Targeting cFLIP or NF-κB activity in LMP1$^+$ EBV-infected B cell tumors may also improve current therapeutic approaches for eliminating such lymphomas.

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Disclosures

The authors have no financial conflict of interest.

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