Up-Regulation of Activating Transcription Factor-5 Suppresses SAP Expression to Activate T Cells in Hemophagocytic Syndrome Associated with Epstein-Barr Virus Infection and Immune Disorders

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Hemophagocytic syndrome (HPS) is a fatal, pro-inflammatory cytokine disorder that is associated with viral infections and immune disorders. Previously, we demonstrated that Epstein-Barr virus latent membrane protein-1 (LMP-1) could down-regulate the *SAP* gene, enhancing Th1 cytokine secretion in T cells and leading to HPS. The exact mechanism of *SAP* gene regulation by LMP-1 remains to be clarified. In this study, using cDNA microarray analysis, we identified ATF5 as the candidate transcriptional repressor for *SAP* expression in LMP-1-expressing T cells. LMP-1 up-regulated ATF5 via TRAF2,5/NF- κ B signals to suppress *SAP* gene expression. Reporter assays and electrophoretic mobility shift assays revealed that ATF5 bound differentially to two sites of the *SAP* promoter. In resting T cells, ATF5 bound predominantly to the high-affinity site in the -81 to -74 region while additionally binding to the low-affinity site at -305 to -296 in LMP-1-expressing T cells. Such binding subsequently disrupted the transcription of the *SAP* gene. At the same time, Th1 cytokine secretion was enhanced. This phenomenon was also observed in conditions such as ATF5 overexpression, phytohemagglutinin stimulation of primary T cells, and ligand engagement of T-cell lines. Therefore, the down-regulation of the *SAP* gene by ATF5 may represent a common mechanism for the pathogenesis of HPS that is associated with either Epstein-Barr virus infection or immune disorders with dysregulated T-

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Hemophagocytic syndrome (HPS) is a fatal pro-inflammatory cytokine disorder frequently associated with severe virus infections such as Epstein-Barr virus (EBV) and recently H5N1 avian influenza.^{1,2} HPS may also occur in immune disorders such as systemic lupus erythematosus and juvenile rheumatoid arthritis.^{3,4} HPS is characterized by hepatosplenomegaly, pancytopenia, coagulopathy, and dysregulation of T cells with enhanced secretion of pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ).^{5–7} These proinflammatory cytokines may subsequently cause systemic tissue damage and macrophage activation with phagocytosis of blood cells in the presence of autoantibodies.^{8–11}

The major breakthrough in our understanding of the pathogenesis of HPS comes from the identification of the mutations of the *SH2D1A/SAP* (signals for lymphocyte activation molecule-associated protein) gene in X-linked lymphoproliferative disorder (XLP).^{12,13} The mutations of the *SAP* gene will lead to the activation of the SLAM/ERK pathway for T-cell activation and enhanced secretion of Th1 cytokines as is observed in XLP patients with HPS.^{14,15} The exact regulatory mechanism for *SAP* gene regulation, however, remains to be clarified. Previously, we demonstrated that EBV latent membrane protein-1 (LMP-1) could suppress the *SAP* gene expression mediated via the TNF-associated factors (TRAFs)/nuclear factor- κ B (NF- κ B) signaling pathway.¹⁶ The suppression of *SAP* by NF- κ B-mediated signal pathway represents a

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novel phenomenon, because the activation of NF- κ B usually operates in a positive manner to promote transcription of genes associated with inflammation or tumorigenesis.¹⁷ Several virus proteins have been shown to suppress the transcription of host genes via transcriptional repressors.^{18–22} These examples suggest to us that EBV LMP1 may elicit a transcriptional repressor via NF- κ B to suppress the expression of the *SAP* gene. Identifying this transcriptional repressor will assist us in clarifying the regulatory mechanism of *SAP* gene in T-cell activation and also help us understand the pathogenesis of HPS. Thus, we have performed cDNA microarray in LMP-1-expressed H9 T cell line and successfully identified the activating transcription factor-5 (ATF5) as the transcriptional repressor that regulates *SAP* expression. Noticeably, the down-regulation of *SAP* gene by ATF5 could also be observed in other conditions such as ATF5 overexpression, phytohemagglutinin (PHA) stimulation of primary T cells, or ligand engaging of T cell lines, suggesting that the down-regulation of *SAP* by ATF5 may represent a common mechanism for the pathogenesis of HPS associated with either severe virus infections or immune disorders related to dysregulated T-cell activation.

Materials and Methods

Lymphoma Cell Lines and Stable Cell Lines Expressing LMP1

The EBV-negative T-cell lines H9 and Jurkat were used in this study and maintained in RPMI 1640 medium (JRH, Lenexa, NY) supplemented with 10% (v/v) fetal bovine serum (JRH). Cells used for the following experiments were in the exponential phase of growth and limit-cultured for 1 month. The stable pSG5-LMP1-expressed H9 cell line and TRAF2/5 dominant-negative mutation co-expressed lines have previously been well established.¹⁶ The cells were maintained in RPMI containing 400 μ g/ml of neomycin. The purification of primary T cells was performed as previously described.²³

Transient Transfection

To suppress ATF3 or ATF5 expression, ATF3 siRNA sequences (Santa Cruz Biotechnology, Santa Cruz, CA), ATF5 siRNA sequences (Invitrogen, Carlsbad, CA), ATF7 siRNA sequences (Santa Cruz Biotechnology) were constructed to pSUPER RNAi system (Oligoengine, Seattle, WA). These pSUPER-shRNAs and the control vector were used. To perform the luciferase reporter assay, a series of promoter regions –1250 to +11, –420 to +11, –210 to +11, –420 to –210, and –420 to +11 with mutated sites (change to AAAAAAAAAA for M1 and M3, or to TTTTTTTTTT for M2) were constructed to pGL3-basic vector (Promega, Madison, WI). For transient transfection, a total of 1×10^6 cells were transfected by the MicroPorator (Digital Bio Technology, Seoul, Korea) with a total of 2.0 μ g of DNA in 120 μ l of resuspension buffer. The electroporation settings were as follows: 1420 mV, 30 ms, and one

pulse for T-cell lines; 2200 mV, 30 ms, and one pulse for primary T cells. The highest efficiencies were 78% for transient transfection of T-cell lines and 39% for primary T cells.

Microarray Analysis

A total of 1×10^6 pSG5-H9 or LMP1-H9 cells were sorted, and their RNA were extracted with TRIzol (Invitrogen), yielding 30 ng of total for each sample. The cDNA microarray was performed by Egenomic Co (Taipei City, Taiwan), using human 20K-2-JH chips with 20,000 genes from the National Institutes of Health. Labeled cDNA probes were prepared and the microarray was hybridized, according to the protocol from the laboratory of Pat Brown at the Department of Biochemistry, Howard Hughes Medical Institute, Chevy Chase, MD.

Luciferase Reporter Assay

Luciferase reporter assay was performed in Jurkat cells containing higher *SAP* expression than H9 cells. Various reporter plasmids were constructed in vector pGL3-basic (Promega). Renilla expression vector driven by the herpes simplex virus thymidine kinase promoter (pRL-TK, Promega) was co-transfected to standardize each experiment. The cells were washed by phosphate-buffered saline, harvested, and resuspended in RPMI medium (60 μ l) and lysis buffer (60 μ l) (Promega). After incubating for 10 minutes, the luciferase activity was measured. The luciferase-stop/renilla buffer (60 μ l) was added for another 10 minutes, after which the renilla (pRL-TK) activity was measured for normalization. The data represent the mean with SD error bar of luciferase activity relative to the vector control cells.

Chromatin Immunoprecipitation (ChIP)

Cells (1×10^6) were first cross-linked with 27% formaldehyde solution. The cross-linked protein-chromatin material was then sonicated at 95% power for three times/second to obtain ~300-bp DNA fragments, which were then immunoprecipitated with 50 μ l of agarose-L (Sigma, St. Louis, MO) and 1 μ l of anti-ATF5 antibody (Imgenex, San Diego, CA) or normal serum to obtain protein-DNA complexes of interest. The amount of specific DNA immunoprecipitated, was then analyzed by polymerase chain reaction (PCR) amplification using specific primers.

Western Blotting

Transfected cells or stable cell lines were harvested with lysis buffer RIPA, followed by clearing lysates via centrifugation at 13,000 rpm for 25 minutes. Immunoblotting experiments were performed as previously described. Anti-LMP1 mouse monoclonal antibody (CS1-4; DAKO, Glostrup, Denmark), anti-ATF5 antibody (Imgenex), anti-SAP rabbit antibody, anti- α -actin goat antibody (Santa Cruz Biotechnology), and NF- κ B inhibitor

Bay11-7082 (Sigma) were used. ECL chemiluminescence kits (PerkinElmer Life Sciences, Boston, MA) were then used for antibody binding.

Electrophoresis Mobility Shift Assay (EMSA)

Two double-stranded ATF5 consensus oligonucleotides: SAP -309 to -284 5'-caatcttgcaaaatccttctccaat-3' and SAP -90 to -66 5'-tcaggtggttgactgtgcctggct-3' end-labeled with biotin were used. Nuclear extracts (10 μ g) were preincubated for 10 minutes with ATF5 antibody for interference, then incubated in 1 $\times$ binding buffer, 1 μ g poly (dl:dC), 0.5% Nonidet P-40 (Pierce Biotechnology, Rockford, IL), and 1 μ l biotin-labeled probe at 37°C for 20 minutes. When competitors were used, they were added to the reactions 10 minutes before the labeled probe. The samples were subjected to 6% nondenaturing acrylamide gel electrophoresis and visualized with X-ray film.

Results

ATF5, Not ATF3/7, Plays a Key Role in LMP1-Induced Suppression of SAP

To define the crucial repressor for suppressing SAP by LMP1, a cDNA microarray was performed to compare the gene expression in LMP1-expressed H9 T cells with control H9 cells. The microarray data were analyzed by GeneMAPP2 and revealed that compared to control H9 cells, only transcriptional repressors ATF3 and ATF5 were significantly enhanced in LMP1-expressed H9 cells, with 3.06- and 2.55-fold levels, respectively (Figure 1A). Because the expression ratio of other transcriptional repressors was lower than the criteria (twofold), the role of ATF3 and ATF5 were studied below. To confirm the specific repressor in suppressing SAP, the siRNAs of ATF3, ATF5, and the matterless control ATF7 siRNA were separately constructed into pSUPER vector and transfected into stably LMP1-expressed H9 clone. Among these three siRNAs, only ATF5 siRNA could significantly reverse the expression of SAP in LMP1-expressed H9 cells (Figure 1B). To further confirm the effect of ATF5 siRNAs, the luciferase reporter assay was examined in Jurkat T cells, a positive control line for SAP expression. The promoter activity of SAP was down-regulated in LMP1-Jurkat cells, but could be reactivated by ATF5 siRNA (Figure 1C). Although the SAP promoter activity was not fully restored (70.4%), the 78% transfection efficiency on Jurkat cells was significant enough. These results indicate that ATF5 plays a key role in LMP1-mediated suppression of SAP in T cells.

Overexpression of ATF5 Down-Regulates SAP and Enhances Cytokine Secretion

To confirm the down-regulation of SAP by ATF5, ATF5 was overexpressed *in vitro* in Jurkat and H9 T-cell lines, and the expression of SAP was then examined. The ex-

A Identification of transcription repressors ATF3 and ATF5 by cDNA array.

ATF3	3.066
ATF5	2.569
CALR	
CART1	
CBX4	
CSDA	
CSEN	
CTCF	
DDIT3

Figure 1. ATF5, not ATF3/7, plays an important role in LMP1-mediated suppression of SAP. **A:** The H9 cell line stably transfected with LMP1 and with control vector (pSG5) was harvested to perform cDNA microarray analysis. The results shown were the ratio of transcriptional repressors of LMP1-expressed H9 to pSG5-H9 cells. The criteria for up-regulation and down-regulation were a ratio of up to 2-fold and less than 0.5-fold, respectively. **B:** To confirm and identify the candidate repressor, the shRNA (pSUPER-EGFP) of ATF3, ATF5, and ATF7 were separately transfected to LMP1-expressed H9 cells. The Western blotting showed the expression of SAP only in ATF5 shRNA-transfected H9 T cells. The expression of ATF3, ATF5, and ATF7 revealed the specificity of each shRNA. The expression of actin was shown in the bottom to ensure equal protein loading. **C:** Luciferase reporter assay for the SAP promoter. Equal numbers of Jurkat T cells with high expressing level of SAP were used and transiently transfected with pSG5-LMP1, reporter construct, and shRNA. The fold induction was relative to Jurkat control cells transfected with reporter vector (pGL3-Basic).

pression of SAP was found to be down-regulated in ATF5-overexpressed Jurkat and H9 cells (Figure 2A), which is in parallel with enhanced TNF- α /IFN- γ secretion (Figure 2B). Furthermore, the promoter activity of SAP was also inhibited in ATF5-overexpressed Jurkat cells, similar to that in LMP1-overexpressed H9 cells (Figure 2C). These results indicate that enhanced expression of ATF5 by LMP1 or by *in vitro* ATF5 expression contributes

Figure 2. Overexpression of ATF5 induced secretion of Th1 cytokines via TRAF/NF-κB pathway. **A:** Jurkat and H9 T cell lines were transiently transfected with ATF5 or LMP1 plasmids. After 48 hours, cells were harvested and protein lysates were collected. Overexpression of ATF5 and suppression of SAP were observed by Western blotting assay. **B:** Jurkat and H9 cells (5×10^5 cells/5 ml of medium) were transiently transfected with ATF5 and LMP1 plasmids. After 48 hours, supernatants were collected to perform ELISA assay of TNF-α and IFN-γ. The results shown are representative of three independent experiments. **C:** Jurkat cells were co-transfected with reconstituted plasmid, SAP promoter-driven luciferase reporter construct, and control plasmid, and performed luciferase reporter assay. The fold induction was relative to control reporter plasmid (pGL3-Basic). **D:** The TRAF2/5 dominant-negative plasmid (TRAF2DN/5DN) could express mutated protein and effectively block the effect of LMP1 signaling on the up-regulation of ATF5 and suppression of SAP. **E:** LMP1-expressed H9 cells were treated with indicated dose Bay 11-7082 for 24 hours, and expression levels ATF5, SAP, and LMP1 were examined by Western blotting assay. Actin level was used for loading control. **F:** LMP1-expressed H9 cells were transfected with IκB dominant-negative plasmid (IκBDN) for 24 hours to inhibit NF-κB activation. The expression levels of ATF5, SAP, and LMP1 detected by Western blotting assay were also showed here.

to the suppression of *SAP* gene transcription and expression, leading to T-cell activation and enhanced cytokine secretion as observed in EBV-associated HPS.

LMP1 Up-Regulates ATF5 via TRAF2,5/NF-κB Signal Pathway

To study the ATF5 enhancement by signal transduction of LMP1, the dominant-negative mutants of TRAF2 and TRAF5 were individually co-expressed with LMP1 in H9 cells. Both dominant-negative mutants could block the ATF5 up-regulation by LMP1 and at the same time restore the SAP expression (Figure 2D). Next, NF-κB inhibitor Bay11-7082 was used to inhibit NF-κB activity. After treating with Bay 11-7082 for 24 hours, the ATF5 expression was suppressed in LMP1-expressed H9 cells in a dose-dependent manner (Figure 2E). The SAP expression was also increased after treatment with the NF-κB inhibitor. Consistently, the dominant-negative plasmids of IκB for inhibiting NF-κB activity also inhibited ATF5 expression and reversed SAP expression in LMP1-H9 cells (Figure 2F). These data indicate that LMP1 could activate TRAF2/5 and NF-κB signaling, resulting in ATF5 up-regulation and then suppressing SAP expression.

Activated Primary T Cells Show Up-Regulation of ATF5 and Down-Regulation of SAP

Since the association between ATF5-regulated SAP and T-cell activation was first reported here, we further clari-

fied the physiological relevance of LMP1-induced ATF5 regulation of SAP in primary T cells and the effects of ligands engaging in Jurkat and H9 T-cell lines. We demonstrated that ATF5 was up-regulated by LMP1 in primary T cells (Figure 3A). Although ATF5 was certainly expressed, SAP expression was synchronously suppressed. LMP1-expressed resting T cells also secreted high levels of TNF-α/IFN-γ (Figure 3B) as did phytohemagglutinin (PHA)-stimulated T cells. In PHA-stimulated T cells, LMP1 induced a higher level of ATF5 and TNF-α/IFN-γ secretion than PHA-stimulated T cells (Figure 3, A and B). To further confirm the correlation of T-cell activation and ATF5 expression, the stimulation of PHA to primary T cells was examined with a time course. Although the resting T cells showed few ATF5 expressions, the ATF5 expression was up-regulated by PHA stimulation immediately and gradually decreased throughout time, from 6 to 48 hours (Figure 3C). After the up-regulation of ATF5, the SAP expression was suppressed in 2 to 24 hours. Although the expression level of ATF5 decreased to normal level, the SAP expression was subsequently restored at 48 hours. Moreover, the TNF-α and IFN-γ secretion were unexpectedly enhanced without SAP expression after treating with PHA for 2 hours (Figure 3D). Looking at ATF5 down-regulation and SAP restoration at 48 hours, the trends of cytokine production were reversed. To perform the T-cell activation in cell lines, Jurkat and H9 cells were stimulated by engaging the T-cell receptor. The anti-CD3/CD28-co-treated T-cell lines also showed similar up-regulation of ATF5 and suppression of SAP (Figure

Figure 3. Activated primary T cells or engaged T-cell lines showed up-regulation of ATF5. **A:** Primary T cells were isolated from whole blood by anti-CD3 beads. A half of primary T cells were treated with 2 μ mol/L PHA to stimulate and activate T cells for 18 hours. LMP1 was transfected into naive or PHA-stimulated primary T cells. After another 24 hours, protein lysates were collected for Western blotting, and supernatants were subjected to ELISA assay for IFN- γ and TNF- α . ATF5 up-regulation and SAP down-regulation in PHA-stimulated and LMP1-expressed T cells were shown by Western blotting analysis. **B:** The IFN- γ and TNF- α were dramatically induced by PHA and LMP1. **C and D:** The primary T cells were treated with or without 2 μ mol/L PHA for different time courses. The cell lysates and supernatants were collected to perform Western blotting and ELISA assay. The ATF5 was enhanced immediately, but decreased after treating PHA for 2 hours. The SAP expression was suppressed by PHA and restored at 48 hours. After the ATF5 up-regulation and SAP down-regulation, the IFN- γ and TNF- α secretion were induced. **E:** Jurkat and H9 cell lines were co-treated with anti-CD3 and anti-CD28 antibodies for 6 hours to activate T cells by engaging T-cell receptor. The induction of ATF5 and inhibition of SAP were observed in engaged T-cell lines and LMP1-expressed cells (**left**). While pretreating with ATF5-shRNA for 72 hours, the CD3/CD28-induced ATF5 up-regulation and SAP suppression were reversed in Jurkat T cells (**right**).

3E). Furthermore, the CD3/CD28-stimulated down-regulation of SAP could be blocked by ATF5 shRNA (Figure 3E, right). Therefore, the transient expression of ATF5 is effective in suppressing SAP expression. These results suggest that the regulatory role of ATF5 on SAP expression not only operates in EBV-associated HPS but may also play an important role in T-cell activation and enhanced cytokine secretion in other immune disorders.

The Region -420 to -210 of SAP Promoter Is Responsible for Inhibition of SAP Expression by ATF5

To identify the responsible region for ATF5 on the promoter of SAP, a series of promoter regions, -1250 to +11, -420 to +11 (a+b), -210 to +11 (b), and -420 to -210 (a) (Figure 4A) were constructed to form reporter

Figure 4. The region -420 to -210 of SAP promoter was responsible for inhibition by ATF5. **A:** A series of promoter regions -1250 to +11, -420 to +11 (a+b), -210 to +11 (b), and -420 to -210 (a) of SAP promoter were constructed to perform reporter assay. **B:** Luciferase reporter assay of the SAP promoter were analyzed for the LMP1-repressable region. Data were relative to control cells and expressed as the mean $\pm$ SD of quadruplicate sample. **C:** ChIP assay demonstrated binding of ATF5 to the promoter region of SAP. Control H9 and LMP1-expressed H9 cells were collected and ChIP assay was performed with anti-ATF5 and normal serum (negative control for precipitation). Precipitated DNA fragments were amplified by PCR using specific primers for the region -420 to -210 and region -210 to +11 of SAP promoter. Input material corresponds to 1% of the DNA material subjected to ChIP assay.

plasmids. The luciferase reporter activities for regions –1250 to +11 and –420 to +11 (a+b) were high (24.1- and 18.6-fold induction, respectively) in the control Jurkat cells, whereas only 33% of the activities were detected in LMP1-expressed Jurkat cells (Figure 4B). The activity of region –210 to +11 (b), devoid of –420 to –210 (a), was of relatively low activity (11.1-fold induction) in control pSG5-Jurkat cells, and the activity still remained in LMP1-Jurkat cells. The construct of region –420 to –210 showed low or insignificant activities (2.9- or 1.9-fold induction). These results suggest that the region –420 to –210 (a) is the candidate region responsible for the inhibition of SAP by LMP1-mediated ATF5. To confirm the direct binding of ATF5 on the SAP promoter, the ChIP assay was performed. It showed that ATF5 could bind to the region –210 to +11 (b) of the SAP promoter in both control and LMP1-expressed H9 cells (Figure 4C), whereas direct binding to region –420 to –210 (a) was only detected in LMP1-H9 cells.

ATF5 Bound to Regions –305 to –296 and –81 to –74 for Suppressing SAP Promoter Activity

Because the region –420 to –210 (a) of the SAP promoter contains the key site for SAP suppression, the software TFSEARCH was used to predict ATF5 binding site on this region (a) by constructing mutation plasmids to block SAP expression (Figure 5A). Within region (a), one predicted site M1: –342 to –335 (TGAGTGCA) was

only 60% similar to CRE, and site M2 mutated at the CCAAT/enhancer-binding protein (CEBP) binding element –305 to –296 (CTTGCAAAAT), a low-affinity site for the CREB family.²⁴ As indicated in Figure 4C, ATF5 could also bind with region –210 to +11 (b) of the SAP promoter, but there is no CEBP site predicted by TFSEARCH in this region (b). We further predicted the binding site of ATF5 on region (b) with another software PROMO. This software indicated the presence of a predicted ATF3 site at –81 to –74 (TGACTTGT) of the SAP promoter, which was then mutated as M3 for reporter assay. The activity of the SAP promoter as measured by luciferase reporter assay (Figure 5B) revealed that regions –1250 to +11, –420 to +11 (a+b), and (a+b) M1 were activated in control Jurkat cells and could be comparably suppressed by LMP1. Similar to the region –210 to +11 (b), the (a+b) M2 and M3 were activated in both control and LMP1-expressed cells. The data suggest that the binding on the two sites is essential for LMP1-mediated suppression of SAP. To further confirm the direct binding on site –305 to –296 and site –81 to –74 of the SAP promoter, EMSA assays were performed. As the EMSA assay showed (Figure 5C, top), LMP1-upregulated ATF5 directly bound to site –305 to –296 of the SAP promoter with high intensity. A weak band was detected in the control H9 cells because excessive 10 μ g of nuclear extract proteins were added *in vitro* in the EMSA assay, distinct from the *in vivo* ChIP assay (Figure 4C). Moreover, on the site –81 to –74 of the SAP promoter (Figure 5C, bottom), ATF5 bound with high affinity in both control and

Figure 5. ATF5 exactly binds to site –305 to –296 and site –81 to –74 for suppressing SAP promoter activity. **A:** Prediction of ATF5 binding sites on region –420 to –210 and region –210 to +11 of the SAP promoter. The three predicted sites were separately mutated (M1, M2, and M3) to identify crucial sites. **B:** Luciferase reporter constructs of the SAP promoter were analyzed for the LMP1-repressible region. Data were relative to control cells and expressed as the mean + SD of quadruplicate sample. **C:** EMSA was done using 10 μ g of nuclear extract proteins from pSG5-H9 or LMP1-H9 cells. Biotin-labeled probes contained ATF5 binding site of regions –305 to –296 (**top**) and –81 to –74 (**bottom**) of SAP promoter. Unlabeled competitor DNA and mutated competitor (M; consisted of the M2 or M3 in **A**) were added as indicated. The anti-ATF5 antibody (A) could recognize the partial DNA binding domain bZIP of ATF5, and interfere the binding of labeled DNA. The anti-histone H1 (H) antibody was added as negative control. **D:** Binding on the region –305 to –296 was also shown in ATF5-overexpressed H9 cells, but less in pSG5-H9 cells.

LMP1-H9 cells, which is consistent with the result of ChIP assay. Furthermore, mutated competitors of either site could not block the interaction between ATF5 and the probe, further confirming the crucial binding site of ATF5. The addition of anti-ATF5 antibody reduced the intensity of ATF5 binding because ATF5 antibody recognizes partial bZIP domain and interferes with DNA binding. Taken together, these results indicate the high-affinity direct binding of ATF5 on sites -81 to -74 of promoter but low affinity on site -305 to -296. To demonstrate that high concentration of ATF5 can bind to the low-affinity site -305 to -296, ATF5-overexpressed H9 cell were studied by EMSA assay (Figure 5D). Overexpressed ATF5 could bind to probe -305 to -296, which showed low affinity on normal concentration in control H9 cells. Taken together, our results suggest that normal or low concentrations of ATF5 at resting or physiological conditions only binds to site -81 to -74, but high levels of ATF5 will bind to both sites and subsequently disrupt the SAP expression.

Discussion

In this study, we demonstrated for the first time that EBV LMP1-induced suppression of SAP gene in T cells is mediated by a transcriptional repressor ATF5 to disrupt the transcription of the SAP gene, subsequently leading to enhanced Th1 cytokine secretion as observed in HPS. Furthermore, the down-regulation of SAP by ATF5 could also be observed in PHA stimulation of primary T cells and ligand (CD3/CD28)-engaging of T-cell lines. These observations indicate that ATF5 is a critical molecule to regulate SAP gene expression and therefore provides a potentially common mechanism for the pathogenesis of HPS associated with either severe virus infections or immune disorders.^{3,4,25,26}

ATF5 is a member of the CREB/ATF family consisting of bZIP domain at C-terminus to bind to CRE element (ATGACGTCAT). Unlike other activators of the CREB/ATF family such as CREB or ATF7, the repressing roles of ATF3 and ATF5 have been previously described. ATF3 can suppress the transcription of insulin receptor substrate 2 in β cells, contributing to pancreatic cell apoptosis.¹¹ ATF5 represses cAMP-induced transcription and inhibited cell apoptosis.^{27,28} Moreover, ATF5 can compete with CREB binding site to suppress CRE transactivation in neuroblast-like PC12 cells.²⁹ These reports indicate that ATF5 is potentially a transcription repressor through different mechanisms, distinct from other activators such as the replacement of activators. In our study, the LMP1-induced ATF5 expression suppressed the transcription activity of SAP promoter through an interesting hitherto undescribed model. Based on a series of studies on mutation analyses and ChIP assay in this study, we demonstrated that there are two binding sites on the promoter region of the SAP gene for ATF5 with differential binding affinity. The region -210 to +11 (b) exhibits high affinity for ATF5/CREB binding and seems to function at basal levels of ATF5 to regulate SAP transcription in physiological or resting conditions, as shown in control T

cells and unstimulated H9 cells. The region -420 to -210 (a), however, exhibits low affinity for ATF5 binding. In conditions of ATF5 overexpression such as LMP-1 expression, *in vitro* expression of ATF5, or ligand engaging, ATF5 bound to both (a) and (b) regions as revealed in ChIP assay (Figure 4C). The SAP expression was unexpectedly suppressed at this ATF5-overexpressed status. Therefore, the dual binding of ATF5 at sufficient levels of ATF5 to both regions of the SAP promoter appears to be critical for the suppression of SAP transcription. Because mutation of either site lost the suppression effect of ATF5 on SAP transcription in LMP-1-expressed T cells, these two sites contributed equally to regulate the SAP gene (Figure 5B).

There exist several potential mechanisms that can explain the ATF5-suppressed SAP transcription. Although methylation would be one possibility, our preliminary data excluded the contribution of methyltransferase in the suppression of SAP by using 5'-aza-2'-deoxycytidine (unpublished data). Alternatively, the binding of ATF5 on both sites may potentially recruit acetyltransferases or other transcriptional co-factors to tie with chromosome and thereby suppress SAP transcription.^{30,31} In our microarray data, acetyltransferases were not significantly up-regulated in LMP1-expressed H9 cells (unpublished data) and the possibility remains to be clarified. Finally and most preferably, ATF5 may potentially dimerize or tetramerize with each other to form a loop at the SAP promoter, resulting in the disruption of SAP transcription, because the CREB can form homodimer to bind to DNA in a preferable way²⁴ and ATF5 is a member of the CREB family. A previous study reported that wild-type p53 may bind to region -2209 to -2160 of the SAP promoter to induce SAP expression.³² Our data on deletions at site -2209 to -2160 showed no such effect on SAP expression by ATF5. Therefore, different factors may use different mechanisms to regulate SAP expression.

Although the consensus of ATF5 binding site (-305 to -296; CTTGCAAAAT) to CEBP site (ATTGCGCAAT) was only 70% based on previous reports, it should be sufficient for certain ATF5 binding. It has been demonstrated that a high concentration of CREB homodimer bound to CEBP/CRE chimeric site (ATTGCGTCAT), which completely contains the half site of CEBP or CRE.²⁴ Moreover, CREB/ATF also interacts with CRE/CEBP (GTGACGCAAT) or CEBP/CRE (GTTACGTCAG)^{33,34} and (CTTACTTCAC, 50% consensus).³⁵ Furthermore, the hepatitis B virus X protein gene was also regulated by CREB on the chimeric CRE/CEBP site (CTGACGCAAC) with 80% similarity.³⁶ Therefore, the affinity between imperfect CEBP site and ATF5 is low. A high concentration of ATF5 is, however, sufficient for binding to CEBP-like site CTTGCAAAAT on region -420 to -210 (a) of the SAP promoter. In the region -210 to +11 (b) of the SAP promoter, site -81 to -74 (TGACTTG7) is similar to ATF3 (TGACTCAT) binding site. This binding site also reveals an unconventional binding mechanism, which is consistent with the variant binding sites of CREB family as previously reported. Although this site -81 to -74 is near the start site of transcription and is related to promoting activity, the promoter activity was still reversed by muta-

tion on -81 to -74 in LMP1-expressed T cells. The results further support the theory that this binding site was also essential for suppressing SAP transcription and sufficient for forming dimer or tetramer of ATF5.

One important implication of the results in this study is the potential regulation of SAP expression by ATF5 in immune conditions other than EBV-associated HPS. Previous studies have shown that SAP controls T-cell responses to virus infection and the SAP knockout mice had altered lymphocyte responses and enhanced cytokine production as observed in HPS of XLP patients.^{14,15} The identification of ATF5 as a transcriptional repressor to regulate SAP in this study will provide an important mechanism for SAP gene regulation in immune responses. Besides LMP-1 expression, the same phenomenon of ATF5 up-regulation and SAP down-regulation was observed in conditions such as ATF5 overexpression, PHA stimulation of primary T cells, and ligand engaging of T-cell lines. Interestingly, the enhanced expression of ATF5 and suppression of the SAP gene by PHA stimulation of primary T cells declined to the unstimulated levels at 24 hours of stimulation (Figure 3C), suggesting that this regulatory machinery of SAP and ATF5 is tightly regulated in normal T cells. Recently, Nagy and colleagues³² reported that the wild-type p53 activates SAP expression in Burkitt's lymphoma cells and lymphoblastoid cell lines, providing another regulatory mechanism for the regulation of SAP gene expression. Further studies are needed to clarify the role of ATF5 in the regulation of SAP expression in normal immune responses.

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