Growth Factors, Cytokines, Cell Cycle Molecules

Epstein-Barr Virus (EBV) Latent Membrane Protein-1 Down-Regulates Tumor Necrosis Factor- α (TNF- α) Receptor-1 and Confers Resistance to TNF- α -Induced Apoptosis in T Cells

Implication for the Progression to T-Cell Lymphoma in EBV-Associated Hemophagocytic Syndrome

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The infection of T cells by Epstein-Barr virus (EBV) may result in hemophagocytic syndrome (HPS) through enhanced cytokine secretion, particularly tumor necrosis factor- α (TNF- α), by EBV latent membrane protein-1 (LMP-1). One bewildering observation of HPS patients is relapsing disease or progression to T-cell lymphoma. This finding raises the question whether EBV LMP-1-expressing T cells may survive and proliferate in the cytokine milieu of HPS. To explore this possibility, we tested the sensitivity of LMP-1-expressing T cells to apoptosis in the presence of TNF- α . LMP-1 up-regulated TNF- α through TRAF2,⁵ and nuclear factor- κ B pathway in T cells. The LMP-1-expressing T cells then became resistant to TNF- α -induced apoptosis. Interestingly, the expression of TNFR1 was remarkably down-regulated by LMP-1 in T cells. Furthermore, the TNF- α /TNFR1 downstream death signal TNFR1-associated death domain protein was constitutively recruited by LMP-1, and the activities of apoptotic caspases 3, 8, and 9 were suppressed. Reconstitution of TNFR1 successfully reversed the TNF- α -induced apoptotic cascades. Therefore, EBV LMP-1 not only activates T cells to pro-

liferate but also confers resistance to TNF- α -mediated apoptosis via down-regulation of TNFR1 in the cytokine milieu of HPS. This finding provides a potential mechanism to explain the disease persistence or progression to T-cell lymphoma in HPS patients. (Am J Pathol 2007, 170:1607–1617; DOI: 10.2353/ajpath.2007.061026)

The Epstein-Barr virus (EBV) may infect B cells, epithelial cells, or NK/T cells and lead to the development of a spectrum of benign and malignant human diseases.^{1–4} Distinct from other EBV-associated disorders, the infection of T cells by EBV may manifest a fatal form of infectious mononucleosis or hemophagocytic syndrome (HPS) in young children, characterized by hepatosplenomegaly, pancytopenia, coagulopathy, and a systemic proliferation of T cells and macrophages with enhanced cytokine secretion, particularly tumor necrosis factor- α (TNF- α) and interferon- γ .^{5–8} The enhanced cytokine secretion has been presumed to play a key role in the pathology of HPS, which includes apoptosis and deple-

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tion of the immune system and the impairment of hepatic and pulmonary functions.^{9–11} TNF- α may induce apoptosis and cell injuries via binding to TNF- α receptor-1 (TNFR1) to activate the TRAF2/TRADD/FADD (TNFR-associated factor 2/TNFR1-associated death domain/Fas-associated DD) signaling and caspase activities.^{12,13}

The EBV latent membrane protein-1 (LMP-1) has been shown to be the gene product responsible for the up-regulation of TNF- α and subsequent macrophage activation in T cells, but not in B cells or epithelial cells.¹⁴ LMP-1 has been shown to be constitutively expressed in EBV-infected T cells.^{15–17} LMP-1 belongs to the protein superfamily of TNFR and is reported to be a strong transactivator of viral and cellular genes.^{18–21} In B cells and epithelial cells, the signaling pathway of LMP-1 has been extensively studied. LMP-1 can mediate its function via two effector regions at its C-terminal cytoplasmic domains, CTAR-1 and CTAR-2. CTAR-1 and CTAR-2 can separately bind TRAFs and TRADD, resulting in the activation of transcription factors nuclear factor (NF)- κ B and c-Jun N-terminal kinase (JNK).^{22,23} The activation of NF- κ B provides the molecular mechanism for LMP-1-induced cell proliferation and transformation.^{24,25} The molecular and biological effects of LMP-1 in T cells, however, are relatively poorly understood. We recently demonstrated that LMP-1 can up-regulate Th1 cytokines such as TNF- α and interferon- γ through the TRAFs/NF- κ B/SAP/ERK signal pathway in T cells, subsequently leading to cytokine storm and tissue injuries as observed in patients with HPS.²⁶

Although current therapy has been successfully used to control HPS in patients,^{27,28} a substantial percentage of patients who received initial treatment may develop relapsing disease or even progress to T-cell lymphoma.^{29,30} Therefore, it is reasonable to speculate whether EBV-infected T cells are less sensitive to the cytokine-mediated cytotoxicity than the surrounding uninfected or bystander cells and hence survive or proliferate in the cytokine milieu of HPS. In this study, we performed a series of experiments to test this hypothesis. First, we tested whether LMP-1-expressing T cells are relatively resistant to TNF- α -induced apoptosis, compared with control T cells. The activities of caspases 3, 8, and 9 and cytochrome *c* were then examined. Second, the regulation of TNFR1 and TRADD by LMP-1 was studied with or without the presence of exogenous TNF- α . We demonstrated that LMP-1 expression could down-regulate TNFR1 and recruit TRADD to block the TNF- α -mediated apoptotic pathway. These findings provide a potential mechanism to explain the persistent disease or the progression to T-cell lymphoma in HPS patients.

Materials and Methods

Lymphoma Cell Lines and Human Primary T Cells

The EBV-negative T-cell lines H9 and HUT78 were used in this study. These cell lines were maintained in RPMI 1640 medium (JRH, Lenexa, NY) supplemented with 10% (v/v) fetal bovine serum (JRH) in 250-ml tissue culture flasks (Becton, Dickinson and Company,

Franklin Lakes, NJ) and were grown at 37°C in a 5% CO₂/95% air atmosphere with twice weekly feeding. Cells used for the following experiments were in the exponential phase of growth. For human primary T cells, CD3⁺ T cells were isolated from peripheral blood mononuclear cells by density centrifugation on Ficoll-Paque Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Cells labeled with anti-CD3 microbeads (20 μ l per 10 million cells; Miltenyi Biotec, Bergisch Gladbach, Germany) and separated on MACS LS column (Miltenyi Biotec) were collected for studies. All of the procedures were performed according to the manufacturer's instructions. The purity of sorted CD3⁺ cells was further evaluated by flow cytometry.

Transiently Transfected and Stable Cell Lines Expressed LMP-1

The LMP-1-expressing constructs were based on B95.8 EBV-derived LMP-1 sequences³¹ in the pSG5 vector, which contain the SV40 early promoter and a β -globin intron upstream of the cloning site. These vectors contain the selection markers Amp^r and Neo^r expressed from the SV40 *ori*. The stable LMP-1-expressing H9 and HUT78 cell lines have been well established previously.¹⁴ The transient LMP-1-expressing H9 cells were transfected by electroporation (Bio-Rad, Hercules, CA) using 0, 2, 5, or 7.5 μ g of plasmids and 2×10^6 cells in 300 μ l of serum-free RPMI 1640 (see Figures 3B and 4B). The highest transfection efficiency was 30% in transient expression. For transient expression of LMP-1 in human primary T cells, the purified CD3⁺ T cells were transfected by Nucleofector with program V-24 (Amaxa Biosystem, Cologne, Germany). To define the signal of LMP-1 that is responsible for TRAFs/NF- κ B/TNF- α transactivation, expression plasmids of dominant-negative TRAF2/TRAF5/ κ B mutants (kindly provided by Prof. T. Watanabe, University of Tokyo, Tokyo, Japan) were used.^{32,33} To restore the expression of TNFR1, the constructs containing full-length cDNA of TNFR1 (kindly provided by Professor Shie-Liang Hsieh, National Yang-Ming University, Taipei, Taiwan) and control vector were used.³⁴ For co-transfection, a total of 2×10^6 cells and 5.0 μ g of DNA were used with electroporation. The co-transfected cells were then selected by diluting 1:10 in RPMI 1640 medium containing 800 μ g/ml neomycin. The concentration of neomycin was subsequently reduced to 400 μ g/ml after 1 week of selection. The surviving cells were collected and expanded for further analysis.

Detection of Apoptosis

To detect the apoptosis rate of cells with or without LMP-1 expression, cells were spun down and resuspended in 200 μ l of phosphate-buffered saline buffer. DNA fragmentation in apoptotic cells was detected using DNA fragmentation assay kits (Clontech, Palo Alto, CA). According to the manufacturer's instructions, T-lymphoid cells were fixed on poly-L-lysine-coated slides. For TUNEL (terminal dUTP nick-end labeling)/DAPI (4',6-diamidino-2-phenylindole-dihydrochloride) staining, cells

were covered by TUNEL/DAPI solution (0.01 $\mu\text{g/ml}$) and incubated at room temperature for 15 minutes. Cells were then photographed by fluorescence microscopy (BX51; Olympus Optical, Tokyo, Japan). Finally, aliquots of 5 μl of annexin V-fluorescein isothiocyanate (Pharmingen, San Diego, CA) and 5 μl of propidium iodide (PI, 50 $\mu\text{g/ml}$ stock solution; Pharmingen) were added, and the cells were incubated for 15 minutes at room temperature in the dark. Then, 400 μl of binding buffer (10 mmol/L Hepes/NaOH, pH 7.4, 140 mmol/L NaCl, and 2.5 mmol/L CaCl_2) was added to each tube, and the samples were analyzed by flow cytometry analysis (FACSCalibur; Becton Dickinson Immunocytometry Systems, San Jose, CA).

Enzyme-Linked Immunosorbent Assay (ELISA) and Cytotoxicity Bioassay of TNF- α Secreted in the Culture Supernatant

To further verify the TRAFs/NF- κB signaling in LMP-1-induced TNF- α secretion, the effects of TRAF2/5- or I κB -dominant-negative mutants on TNF- α secretion were examined. The TNF- α levels in culture supernatants of LMP-1-transfected T cells were measured as previously described.⁸ Monoclonal antibodies that react to specific epitopes on the TNF- α molecules were used in the ELISA tests (Bender MedSystems, Vienna, Austria). Aliquots of the volume of the supernatants of LMP-1-expressing T cells were obtained from 10^6 viable cells. Supernatants were harvested by filtration through a 0.45- μm pore size filter (Costar, Cambridge, MA) to exclude cell debris. The standard curve was created by plotting the mean absorbance for each standard concentration. The sensitivity of the ELISA test for TNF- α was 1.5 pg/ml.

The activity of TNF- α produced by LMP-1-expressing H9 cells could be further determined by bioassays based on cytokine-induced cytotoxicity of a TNF-sensitive murine WEHI164-13 fibrosarcoma line. A serial dilution of filtered conditioned medium of LMP-1-expressing H9 cells, leaving a final volume of 50 μl , were cocultured with 50 μl of WEHI164-13 cells at a concentration of 5×10^5 cells/ml for an additional 36 hours, and cell viabilities were counted by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The specificity of the response was assessed by adding neutralizing anti-TNF- α antibody (Santa Cruz Biotechnology, Santa Cruz, CA) to the dilutions of the samples.

Reporter Assays for NF- κB and TNF- α

To confirm further the importance of the NF- κB signal pathway in the enhancement of TNF- α in LMP-1-H9 cells, a reporter gene assay to detect NF- κB activity and TNF- α promotion was performed. A NF- κB site-dependent luciferase vector p6x- κB -Luc as described previously was used for NF- κB activity assay.³⁵ The genomic DNA products of the TNF- α promoter region were directly amplified by the polymerase chain reaction method. Primers for the promoter sequences (from -1052 to +126) of TNF- α

were used. Sequencing was applied to check the accuracy of polymerase chain reaction amplification and the orientation of the inserted sequence.¹⁴ Luciferase expression vector driven by the herpes simplex virus thymidine kinase promoter (pRL-TK; Promega, Madison, WI) was co-transfected to standardize each experiment as previously described.²⁶ For H9 T-cell lines, 5×10^4 cells were transfected using electroporation. Co-transfection of unrelated proteins such as β -galactosidase showed no effects on the NF- κB -driven luciferase activity. Representative results of triplicated experiments with mean and SD are shown in the figures.

Coimmunoprecipitation and Immunoblotting

To clarify the signal transduction of TNF- α /TNFR1 pathway in LMP-1-expressing H9 cells, the interaction between TRADD and TNFR1 or LMP-1 was studied. Immunoblotting experiments to detect the expression of the transduced gene and coimmunoprecipitation were done as previously described.³³ When indicated, aliquots of whole cell lysates were removed for immunoblots. Anti-LMP-1 mouse monoclonal antibody (CS1-4; DAKO, Glostrup, Denmark), anti-pan-caspase-3 mouse antibody (C5737; Sigma, St. Louis, MO), anti-caspase-3 p17 goat antibody, anti-caspase-9 p10 mouse antibody, anti-cytochrome c mouse antibody, anti-TNFR1 mouse antibody, anti-TRAF2 rabbit antibody, anti-TRAF5 goat antibody, anti-TRADD rabbit antibody, and anti- α -actin goat antibody (Santa Cruz Biotechnology) were used. Enhanced chemiluminescence kits (Perkin-Elmer Life Sciences, Boston, MA) were then used for antibody binding.

Immunofluorescence Staining

To define the subcellular location of TRADD in LMP-1-H9 cells, double-labeling immunostaining was done. Cells in suspension culture were first washed twice in Tris-buffered saline with 2% bovine serum albumin. Cytospin samples were prepared using 10^4 cells and fixed with 100% methanol for 10 minutes. The staining procedure was performed as previously described.²⁶ The fluorescence-labeled secondary antibodies, bovine anti-mouse fluorescein isothiocyanate and rabbit anti-goat rhodamine (Santa Cruz Biotechnology), were used. Signals were detected by fluorescence microscopy (BX51; Olympus Optical).

Statistical Analysis

Statistical evaluation was performed by analysis of variance ($m \times n$ factorial design), and multiple comparisons between two groups were evaluated by means of Duncan's new multiple range test. All data were expressed as SEM, and statistical significance was defined as $*P < 0.05$ and $**P < 0.01$.

Figure 1. LMP-1 up-regulated TNF- α via the TRAF2/5/NF- κ B signal pathway. **A:** The expression and secretion of TNF- α in LMP-1 transfectants were measured by Western blotting and ELISA. pSG6-transfectant, negative control for stable lines; LMP-1-H9/TRAF2,5DN, TRAF2,5 dominant-negative (DN) plasmids in LMP-1 H9 cells. Each column represents the data from multiple independent experiments with SEM. **B:** Reporter assay for TNF- α and NF- κ B promoters. TPA: (tetradecanoyl-phorbol-acetate, 10 ng/ml), positive control for reporter assay. **C:** TNF- α cytotoxicity assay in LMP-1 H9 cells. The murine WEHI164-13 cells were cultured with the culture soup of H9 T cells transfected with various constructs. The viability of WEHI164-13 was examined by MTT assay. **D:** The viability of LMP-1 transfectants was tested by MTT assay under exogenous TNF- α (25 ng/ml) for 72 hours.

Results

EBV LMP-1 Enhanced the Production and Secretion of TNF- α via the TRAF2/TRAF5/NF- κ B Signal Pathway in T Cells

To confirm further the observation on the up-regulation of TNF- α by LMP-1 in previous studies,²⁶ Western blotting and ELISA assays were performed to assess the production and secretion of TNF- α by EBV LMP-1. To clarify the signal pathway involving in the up-regulation of TNF- α by LMP-1, dominant-negative constructs of TRAF2, TRAF5, or I κ B plasmids were separately transfected into LMP-1-expressing H9 cells. The results showed that LMP-1 expression could significantly enhance the production and

secretion of TNF- α (Figure 1A). In both studies, transfection of dominant-negative constructs of TRAF2, TRAF5, and I κ B significantly inhibited the production and secretion of TNF- α up-regulated by LMP-1 as determined by immunoblotting (Figure 1A, top; 65% inhibition for pTRAF2DN, 81% inhibition for pTRAF5DN, and 69% inhibition for pI κ BDN, as compared with pLMP-1-transfectant) and ELISA test (Figure 1A, bottom; 72% inhibition for pTRAF2DN, 43% inhibition for pTRAF5DN, and 57% inhibition for I κ BDN) in LMP-1-transfected H9 T cells. To confirm further the transcriptional regulation of TNF- α under LMP-1 signaling, a reporter plasmid containing TNF- α promoter and NF- κ B driving sequence were constructed and co-transfected in the LMP-1-H9 sublines. Consistently, the transcriptional activities of TNF- α pro-

Figure 2. Resistance of LMP-1-expressing T cells to TNF- α -induced apoptosis. **A:** The percentage of TNF- α -induced apoptotic cells was measured by DNA fragmentation, double-staining TUNEL/DAPI assay, and annexin V/PI stain. The cells were treated with exogenous TNF- α (25 ng/ml for 48 hours after 1 day of incubation with serum starvation) before examination. The **arrowheads** indicate apoptotic cells. Cells were observed under an immunofluorescence microscopy. For annexin V/PI staining, the percentages of apoptotic cells were detected by flow cytometry as shown in the right column. **B:** The molecules of apoptosis cascades in H9 T cells were shown by Western blotting with or without TNF- α treatment (50 ng/ml for 48 hours). In the presence of exogenous TNF- α , the control pSG5-H9 cells showed activation of apoptotic caspases and cytochrome c, whereas no change was detectable in LMP-1 H9. Dominant-negative construct of TRAF2 recovered the activities of apoptotic enzymes. **C:** The activities of caspase-8 as examined by luminescent assay in the control LMP-1 transfectant, and LMP-1/TRA2DN constructs with or without exogenous TNF- α (50 ng/ml for 48 hours). ** $P < 0.01$. Original magnifications, $\times 1000$.

motor by LMP-1 could be abolished by three dominant-negative plasmids as shown in Figure 1B.

The biological activities of TNF- α produced by LMP-1-expressing T cells were further determined by bioassays based on cytokine-induced cytotoxicity of a TNF- α -sensitive murine WEHI164-13 fibrosarcoma line. The cell viability of WEHI164-13 was significantly decreased (45%) by treating with culture supernatant obtained from LMP-1 H9 cells (Figure 1C). This decrease could be reversed by the expression of three dominant-negative plasmids. For

the study of TNF- α -induced cytotoxicity, the T cells were treated with exogenous TNF- α for 3 days, and viability was measured by MTT assay (Figure 1D). As compared with mock control T cells, a 42% increase of cell viability of LMP-1-transfected T cells was observed. Co-transfection of p κ B/TRA2/TRA5 dominant-negative constructs resulted in an increase of apoptotic death of LMP-1-transfected T cells. Likewise, the cell viabilities of dominant-negative transfectants decreased to 68% for TRA2DN, 77% for TRA5DN, and 46% for κ BDN, lower

Figure 3. Suppression of TNFR1 expression by LMP-1 signaling in T-cell lines and primary T cells. **A:** Expression of TNFR1 in two LMP-1-expressing stable T-cell lines and primary T cells as shown by Western blotting. Compared with the controls, the expression of LMP-1 significantly reduced the levels of TNFR1. **B:** TNFR1 expression at different levels of LMP-1 in transiently transfected line. **C:** The TRAF2/5 dominant-negative plasmids could effectively block the effect of LMP-1 signaling on the expression of TNFR1.

than the 98% of LMP-1-expressing T cells. Taken together, the results shown above indicated that EBV LMP-1 could specifically up-regulate TNF- α via the TRAF2,5/NF- κ B pathway in LMP-1-expressing T cells.

LMP-1-Expressing T Cells Were Resistant to Apoptosis Induced by Exogenous TNF- α

To clarify whether the EBV-infected T cells are resistant to TNF- α -induced apoptosis in a paracrine or autocrine manner, we first assessed the apoptosis of LMP-1-expressing and mock control-transfected H9 T cells under stimulation with exogenous recombinant TNF- α (25 ng/ml for 48 hours after 1 day of incubation with serum starvation) by oligonucleosomal DNA fragmentation assay (Figure 2A, left). In the presence of exogenous TNF- α , the degree of DNA fragmentation was enhanced in the mock pSG5-H9 cells, although only mildly detected in the LMP-1-expressing cells. Similar observation of the apoptotic phenomenon was demonstrated by DAPI and TUNEL double staining (Figure 2A, middle). The percentages of apoptotic cells of LMP-1 transfectants were significantly

Figure 4. Constitutive recruitment of TRADD by LMP-1 in H9 T cells. **A:** The association between TRADD and LMP-1 in H9 parental cells or LMP-1 H9 cells with or without exogenous TNF- α (25 ng/ml for 24 hours). Cell lysates were immunoprecipitated with antibody against TRADD. The immunoprecipitates were examined by immunoblot with TNFR1, LMP-1, and TRADD antibodies. The LMP-1-1 and LMP-1-2 were two stable LMP-1-expressing H9 lines. **B:** Constitutive interaction of LMP-1 and adaptor protein TRADD, TRAF2, and TRAF5 at different expression levels of LMP-1 without exogenous TNF- α . The quantitative data (x-fold induction) of each adaptor protein I were shown below.

lower than that of mock-H9 cells (LMP-1 H9, 57.5%; pSG5 H9, 24.6%; $P < 0.05$). Apoptosis was further assessed by annexin V and PI staining and flow cytometry analysis. The percentage of late apoptosis cells was reduced in LMP-1-expressing cells (Figure 2A, right).

We next examined the caspase cascade activities of LMP-1-expressing and pSG5-control T cells. As shown in Figure 2B, the released levels of cytochrome c and activities of caspase-3 and caspase-9, induced by treating with recombinant TNF- α , were significantly suppressed by LMP-1 transfection. The expression of LMP-1 led to the reduction of processed 32-kd fragment for caspase-9 and 16.5-kd fragment for caspase-3 together with the reappearance of the intact 47-kd caspase-9 in the immunoblotting. The transfection of dominant-negative TRAF2 could reverse the release levels of cytochrome c and the activities of caspases 3 and 9, indicating that caspases 3 and 9 were indeed activated during the TNF- α -induced cell death and suppressed by LMP-1 expression. Similar inhibitory effect was also demonstrated in the activity of caspase-8 as measured by luminescent assay (Figure 2C). Synthesizing all of the data in this study, it is concluded that the TNF- α -induced cell death could be inhibited by LMP-1 expression in T cells.

Down-Regulation of p55 TNFR1 by LMP-1 in T-Cell Lines and in Primary T Cells

Next, we tried to clarify the protection mechanism of TNF- α -induced apoptosis by examining the expression

Figure 5. Co-localization of TRADD with TNFR1 and LMP-1 in H9 T cells. The cells were treated with 25 ng/ml of exogenous TNF- α for 24 hours before examination. Double immunofluorescence staining revealed co-localization of TRADD and LMP-1 (**A** and **C**) or TRADD and TNFR1 (**B** and **D**) on H9 T cells. Cells were immunostained with antibodies for TRADD, LMP-1, and TNFR1, and were observed by immunofluorescence microscopy. Original magnifications, $\times 1000$.

of TNFR1 in LMP-1-expressing T cells. The receptor of TNF- α , p55 TNFR1, was first examined by Western blotting in LMP-1-expressing T-cell lines H9 and HUT78. To observe the phenomenon in normal condition, human primary T cells were also included in this study. Abundant p55 TNFR1 protein was detected in both control T-cell lines and human primary T cells (Figure 3A). In LMP-1-expressing T cells, however, the expression levels of TNFR1 were remarkably suppressed, especially in H9 T cells (0.1-fold for H9 and 0.3-fold for HUT78, respectively). The primary T cells revealed similar findings with reduction of TNFR1 in the

presence of LMP-1 expression. The inhibitory response of TNFR1 expression was specific and dependent on the expression levels of LMP-1 (Figure 3B). However, the LMP-1-expressing B-cell line BJAB, in which TNF- α production was not up-regulated by LMP-1, showed no change of TNFR1 expression (data not shown). Furthermore, the TNFR1 expression could be recovered by the transfection of dominant-negative plasmids of TRAF2/TRAF5 in LMP-1 H9 cells (Figure 3C). Together, these data indicated that EBV LMP-1 could specifically down-regulate TNFR1 in T cells but not in B cells. The decreased expression of TNFR1 could lead to agnosia

Figure 6. Restoration of TNF- α -induced apoptosis by reconstituted TNFR1 in LMP-1-expressing H9 T cells. The cells were transfected with TNFR1 or control plasmids (pTNFR1 or pCMV-1) by electroporation and harvested at 24 and 36 hours. **A:** Reconstitution of TNFR1 plasmid led to the subsequent activation of the key apoptotic enzyme caspase-3 in LMP-1-expressing T cells, as detected with antibody of pan-caspase-3 by Western blotting. **B:** The percentages of apoptotic cells were measured with annexin V/PI stain by flow cytometry. The reconstituted TNFR1 could induce apoptosis in LMP-1 H9 again. **C:** Reconstitution of TNFR1 maintained high-level secretion of endogenous TNF- α in LMP-1 H9 cells. **D:** The addition of anti-TNF- α antibody at the concentration of 20 μ g/ml, an excessive concentration for blocking endogenous TNF- α , could rescue the TNFR1-reconstituted LMP-1-expressing T cells from apoptosis. The mouse anti-actin antibody was used as the control reagent.

for the ligand binding in EBV-infected T cells and hence escape from the TNF- α -mediated cytotoxicity in the cytokine-rich milieu of HPS.

Constitutive Recruitment of TRADD by LMP-1 in H9 T Cells

To investigate further the mechanism of protection from cell death by LMP-1, we tested the possible association of LMP-1 with TRADD protein, which contains a 111-amino acid death domain with sequence similarity to that of

TNFR1. Cell lysates were immunoprecipitated with an anti-TRADD antibody. The immunoprecipitates were examined by immunoblot with anti-TNFR1, anti-LMP-1, and anti-TRADD in the presence of exogenous TNF- α or control medium. As shown in Figure 4A, the recruitment of endogenous TRADD by TNFR1 in control T cells could be demonstrated only after treatment with exogenous TNF- α . However, this recruitment of TRADD by treatment with TNF- α was significantly blocked by LMP-1 protein (Figure 4A, top lane). Furthermore, LMP-1 could constitutively engage with TRADD even in the absence of exogenous TNF- α (Figure

Figure 7. Schematic depiction of the molecular mechanism for the progression from HPS to chronic active disease or T-cell lymphoma in EBV-infected T cells. EBV LMP-1 up-regulates TNF- α via the TRAFs/NF- κ B signals in one way and down-regulates TNFR1 in the other way to suppress the apoptotic signal pathway, allowing the LMP-1-expressing T cells to survive from TNF- α -induced apoptosis, whereas the bystander cells undergo apoptosis and lead to systemic cytokine injuries. This model may represent a prototype example for the pathogenesis of virus- or inflammation-associated cancer.

4A, middle lane). The engagement with anti-TRADD assured the equal amount of loading volume (Figure 4A, bottom lane). Comparable immunoprecipitation experiments were performed by using anti-LMP-1 to precipitate the protein lysates of T cells expressed an increasing level of LMP-1. Likewise, LMP-1 was found to co-immunoprecipitate with TRADD, TRAF2, and TRAF5 protein (Figure 4B).

To confirm further the recruitment of TRADD proteins by LMP-1, we examined the subcellular localization of endogenous TRADD and TNFR1 proteins in LMP-1-transfected T cells under TNF- α stimulation by immunofluorescence microscopy. In control T cells, most of the TRADD proteins were present in the cytoplasm or membranous region, whereas TNFR1 was expressed on the membrane of T cells (Figure 5, A and B). In the merged figures (Figure 5B, right), TNFR1 and TRADD proteins were found to co-localize. In LMP-1-expressing T cells, however, the engagement of TNFR1 and TRADD diminished to a minimal level even after stimulating with exogenous TNF- α (Figure 5D), whereas the co-localization of TRADD with LMP-1 was clearly demonstrated in a manner of capping (dot-like) or along the cell membrane (Figure 5C). These observations were consistent with the data obtained in the co-immunoprecipitation experiment.

Reconstitution of TNFR1 Restored Apoptosis in LMP-1-Expressing H9 T Cells

To confirm further that the down-regulation of TNFR1 represents the critical event for the LMP-1-expressing T

cells to escape from TNF- α -induced apoptosis, reconstitution of TNFR1 and assessment of its effect on apoptosis was examined. The cytomegalovirus-derived TNFR1 plasmid and control vector were transiently transfected to the LMP-1 H9 cells. In parallel with TNF- α secretion, the activity of the key enzyme, caspase-3, in the apoptosis pathway was found to be activated again by reconstituting TNFR1 in 24 hours on LMP-1 H9 cells (Figure 6A). Furthermore, the reconstitution of TNFR1 could properly induce apoptosis on LMP-1 H9 cells (Figure 6B). The reconstitution of TNFR1 showed no influence on the secretion of endogenous TNF- α in LMP-1-expressing T cells (Figure 6C). By the addition of neutralizing anti-TNF- α antibody to block the endogenous TNF- α stimulation, the TNFR1-reconstituted LMP-1 H9 cells could again escape from endogenous TNF- α -induced apoptosis (Figure 6D). Overall, these data indicated that the down-regulation of TNFR1 by LMP-1 in T cells represents a critical event in the resistance of LMP-1-expressing T cells to apoptosis induced by TNF- α , either endogenous or exogenous.

Discussion

In the past decades, physicians were puzzled by the observation that a substantial percentage of HPS patients developed relapsing or progressive disease after receiving the initial therapy.^{7,29,30} These findings suggested to us that the EBV-infected T cells may survive or even

proliferate in the cytokine milieu of HPS, in which TNF- α is presumed to be the culprit cytokine.⁸ Consistent with previous studies,²⁶ we demonstrated here that EBV LMP-1 could up-regulate TNF- α via a TRAFs/NF- κ B pathway in EBV-infected T cells. Importantly, LMP-1-expressing T cells could escape and survive from TNF- α -induced apoptosis through down-regulation of TNFR1, providing a potential mechanism to explain the relapsing or progression to T-cell lymphoma in HPS patients.

The most compelling observation of this study is the competition and consequence of two death and survival signal pathways initiated by two members of TNFR superfamily, ie, TNFR1 and LMP-1, in T cells. Although both molecules can interact with TRADD and TRAF2, they exhibit entirely different downstream signaling and biological responses.^{13,36} The TNF- α -TNFR1 complex elicits an apoptosis process in most events, whereas the LMP-1/TRAFs/NF- κ B signal usually protects cells from apoptosis and leads to proliferation and transformation in B cells.³⁷⁻³⁹ For T-cell-type in this study, the LMP-1-induced signal appears to be dominated over the TNF- α /TNFR1 signals in a unique manner through the down-regulation of TNFR1 and recruitment of TRADD by LMP-1. In the presence of TNF- α , the control T cells showed aggregation of TNFR1 and TRADD, leading to the activation of proapoptotic caspases. In LMP-1-expressing T cells, however, the TRADD molecule was constitutively recruited by LMP-1 and dissociated from TNFR1 independent of TNF- α . In LMP-1-expressing T cells, the activities of the downstream molecules of TNF- α /TNFR1/TRADD apoptotic signals such as caspases 3, 8, and 9 and cytochrome *c* were remarkably suppressed. Reconstitution of TNFR1 could effectively reverse the caspase activities, resulting in apoptosis of LMP-1-expressing T cells and further supporting that the down-regulation of TNFR1 is the critical event in the survival of LMP-1-expressing T cells from TNF- α -induced apoptosis.

The suppression of TNFR1 by viral proteins appears to be a common mechanism for virus-infected cells to escape from cytokine injuries in virus infection. TNFR1 can also be transcriptionally inhibited by EBV immediate-early protein BZLF1 in HeLa and A549 cell lines.⁴⁰ EBV may therefore use different viral proteins or strategies to regulate the expression of TNFR1 in both lytic and latent programs to escape from TNF- α -induced cytotoxicity. In recent years, similar findings have been observed in other virus infection-associated immunodeficiencies. The HIV accessory protein Nef can also protect HIV-infected T cells from cell death by inhibiting the expression of TNFR1 or FAS.^{41,42}

The other members of TNFR superfamily, CD30 and CD40, appear to behave in a different manner, although also signaling via the TRAFs/NF- κ B pathway. CD40 has been shown to be overexpressing in EBV-infected T or NK cells.⁴³ In Hodgkin's lymphoma, there is constitutive overexpression and activation of CD30.⁴⁴ Distinct from TNFR1 and LMP-1, the adaptor protein TRAFs for CD30 and CD40 do not recruit the death domain protein TRADD and may explain the differential biological responses for the members of TNFR superfamily.⁴⁵ Preliminary data in our laboratory revealed that CD30 was down-regulated, whereas CD40 was up-regulated, in

LMP-1-expressing T cells (H.-C.C. and I.-J.S., unpublished data). The exact role of TNFR superfamily members in EBV-infected cells remains to be clarified in the future.

The findings we observed in the studies of LMP-1-expressing T cells appear to be consistent with the current concept of molecular mechanism of inflammation-associated cancers, involving inflammatory cytokines and NF- κ B activation.⁴⁶⁻⁴⁹ As summarized in Figure 7, the microbial infections may trigger an inflammatory response. The cytokine secretion may result in injuries or apoptosis of the bystander cells, representing the initial events or early phase of inflammation-associated cancers. The activation of NF- κ B and subsequent suppression of apoptosis in virus-infected cells, through down-regulation of TNFR1 in this case, may then lead to persistent disease and progress finally to malignancies.⁵⁰ Therapeutic target on the inhibition of NF- κ B signal pathway may therefore be designed for patients with HPS or virus-associated T-cell lymphoma.

References

1. Klein G, Pearson G, Nadkarni JS, Nadkarni JJ, Klein E, Henle G, Henle W, Clifford P: Relation between Epstein-Barr viral and cell membrane immunofluorescence of Burkitt tumor cells. I. Dependence of cell membrane immunofluorescence on presence of EB virus. *J Exp Med* 1968, 128:1011-1020
2. Rickinson AB: Epstein-Barr virus in action in vivo. *N Engl J Med* 1998, 338:1461-1463
3. Su IJ, Hsieh HC, Lin KH, Uen WC, Kao CL, Chen CJ, Cheng AL, Kadin ME, Chen JY: Aggressive peripheral T-cell lymphomas containing Epstein-Barr viral DNA: a clinicopathologic and molecular analysis. *Blood* 1991, 77:799-808
4. Andersson-Anvret M, Forsby N, Klein G, Henle W: Relationship between the Epstein-Barr virus and undifferentiated nasopharyngeal carcinoma: correlated nucleic acid hybridization and histopathological examination. *Int J Cancer* 1977, 20:486-494
5. Imashuku S, Hibi S, Fujiwara F, Ikushima S, Todo S: Hemophagocytic lymphohistiocytosis, interferon-gamma-naemia and Epstein-Barr virus involvement. *Br J Haematol* 1994, 88:656-658
6. Su IJ, Chen RL, Lin DT, Lin KS, Chen CC: Epstein-Barr virus (EBV) infects T lymphocytes in childhood EBV-associated hemophagocytic syndrome in Taiwan. *Am J Pathol* 1994, 144:1219-1225
7. Su IJ, Wang CH, Cheng AL, Chen RL: Hemophagocytic syndrome in Epstein-Barr virus-associated T-lymphoproliferative disorders: disease spectrum, pathogenesis, and management. *Leuk Lymphoma* 1995, 19:401-406
8. Lay JD, Tsao CJ, Chen JY, Kadin ME, Su IJ: Upregulation of tumor necrosis factor-alpha gene by Epstein-Barr virus and activation of macrophages in Epstein-Barr virus-infected T cells in the pathogenesis of hemophagocytic syndrome. *J Clin Invest* 1997, 100:1969-1979
9. Gharaee-Kermani M, Phan SH: The role of eosinophils in pulmonary fibrosis. *Int J Mol Med* 1998, 1:43-53
10. Egeler RM, Favara BE, van Meurs M, Laman JD, Claassen E: Differential in situ cytokine profiles of Langerhans-like cells and T cells in Langerhans cell histiocytosis: abundant expression of cytokines relevant to disease and treatment. *Blood* 1999, 94:4195-4201
11. Collin L, Moulin P, Jungers M, Geubel AP: Epstein-Barr virus (EBV)-induced liver failure in the absence of extensive liver-cell necrosis: a case for cytokine-induced liver dysfunction? *J Hepatol* 2004, 41:174-175
12. Aggarwal S, Gollapudi S, Yel L, Gupta AS, Gupta S: TNF-alpha-induced apoptosis in neonatal lymphocytes: TNFRp55 expression and downstream pathways of apoptosis. *Genes Immun* 2000, 1:271-279
13. Micheau O, Tschopp J: Induction of TNF receptor I-mediated

- apoptosis via two sequential signaling complexes. *Cell* 2003, 114:181–190
14. Lay JD, Chuang SE, Rowe M, Su IJ: Epstein-Barr virus latent membrane protein-1 mediates upregulation of tumor necrosis factor- α in EBV-infected T cells: implications for the pathogenesis of hemophagocytic syndrome. *J Biomed Sci* 2003, 10:146–155
15. Chen CL, Sadler RH, Walling DM, Su IJ, Hsieh HC, Raab-Traub N: Epstein-Barr virus (EBV) gene expression in EBV-positive peripheral T-cell lymphomas. *J Virol* 1993, 67:6303–6308
16. Anagnostopoulos I, Hummel M, Stein H: Frequent presence of latent Epstein-Barr virus infection in peripheral T cell lymphomas. A review. *Leuk Lymphoma* 1995, 19:1–12
17. van Gorp J, Brink A, Oudejans JJ, van den Brule AJ, van den Tweel JG, Jiwa NM, de Bruin PC, Meijer CJ: Expression of Epstein-Barr virus encoded latent genes in nasal T cell lymphomas. *J Clin Pathol* 1996, 49:72–76
18. Peng M, Lundgren E: Transient expression of the Epstein-Barr virus LMP-1 gene in human primary B cells induces cellular activation and DNA synthesis. *Oncogene* 1992, 7:1775–1782
19. Mosialos G, Birkenbach M, Yalamanchili R, VanArsdale T, Ware C, Kieff E: The Epstein-Barr virus transforming protein LMP-1 engages signaling proteins for the tumor necrosis factor receptor family. *Cell* 1995, 80:389–399
20. Gires O, Zimmer-Strobl U, Gonnella R, Ueffing M, Marschall G, Zeidler R, Pich D, Hammerschmidt W: Latent membrane protein 1 of Epstein-Barr virus mimics a constitutively active receptor molecule. *EMBO J* 1997, 16:6131–6140
21. Cahir McFarland ED, Izumi KM, Mosialos G: Epstein-Barr virus transformation: involvement of latent membrane protein 1-mediated activation of NF- κ B. *Oncogene* 1999, 18:6959–6964
22. Huen DS, Henderson SA, Croom-Carter D, Rowe M: The Epstein-Barr virus latent membrane protein-1 (LMP-1) mediates activation of NF- κ B and cell surface phenotype via two effector regions in its carboxy-terminal cytoplasmic domain. *Oncogene* 1995, 10:549–560
23. Eliopoulos AG, Waites ER, Blake SM, Davies C, Murray P, Young LS: TRAF1 is a critical regulator of JNK signaling by the TRAF-binding domain of the Epstein-Barr virus-encoded latent infection membrane protein 1 but not CD40. *J Virol* 2003, 77:1316–1328
24. Li HM, Zhuang ZH, Wang Q, Pang JC, Wang XH, Wong HL, Feng HC, Jin DY, Ling MT, Wong YC, Eliopoulos AG, Young LS, Huang DP, Tsao SW: Epstein-Barr virus latent membrane protein 1 (LMP-1) up-regulates Id1 expression in nasopharyngeal epithelial cells. *Oncogene* 2004, 23:4488–4494
25. Thornburg NJ, Kulwichit W, Edwards RH, Shair KH, Bendt KM, Raab-Traub N: LMP-1 signaling and activation of NF- κ B in LMP-1 transgenic mice. *Oncogene* 2006, 25:288–297
26. Chuang HC, Lay JD, Hsieh WC, Wang HC, Chang Y, Chuang SE, Su IJ: Epstein-Barr virus LMP-1 inhibits the expression of SAP gene and upregulates Th1 cytokines in the pathogenesis of hemophagocytic syndrome. *Blood* 2005, 106:3090–3096
27. Imashuku S, Teramura T, Tauchi H, Ishida Y, Otoh Y, Sawada M, Tanaka H, Watanabe A, Tabata Y, Morimoto A, Hibi S, Henter JI: Longitudinal follow-up of patients with Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis. *Haematologica* 2004, 89:183–188
28. Hayashi K, Joko H, Koiraia TR, Onoda S, Jin ZS, Munemasa M, Ohara N, Oda W, Tanaka T, Oka T, Kondo E, Yoshino T, Takahashi K, Yamada M, Akagi T: Therapeutic trials for a rabbit model of EBV-associated hemophagocytic syndrome (HPS): effects of vidarabine or CHOP, and development of Herpesvirus papio (HVP)-negative lymphomas surrounded by HVP-infected lymphoproliferative disease. *Histol Histopathol* 2003, 18:1155–1168
29. Chen JS, Lin KH, Lin DT, Chen RL, Jou ST, Su IJ: Longitudinal observation and outcome of nonfamilial childhood haemophagocytic syndrome receiving etoposide-containing regimens. *Br J Haematol* 1998, 103:756–762
30. Takeoka Y, Nakao Y, Ueda M, Koh KR, Aoyama Y, Nakamae H, Yamamura R, Ohta K, Takubo T, Yamane T, Hino M, Tokura Y, Ishihara S, Oshima K, Kimura H, Imashuku S: A case of a long-time survivor with chronic active Epstein-Barr virus infection. *Eur J Haematol* 2004, 72:73–76
31. Baer R, Bankier AT, Biggin MD, Deininger PL, Farrell PJ, Gibson TJ, Hatfull G, Hudson GS, Satchwell SC, Séguin C, Tuffnell PS, Barrell BG: DNA sequence and expression of the B95-8 Epstein-Barr virus genome. *Nature* 1984, 310:207–211
32. Aizawa S, Nakano H, Ishida T, Horie R, Nagai M, Ito K, Yagita H, Okumura K, Inoue J, Watanabe T: Tumor necrosis factor receptor-associated factor (TRAF) 5 and TRAF2 are involved in CD30-mediated NF- κ B activation. *J Biol Chem* 1997, 272:2042–2045
33. Horie R, Watanabe T, Ito K, Morisita Y, Watanabe M, Ishida T, Higashihara M, Kadin M, Watanabe T: Cytoplasmic aggregation of TRAF2 and TRAF5 proteins in the Hodgkin-Reed-Sternberg cells. *Am J Pathol* 2002, 160:1647–1654
34. Su WB, Chang YH, Lin WW, Hsieh SL: Differential regulation of interleukin-8 gene transcription by death receptor 3 (DR3) and type I TNF receptor (TNFRI). *Exp Cell Res* 2006, 312:266–277
35. Chuang SE, Yeh PY, Lu YS, Lai GM, Liao CM, Gao M, Cheng AL: Basal levels and patterns of anticancer drug-induced activation of nuclear factor- κ B (NF- κ B), and its attenuation by tamoxifen, dexamethasone, and curcumin in carcinoma cells. *Biochem Pharmacol* 2002, 63:1709–1716
36. Kieser A, Kaiser C, Hammerschmidt W: LMP-1 signal transduction differs substantially from TNF receptor 1 signaling in the molecular functions of TRADD and TRAF2. *EMBO J* 1999, 18:2511–2521
37. Mancao C, Altmann M, Jungnickel B, Hammerschmidt W: Rescue of “crippled” germinal center B cells from apoptosis by Epstein-Barr virus. *Blood* 2005, 106:4339–4344
38. Dirmeier U, Hoffmann R, Kilger E, Schultheiss U, Brisen C, Gires O, Kieser A, Eick D, Sugden B, Hammerschmidt W: Latent membrane protein 1 of Epstein-Barr virus coordinately regulates proliferation with control of apoptosis. *Oncogene* 2005, 24:1711–1717
39. Grimm T, Schneider S, Naschberger E, Huber J, Guenzi E, Kieser A, Reitmair P, Schulz TF, Morris CA, Sturzl M: EBV latent membrane protein-1 protects B cells from apoptosis by inhibition of BAX. *Blood* 2005, 105:3263–3269
40. Morrison TE, Mauser A, Klingelutz A, Kenney SC: Epstein-Barr virus immediate-early protein BZLF1 inhibits tumor necrosis factor α -induced signaling and apoptosis by downregulating tumor necrosis factor receptor 1. *J Virol* 2004, 78:544–549
41. Geleziunas R, Xu W, Takeda K, Ichijo H, Greene WC: HIV-1 Nef inhibits ASK1-dependent death signalling providing a potential mechanism for protecting the infected host cell. *Nature* 2001, 410:834–838
42. Xu XN, Screaton G: HIV-1 Nef: negative effector of Fas? *Nat Immunol* 2001, 2:384–385
43. Imadome K, Shimizu N, Arai A, Miura O, Watanabe K, Nakamura H, Nonoyama S, Yamamoto K, Fujiwara S: Coexpression of CD40 and CD40 ligand in Epstein-Barr virus-infected T and NK cells and their role in cell survival. *J Infect Dis* 2005, 192:1340–1348
44. Horie R, Watanabe T, Morishita Y, Ito K, Ishida T, Kanegae Y, Saito I, Higashihara M, Mori S, Kadin ME, Watanabe T: Ligand-independent signaling by overexpressed CD30 drives NF- κ B activation in Hodgkin-Reed-Sternberg cells. *Oncogene* 2002, 21:2493–2503
45. Lam N, Sugden B: CD40 and its viral mimic, LMP-1: similar means to different ends. *Cell Signal* 2003, 15:9–16
46. Pikarsky E, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, Gutkovich-Pyest E, Urieli-Shoval S, Galun E, Ben-Neriah Y: NF- κ B functions as a tumour promoter in inflammation-associated cancer. *Nature* 2004, 431:461–466
47. Huber MA, Azoitei N, Baumann B, Grunert S, Sommer A, Pehamberger H, Kraut N, Beug H, Wirth T: NF- κ B is essential for epithelial-mesenchymal transition and metastasis in a model of breast cancer progression. *J Clin Invest* 2004, 114:569–581
48. Chen G, Bhojani MS, Heaford AC, Chang DC, Laxman B, Thomas DG, Griffin LB, Yu J, Coppola JM, Giordano TJ, Lin L, Adams D, Orringer MB, Ross BD, Beer DG, Rehmertulla A: Phosphorylated FADD induces NF- κ B, perturbs cell cycle, and is associated with poor outcome in lung adenocarcinomas. *Proc Natl Acad Sci USA* 2005, 102:12507–12512
49. Aggarwal BB: Nuclear factor- κ B: the enemy within. *Cancer Cell* 2004, 6:203–208
50. Yoshimura A: Signal transduction of inflammatory cytokines and tumor development. *Cancer Sci* 2006, 97:439–447