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Detection of IgG directed against a recombinant form of Epstein-Barr virus BALF0/1 protein in patients with nasopharyngeal carcinoma

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ABSTRACT

BALF0/1 is a putative Epstein-Barr virus (EBV) protein that has been described as a modulator of apoptosis. So far, the lack of specific immunological reagents impaired the detection of native BALF0/1 in EBV-infected cells. This study describes the expression and purification of a truncated form of BALF0/1 (tBALF0) using a heterologous bacterial expression system. tBALF0 was further used as an antigen in an indirect Enzyme-linked Immunosorbent Assay (ELISA) that unraveled the presence of low titer IgGs to BALF0/1 during primary (10.0%) and past (13.3%) EBV infection. Conversely high-titer IgGs to BALF0/1 were detected in 33.3% of nasopharyngeal carcinoma (NPC) patients suggesting that BALF0/1 and/or humoral response against it may contribute to NPC pathogenesis.

1. Introduction

Epstein-Barr virus (EBV) is a ubiquitous human γ-herpesvirus which infects about 95% of adults worldwide [1]. EBV primary infection is asymptomatic when occurring in children whereas it may be responsible for infectious mononucleosis in young adults [2]. EBV is transmitted by saliva mainly and establishes lifelong infection after primary infection. This persistent infection alternates latent infection, mainly in memory B cells, and sporadic reactivations that eventually lead to virus production and subsequent transmission [3]. EBV infection is associated with the pathogenesis of a number of human malignancies of lymphoid and epithelial origin including Burkitt's lymphoma (BL), Hodgkin's lymphoma (HD) and nasopharyngeal carcinoma (NPC) [4,5]. The close association between EBV and NPC is illustrated by the consistent expression of EBV gene products in epithelial NPC cells [6] and the distinct serological responses to defined EBV antigens in NPC patients [7–10].

Cellular Bcl-2 is an anti-apoptotic protein of the B-cell lymphoma-2 (Bcl-2) family [11]. Whereas members of γ-herpesviruses usually encode for only one viral Bcl-2 homolog (vBcl-2), EBV potentially encodes for two vBcl-2 proteins, namely BHRF1 and BALF0/1 [12,13]. Two in-frame methionine codons were found near the beginning of BALF1 open reading frame (ORF) suggesting that several proteins may be encoded with different N-termini. BALF1 protein would be encoded by the shorter ORF, while the protein encoded from the first non-conserved methionine is referred to as BALF0. Importantly only BALF1 would be conserved among primate lymphocryptoviruses [14]. The genetic inactivation of both vBcl-2 genes severely impairs the ability of EBV to transform primary resting B lymphocytes and evade apoptosis in infected cells [15]. BHRF1 has been characterized as an anti-apoptotic protein [16] whereas the function of BALF0/1 is still equivocal. BALF0/1 is transcribed both in lytic stage and latency in EBV-positive Burkitt lymphoma's cell lines and NPC biopsies [17] and promotes tumor formation and metastasis in nude mice [18]. BALF0/1 has been proposed to play a role in inhibiting apoptosis through association with Bax and Bak [13]. Conversely BALF1 fails to protect against Sindbis virus- or BAX-induced apoptosis and antagonizes the anti-apoptotic activity of BHRF1 [14]. BALF0 may also antagonize the anti-apoptotic activity of BHRF1 but does not co-immunoprecipitate with BHRF1 as BALF1 [14]. Previous work has shown that NPC patients may produce antibodies recognizing a 31 kDa protein in BALF0/1-transfected NIH3T3 cells [17], which was compatible with BALF0 expected size. Nonetheless the existence of BALF1 could not be confirmed in the same context. So far, the existence of BALF0/1 in naturally infected cells cannot be assessed

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due to the lack of specific immunological reagents. An essential prerequisite for generating such reagents is the production of purified soluble forms of BALF0/1 that could eventually be used for immunization.

The present work describes the production of a purified form of BALF0/1 and its subsequent evaluation as an antigen for the seroepidemiological survey in various EBV-infected populations.

2. Material and methods

2.1. Cell line, bacterial strains and media

The EBV-positive B-cell line B95.8 (purchased from ATCC) was grown in RPMI-1640 media (Gibco, USA) supplemented with 10% fetal bovine serum (Thermo Scientific, USA). *E. coli* strains DH5α and Rosetta (DE3) pLysS (EMD Millipore, USA) were used for transformation steps by heat shock method. Bacteria were cultured in Luria-Bertani (LB) broth [1% (w/v) peptone 140, 0.5% (w/v) yeast extract, and 0.5% NaCl], which was supplemented with ampicillin (100 μg/mL) (Sigma-Aldrich, USA) for the production of plasmid DNA in DH5α and both ampicillin and chloramphenicol (34 μg/mL) (Sigma-Aldrich, USA) for the expression of the recombinant protein in Rosetta (DE3) pLysS.

2.2. Plasmid construction

The DNA sequence corresponding to truncated BALF0/1 (tBALF0, amino acid 1 to 140), a mutant with deletion of the C-terminal transmembrane domain, was amplified by PCR using viral genomic DNA (EBV B95.8 strain) as template. Primers were designed according to the manufacturer's instructions of Cold Fusion Cloning Kit (SBI, USA). The sequences of primers were as follows: 5′-GAAGGAGATATACATATGA ACCTGGCCATTGCTCT-3' (forward) and 5′-GTCGACGGAGCTCGAGTT GTACACTGCGCGCAGGA-3' (reverse), with the underlined regions representing the sequences from the expression vector pET-22b (Novagen, USA). Both primers were synthesized and purchased from Eurogentec (Belgium) and used without further purification. The total PCR reaction was prepared according to the manufacturer's instructions of *PfuUltra* DNA polymerase (Agilent Technologies, USA). The PCR was performed under the following conditions: 2 min at 95 °C, 35 cycles (20 s at 95 °C, 20 s at 61 °C, 15 s at 72 °C), and 3 min at 72 °C. Following PCR amplification, the PCR product was analyzed by electrophoresis on a 1% agarose gel, then stained with ethidium bromide and visualized by UV illumination.

The PCR product and the linearized expression vector pET-22b, which was digested with NdeI and EcoRI (NEB, USA), were purified by QIAquick Gel Extraction Kit (Qiagen, Germany). The cloning reaction of tBALF0 DNA insert and linearized pET-22b was incubated 5 min at room temperature, followed by 10 min on ice according to the manufacturer's instructions. The recombinant plasmid pET-22b-tBALF0 was transformed into chemically competent *E. coli* DH5α. Several colonies were screened for the presence of tBALF0 DNA by colony PCR, and then positive colonies were grown in liquid culture and plasmids were purified using QIAprep Spin Miniprep Kit (Qiagen, Germany). The presence of tBALF0 DNA in the purified plasmids was confirmed by restriction digestion by PstI (NEB, USA), and plasmids were verified by DNA sequencing (Eurofins Genomics, Germany).

2.3. Protein expression and purification

The recombinant expression plasmid pET-22b-tBALF0 was transformed into chemically competent Rosetta (DE3) pLysS cells for protein expression. For the small-scale analysis, freshly transformed cells were plated onto solid LB medium (supplemented with ampicillin, 100 μg/mL and chloramphenicol, 34 μg/mL). One positive colony was chosen randomly and inoculated into 2 mL LB medium containing antibiotics, grown at 37 °C overnight with shaking at 300 rpm. Fifty microliters of overnight pre-culture were added to 5 mL of fresh LB media containing antibiotics and was grown at 37 °C, 300 rpm, until an OD_{600} of 0.5 was reached. The expression of tBALF0 was induced by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma-Aldrich, USA). After 3 h induction at 37 °C, bacterial pellets were harvested by centrifugation at 12,000 g for 20 min and resuspended in 500 μL of lysis buffer (100 mM NaH_2PO_4, 10 mM Tris·HCl, 6 M GuHCl, pH 8). To remove cellular debris, lysates were centrifuged for 20 min at 12,000 g, and the supernatants were transferred to fresh tubes. Twenty microliters of Ni-NTA resin (Qiagen, Germany) were added to each tube and incubated with cleared lysate under constant rotation at 4 °C for 2 h. The resins were harvested by centrifugation at 12,000 g for 5 min and washed three times by wash buffer A (100 mM NaH_2PO_4, 10 mM Tris·HCl, 8 M urea, pH 7). After an incubation on ice for 5 min with the addition of wash buffer A containing 100 mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, USA), the eluate was collected following centrifugation at 12,000 g for 5 min. Small-scale preparations of tBALF0 protein were obtained and analyzed by SDS-PAGE.

Large scale production of recombinant tBALF0 protein was performed by inoculating a single colony into 10 mL LB medium containing antibiotics, and cultured overnight at 37 °C under agitation at 300 rpm. The overnight pre-culture was inoculated to 1 L LB medium containing antibiotics in a 2.5 L glass Erlenmeyer flask. The culture was grown at 37 °C, 300 rpm, until an OD_{600} of 0.5 was reached. Protein expression was induced with the addition of 1 mM IPTG. After 3 h of induction at 37 °C, cultures were harvested by centrifugation at 12,000 g for 30 min, 4 °C, and cell pellets were stored at −80 °C until purification. The pellets (around 6 g wet weight from 1 L of liquid culture) were then resuspended in 30 mL of pre-chilled lysis buffer and incubated overnight at 4 °C under constant rotation. The lysate was centrifuged at 4 °C, 12,000 g for 30 min, and the pellet discarded. The supernatant was added to 1 mL 50% Ni-NTA resin slurry pre-equilibrated with lysis buffer, and incubated under constant rotation at 4 °C for 2 h. The resins were harvested by centrifugation at 12,000 g for 5 min and washed extensively by wash buffer B (100 mM NaH_2PO_4, 10 mM Tris·HCl, 8 M urea, pH 6.3). In the presence of a low pH, tBALF0 protein was eluted in batch under denaturing condition. The elution buffer (100 mM NaH_2PO_4, 10 mM Tris·HCl, 8 M urea, pH 4.5) was added to the resin, and the eluates were collected in 1 mL fractions. Fractions were boiled in sample buffer with and without 10% β-mercaptoethanol (Sigma-Aldrich, USA), respectively, prior to be analyzed by SDS-PAGE (15%). The tBALF0-containing fractions were pooled and dialyzed against phosphate-buffered saline (PBS; EUROMEDEX, France). Following dialysis, the protein fractions were centrifuged at 4 °C, 12,000 g for 15 min to remove insoluble aggregates and stored in aliquots at −80 °C.

2.4. Peptide mass fingerprinting

All reagents were purchased from Sigma-Aldrich (Saint Quentin-Fallavier, France) unless otherwise specified. Trypsin Gold (proteomics grade) was obtained from Promega (Charbonnières, France). Purified tBALF0 was subjected to both in-gel and in-solution trypsin digestion. For in-gel digestion, Coomassie Blue stained bands corresponding to tBALF0 monomer and higher molecular weight species were excised manually from 15% acrylamide gels, destained, dried by vacuum and digested as previously described [19]. Briefly, in-gel trypsin digestion was performed overnight at 37 °C in 50 mM ammonium bicarbonate using 125 ng of trypsin per band. Preliminary steps for reduction and alkylation of disulfides bonds were omitted. The supernatants were kept and bands were subjected to a peptides extraction step using 60 μL of a 60% acetonitrile solution containing 1% trifluoroacetic acid. Supernatants were combined and reduced to 10 μL using a centrifugal vacuum concentrator before analysis by Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS). For in-solution digestion, 3 μL of recombinant tBALF0 (900 ng) was first incubated at 25 °C for 30 min with 2.5 mM DTT in 50 mM ammonium

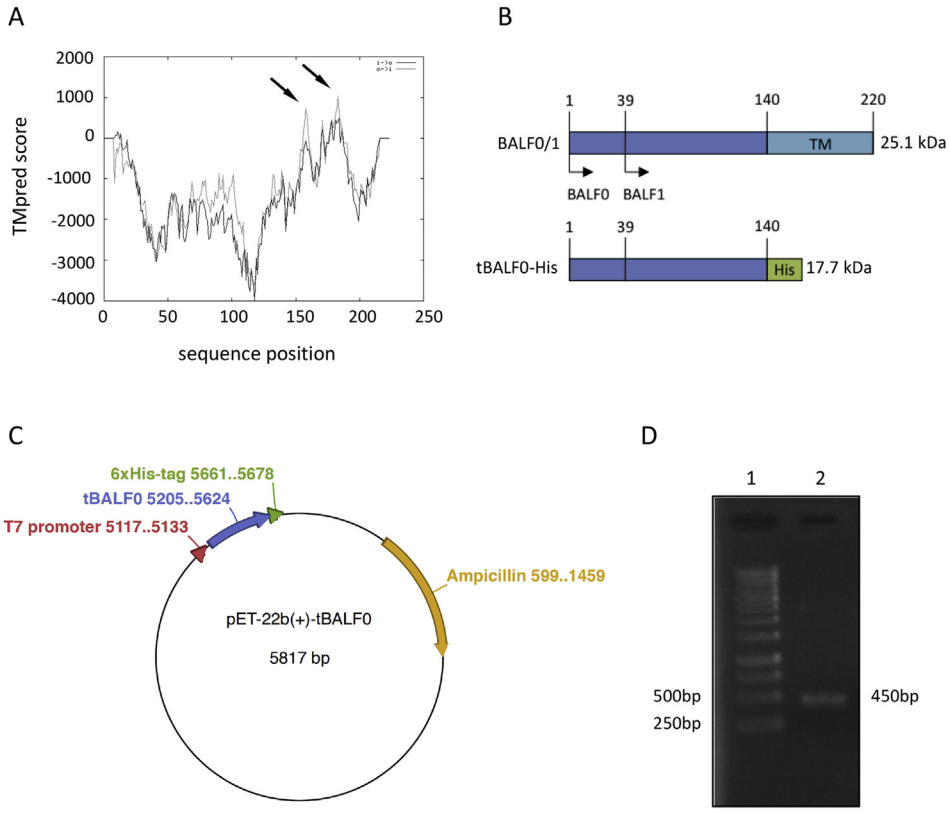

Fig. 1. Construction of an expression plasmid encoding truncated BALF0/1 with a C-terminal histidine tag. (A) Prediction of transmembrane helices in BALF0/1 protein. Hydrophobicity plot of BALF0/1 was obtained by using the TMpred algorithm (https://embnet.vital-it.ch/software/TMPRED_form.html) showing probability of transmembrane domains. The putative membrane-spanning regions are marked by arrows. (B) Schematic representation of the full length BALF0/1 and truncated BALF0 protein. BALF0 consists of 220 amino acids, and contains putative transmembrane (TM) domains at the C-terminus. The BALF1 protein is proposed to be translated from the second methionine at amino acid 39. tBALF0 (amino acid 1 to 140) was obtained by removing the C-terminal TM domain and was fused with a C-terminal poly-histidine tag. (C) Schematic diagram of pET-22b-tBALF0 expression plasmid. The recombinant gene encoding tBALF0 was cloned into a pET-22b(+) vector containing a C-terminal poly-histidine tag and expressed in *E. coli* Rosetta (DE3) pLysS under the control of the T7 promoter. (D) Amplification of the truncated BALF0/1 gene from viral genomic DNA (EBV B95.8 strain) by PCR. The PCR product was analyzed by electrophoresis on a 1% agarose gel; lane 1: 1 kb DNA ladder, lane 2: tBALF0 fragment (450 bp).

bicarbonate (total volume 10 µL). Then, 2 µL of trypsin prepared at 12.5 ng/µL in 1 mM HCl were added. Trypsin digestion was performed at 37 °C for 4h and was diluted 10 times in LC-MS grade water before MALDI-TOF MS analysis. One microliter of tryptic peptides was mixed with 3 µL of a solution of α-cyano-4-hydroxycinnamic acid prepared at half-saturation in a mixture containing 50% acetonitrile, 49.7% grade water and 0.3% trifluoroacetic acid. Two microliters of this premix were spotted onto the sample plate and allowed to dry under a gentle air stream. Spectra were acquired in positive reflectron mode on the Axima Performance MALDI-TOF/TOF mass spectrometer (Shimadzu-Kratos, Manchester, UK) with a pulse extraction fixed at 2500 as previously described [20].

2.5. Mass spectrometry analysis of intact tBALF0

Two microliters of tBALF0 protein (34 pmoles) were incubated at 20 °C for 1 h with 1 µL of 20 mM DTT and 2 µL of 25 mM ammonium bicarbonate. Then, 1 µL was taken and mixed with 2 µL of a solution of sinapinic acid prepared at full saturation in a mixture containing 30% acetonitrile, 49.7% grade water and 0.3% trifluoroacetic acid. Two microliters of this premix were spotted onto the sample plate and allowed to dry under a gentle air stream at room temperature. Spectra were acquired in positive linear mode on a Shimadzu Axima Performance MALDI-TOF/TOF mass spectrometer with a pulse extraction fixed at 20000. Mass determination of tBALF0 was performed after external calibration using mono-charged and dimer ions of equine apomyoglobin.

2.6. Plasmas

A panel of plasma samples from EBV negative controls ($n = 10$), EBV primary infection patients ($n = 10$), and healthy EBV carriers (past infection) ($n = 60$) was collected at Hôpital Saint-Antoine (Paris, France). All these samples were tested for EBV serology as part of their medical follow-up, and BALF0/1 ELISA was performed on excess volume of these samples. The cancer patient panel consisted of plasma samples from 27 histologically-confirmed NPC patients and 8 EBV-positive patients bearing non-EBV-related head and neck squamous cell carcinomas (HNSCCs), which was obtained at Institut Gustave Roussy (Villejuif, France) with informed consent of patients entering a protocol approved by the ethics committee (exoplasma, CPP Tarnier-Cochin N°2746, 2010). All samples were stored at −80 °C prior to be processed anonymously.

2.7. Development of an indirect ELISA assay for detecting antibodies against BALF0/1 in patients with EBV infection and NPC

96-well ELISA plates (BD Biosciences, USA) were coated with 100 ng tBALF0 in denaturing coating buffer (100 mM NaH_2PO_4, 10 mM Tris·HCl, 8 M urea, pH 8). After overnight incubation at room temperature, antigen was discarded and 150 µL blocking buffer [10% skim milk powder (Régilait, France) in PBS containing 0.2% Tween 20 (PBST)] was added to each well. After incubation at 37 °C for 1 h, the wells were emptied and washed five times with PBST. All plasma samples, 1:10-diluted in blocking buffer, were added in duplicate and incubated for 1 h at 37 °C. After five washes with PBST, horseradish peroxidase (HRP) conjugated rabbit anti-human IgG (1:1000-diluted in blocking buffer) (Rockland Immunochemicals, USA) was added and incubated for 1 h at 37 °C. After five washes with PBST, 100 µL of ABTS (Roche Diagnostic, Germany) was added per well and incubated avoiding light for 20 min. The optical density was determined at 405 nm (OD_{405}) using an ELISA plate reader (Infinite 200 PRO; Tecan Life Sciences, Switzerland). All OD_{405} values from wells coated with tBALF0 were normalized by subtracting the background value of wells coated with bovine serum albumin (BSA; EUROMEDEX, France) which loaded with same plasma sample. To discriminate between negative and positive samples, a cut-off value was determined by selecting the mean plus three standard deviations of the normalized OD_{405} of the plasma samples from EBV-negative controls.

3. Results

3.1. Construction of an expression plasmid encoding for tBALF0, a truncated form of BALF0/1

We initially tried to express full length BALF0/1 from two prokaryotic expression vectors (pET-22b and pGEX-2T) with a C-terminal poly-histidine tag and a N-terminal glutathione S-transferase tag, respectively. However, despite trying a wide variety of expression conditions, the growth of the host bacteria was inhibited and the protein was not expressed. We assumed that hydrophobic domains may impair BALF0/1 expression in *E. coli*. Structural analysis predicted the presence of 2 α-helices with high hydrophobicity at BALF0/1 C-terminus (Fig. 1A). Therefore, we constructed an expression plasmid encoding a truncated form of BALF0/1 (tBALF0) in which amino acids 141 to 220 were removed (Fig. 1B). The tBALF0 gene was amplified by PCR from viral genomic DNA (EBV B95.8 strain) and cloned into pET-22b vector (Fig. 1C and D). The recombinant plasmid pET-22b-tBALF0 was further characterized by specific restriction digestion and DNA sequencing which demonstrated that the amplified target sequence was inserted into the correct ORF in the vector.

3.2. Expression and purification of recombinant tBALF0

pET-22b-tBALF0 was transformed into Rosetta (DE3) pLysS. Expression was induced with the addition of 1 mM IPTG at 37 °C. In this condition, tBALF0 expression was associated with a marked reduction in bacterial growth. A protein of 20 kDa apparent mobility could be observed following SDS-PAGE and Coomassie Blue staining (theoretical MW of His-tagged tBALF0 = 17.7 kDa) (Fig. 2A). Large scale protein expression was performed in the same condition and bacterial pellets were harvested 3 h post-induction. tBALF0 was purified under denaturing conditions in the presence of 8 M urea by nickel affinity chromatography and eluted from the Ni-NTA resin by discontinuously decreasing the pH of the solution. Following SDS-PAGE analysis, only one band was observed at the expected MW under reducing condition (Fig. 2B, left panel). Conversely at least seven bands were observed under non-reducing condition (Fig. 2B, right panel) suggesting that oxidization may promote tBALF0 multimerization either during protein expression, purification or gel analysis.

3.3. Characterization of recombinant tBALF0 by mass spectrometry

The band migrating at the expected molecular weight for tBALF0 and the six other bands corresponding to higher molecular weight species were excised from the non-reducing gel (Fig. 2B, right panel). They were submitted to an in-gel trypsin digestion to generate peptide mass fingerprints (PMFs) by MALDI-TOF MS. Mass spectra of the seven bands were highly similar to the one performed after in-solution trypsin digestion of recombinant tBALF0 (Fig. 3A, inset). These PMFs allowed us to cover around 80.4% of the tBALF0 primary sequence (Fig. 3B). They also confirmed that higher molecular weight species were likely disulfide-linked oligomeric forms of tBALF0 as they completely disappeared within the gel performed under reducing conditions (Fig. 2B). We also analyzed the intact protein by MALDI-TOF MS (Fig. 3A) to confirm the integrity of recombinant tBALF0. The experimental mass of tBALF0 was in good agreement with the calculated molecular weight confirming that recombinant tBALF0 was correctly produced and purified.

3.4. Detection of anti-BALF0/1 IgG antibodies by indirect Enzyme linked Immunosorbent Assay (ELISA)

Human antibodies directed against key EBV antigens are commonly used to distinguish between non-infected patients, primary infection or past infection [21]. IgG and IgA antibodies directed against specific EBV antigens have been associated with EBV positive NPC diagnosis and monitoring [22,23]. Therefore, we wondered whether patients with EBV-positive NPC could produce antibodies against BALF0/1.

For this purpose, we designed an ELISA using tBALF0 as an antigen. The baseline OD_{405} for negative samples was calculated as the mean OD_{405} plus three standard deviations obtained on 10 plasmas from EBV negative individuals. Samples with OD_{405} greater than or equal to the cutoff value were considered positive. In order to search for the presence of anti-BALF0/1 IgG antibodies in different forms of EBV infection, ELISA was performed on a panel of plasmas from primary infection patients and healthy EBV carriers (past infection) as well. 10% of the plasmas from primary infection patients showed IgG reactivity to BALF0/1 and a minority of healthy EBV carriers (8 of 60, 13.3%) had a positive IgG response (Fig. 4). We then searched for anti-BALF0/1 IgGs in a panel of NPC patients ($n = 27$). A group of plasma samples collected from EBV-positive patients with HNSCCs (Head and Neck

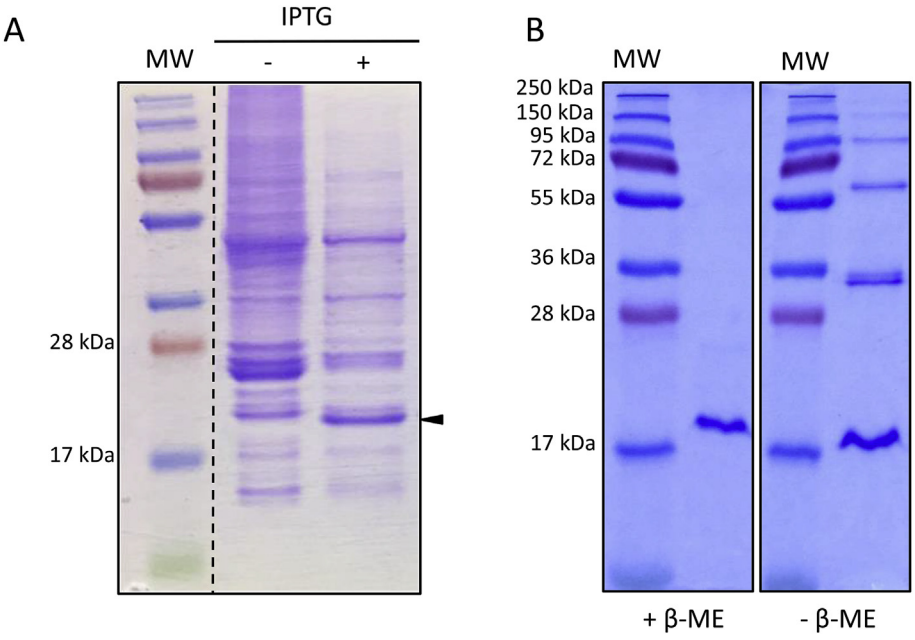

Fig. 2. Expression and purification of recombinant tBALF0 protein. (A) Small-scale expression and purification of His-tagged tBALF0 protein. Protein expression was induced with the addition of 1 mM IPTG at 37 °C for 3 h. The purified fractions of tBALF0 were analyzed by SDS-PAGE (15%) and Coomassie Blue staining. (B) SDS-PAGE analysis of purified fractions of tBALF0. Prior to loading onto gels, fractions were boiled in sample buffer with and without 10% β-mercaptoethanol (β-ME), respectively.

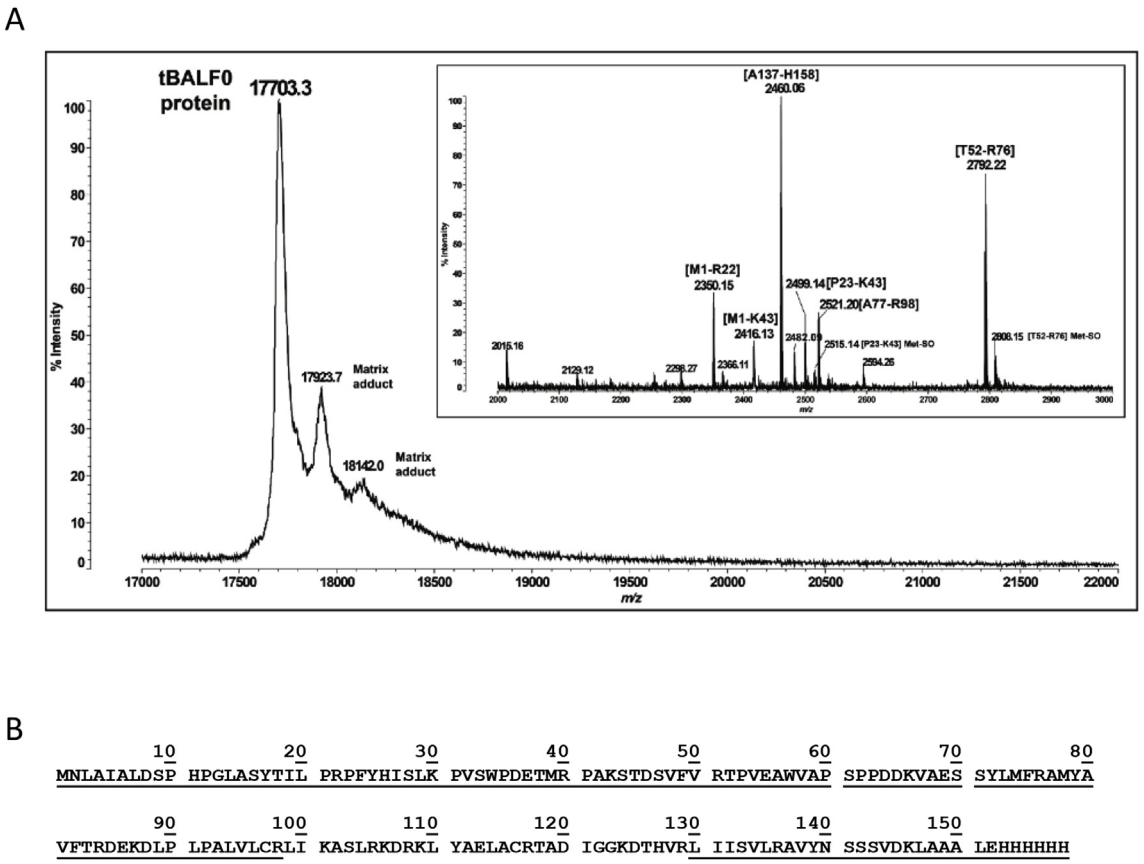

Fig. 3. Mass spectrometry analysis of recombinant tBALF0 protein. (A) Matrix-assisted laser desorption/ionization mass spectrum of intact tBALF0 protein. The experimental mass (17703.3 Da) of tBALF0 is within the expected experimental error (calculated mass: 17722.5 Da). Peaks labelled "Matrix adduct" correspond to tBALF0 with a sinapinic acid adduct. Peptide mass fingerprinting (PMF) of recombinant tBALF0 after in-solution trypsin digestion is shown in the inset. Sequence of peptides belonging to tBALF0 is indicated in brackets. (B) Protein sequence of tBALF0 showing regions covered by MALDI-TOF mass spectrometry analysis.

squamous cell carcinomas) was used as non-NPC controls ($n = 8$). IgG antibodies to BALF0/1 were detectable in 9 of 27 (33.3%) NPC patients, compared to 1 of 8 (12.5%) in non-NPC patient controls (Fig. 4). It is to note that high frequency of IgGs against BALF0/1 in NPC patients was also associated with a significant higher average OD_{405}, suggesting that high level of anti-BALF0/1 IgG antibodies might circulate in this specific subpopulation.

4. Discussion

The growth of bacterial hosts can be inhibited by the expression of heterologous proteins. Gene products that affect the growth rate of bacterial hosts are considered to be toxic. They include membrane proteins, proteins interacting with DNA or interfering with electron transport [24]. Lower transformation efficiency and marked reduction in bacterial growth of constructs encoding full length BALF0/1 were observed (data not shown) suggesting that the BALF0/1 gene product was toxic to the host. Structural analysis predicted the presence of 2 α-helices with high hydrophobicity at BALF0/1 C-terminus showing putative transmembrane domains. A recombinant protein with membrane-spanning domains may have a toxic effect on a bacterial host, probably due to the association between the protein and bacterial membranes [25]. Removal of putative transmembrane domains in BALF0/1 led to a 17.7 kDa polypeptide that could be expressed in *E. coli*. High expression levels of recombinant proteins in bacterial expression systems can lead to the formation of insoluble inclusion bodies [26], which can be completely solubilized by strong denaturing agents, such as 6 M guanidine hydrochloride or 8 M urea. tBALF0 was purified under denaturing conditions in the presence of 8 M urea by nickel affinity chromatography and eluted from the Ni-NTA resin in batch by discontinuously decreasing the pH of the buffer. Several attempts have been done to renature tBALF0 on the resin by gradually decreasing urea concentration until 0 in the washing buffers. However, we could not succeed in eluting native tBALF0 in these conditions even in the presence of 250 mM imidazole. This suggested that tBALF0 may form aggregates in these conditions, which was further confirmed by SDS-PAGE analysis (data not shown). The identity of recombinant tBALF0 in oligomeric form was confirmed by peptide mass fingerprinting. Two cysteines are present in tBALF0 suggesting that disulfide bridges may likely contribute to the multimerization. In the definitive procedure, tBALF0 was purified in batch under denaturing conditions and dialyzed against PBS in the absence of reducing agents. A fraction of the protein precipitated during dialysis and was discarded by centrifugation. We estimated that 2.8 mg tBALF0 could be obtained in a purified and soluble form from a 74 mg (1 L culture) total *E.coli* protein extract.

The existence of EBV BALF0/1 in cells that are infected by EBV has never been confirmed so far due to the absence of dedicated immunological reagents. The presence of antibodies to BALF0/1 in patients infected by EBV could therefore be considered as an important indirect evidence for the existence of BALF0/1 *in vivo*. Seroepidemiological studies were performed by ELISA in EBV primary infection patients, healthy EBV carriers and NPC patients. IgG directed against tBALF0 were detected in different forms of EBV infection (primary and past infections) and in NPC patients suggesting that either BALF0 or BALF1 are expressed under physiological conditions. Among 60 healthy EBV carriers, 13.3% of individuals had a weak positive IgG response to tBALF0. The weak immunogenicity of EBV lytic protein has also been reported in a recent evaluation of IgG antibody responses to a

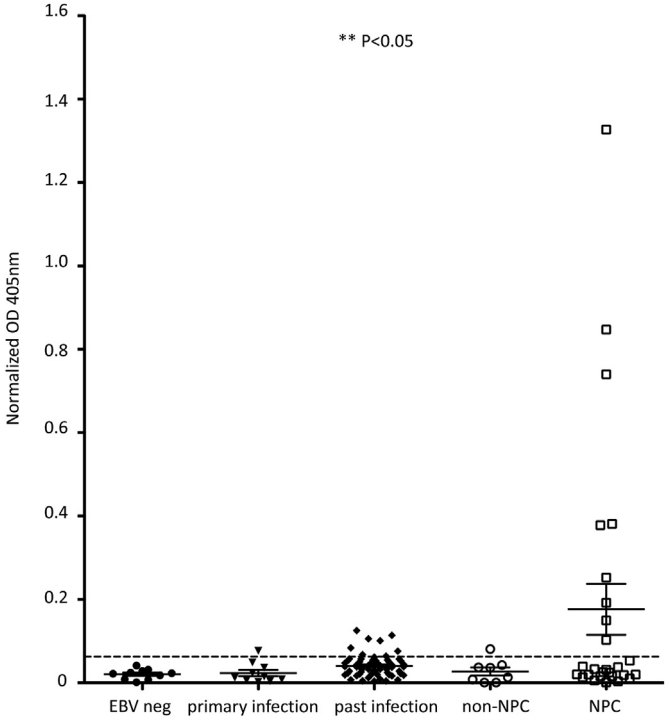

Fig. 4. Detection of anti-BALF0/1 IgG by indirect ELISA in patients with EBV infection and NPC. tBALF0 was used as an antigen to detect anti-BALF0/1 IgG in various groups of patients including EBV negative controls (n = 10), EBV primary infection patients (n = 10), healthy EBV carriers (past infection) (n = 62), non-NPC patient controls (n = 8) and NPC patients (n = 27). One dot represents one patient. Shown are the mean ± S.E.M. of OD_{405} for IgG reactivity. Statistical analysis was performed by *one way ANOVA* nonparametric test by the GraphPad Prism program. The horizontal dotted line represents the cutoff value (0.05) above which the OD_{405} is considered positive.

large spectrum of EBV proteins. In this study one early lytic protein (BMRF1) and 4 late lytic proteins (BGRF1/BDRF1, BFLF2, BRFR1A and BcLF1) elicit positive IgG responses in less than 5% of individuals [27]. NPC patients have strong and characteristic immune responses against EBV proteins, including viral capsid antigen (VCA) and EBV nuclear antigen 1 (EBNA1) [28]. For several decades, IgG and IgA antibodies against EBV antigens have been investigated for their potential as biomarkers for NPC early diagnosis [29]. Results have often been disappointing due to a lack of sensitivity. Currently, a number of researchers give preference to the detection of circulating EBV-DNA as a biomarker suitable for early NPC detection, especially when using next-generation sequencing (NGS) and size assessment of the viral DNA [30]. However, serodiagnosis has probably not said its last word in that a large number of EBV proteins, including early and late lytic proteins, may be the target of circulating antibodies in NPC patients. So far, only 10% of the approximately 90 EBV proteins have been investigated as potential biomarkers for NPC diagnosis [31,32]. The potential of these antibodies especially circulating IgA is well highlighted by a recent report based on peptide micro-arrays. In this study, IgG and IgA antibody responses against 199 sequences from 86 EBV proteins have been measured and combined with the VCA/EBNA1 IgA assay which is the current standard for NPC serodiagnosis. This combination represented a significant improvement in the risk prediction analysis of NPC by comparison with the VCA/EBNA1 IgA assay alone [33]. In the present work, one third of NPC patients were recorded as having high IgG titers to BALF0/1. Due to the mucosal origin of NPC [28], it would be well advised to evaluate the IgA responses to BALF0/1 in further investigations. Even if the circulating antibodies to BALF0/1 do not contribute to the early diagnosis and/or monitoring of EBV-positive NPC, their presence in a fraction of NPC patients will encourage future investigations on the role of BALF0/1 in NPC pathogenesis.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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