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Dewi Kartikawati Paramita, Christien Fatmawati, Hedy Juwana, Frank G. van Schaijk, Jajah Fachiroh, Sophia Mubarika Haryana, Jaap M. Middeldorp

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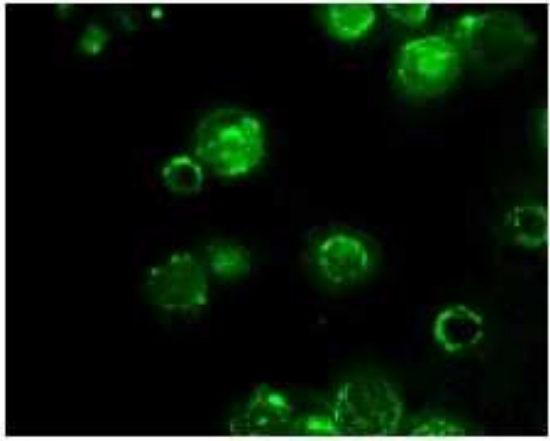


**Humoral Immune Responses to Epstein-Barr virus encoded
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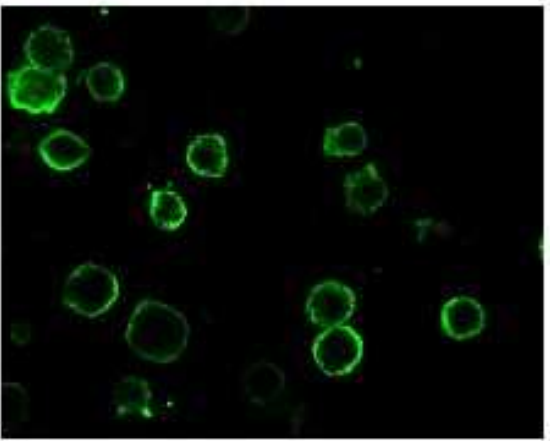
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Keywords:	Epstein-Barr virus, tumor antigens, humoral immunity, immunisation, extracellular domains

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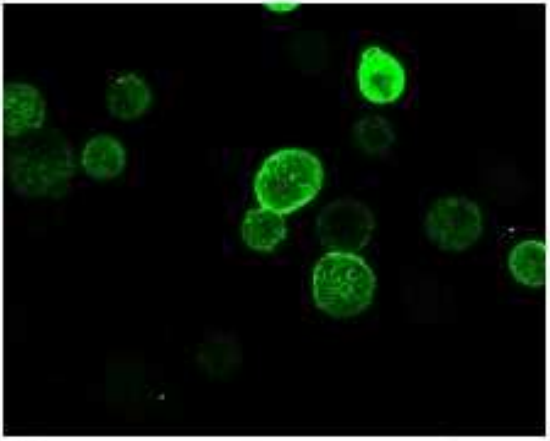
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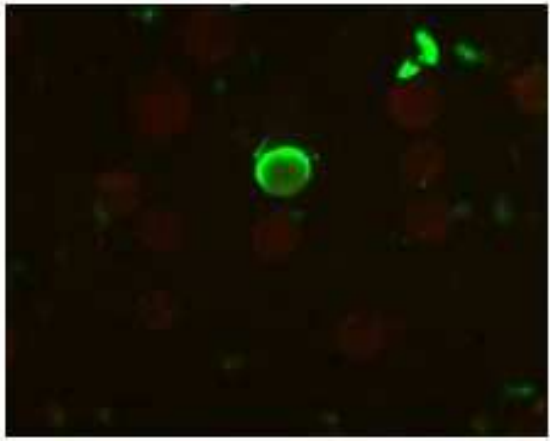
A SF9-EBNA1 OT1X
(1:200)



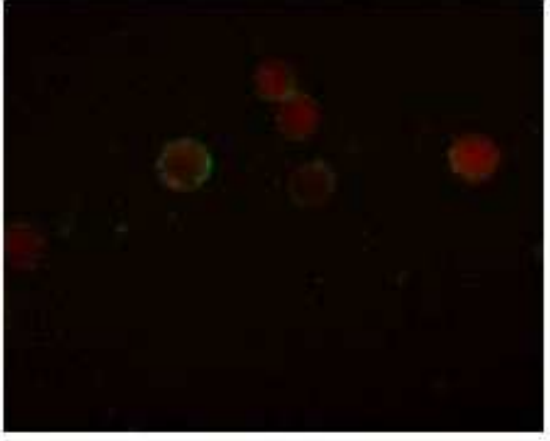
C SF9-LMP1 OT21C
(1:100)



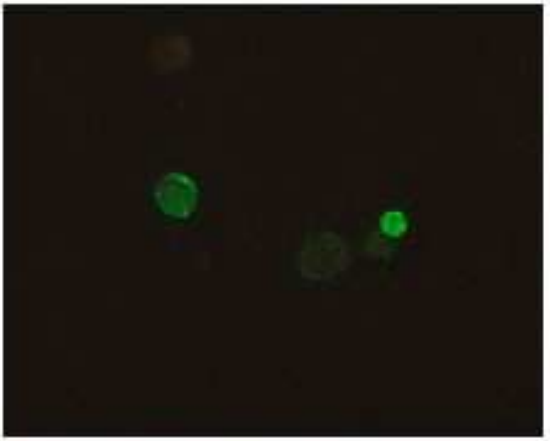
E SF9-LMP2 14B7
(1:100)



B SF9-EBNA1
NPC serum (1:100)

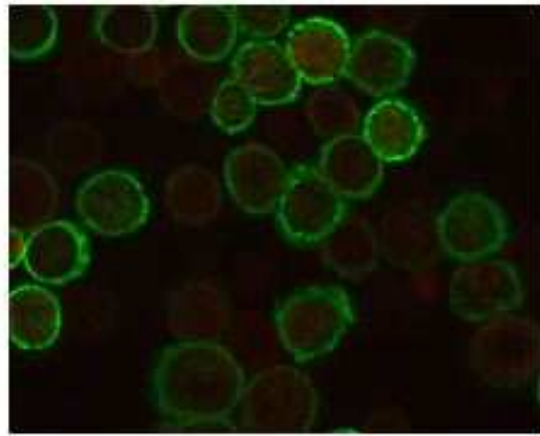


D SF9-LMP1
NPC serum (1:100)



F SF9-LMP2
NPC serum (1:100)

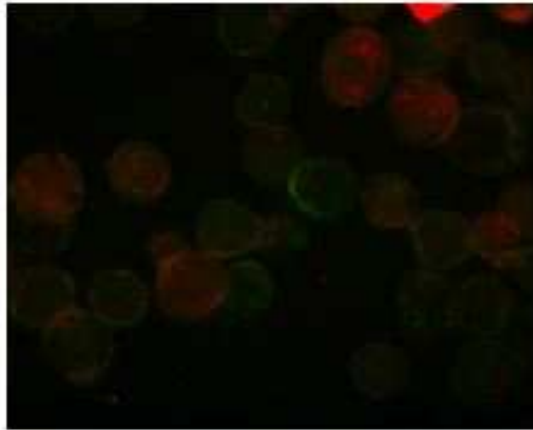
Figure 1



G SF9-BARF1
K150/4A6 (1:10)



I WT-Bac



H SF9-BARF1
NPC serum (1:200)

Figure 1

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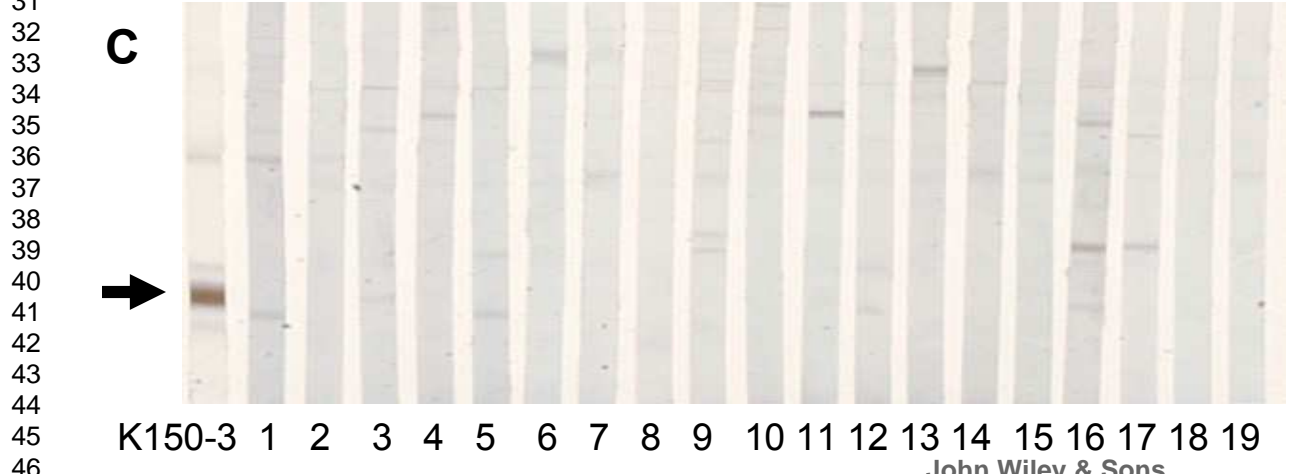
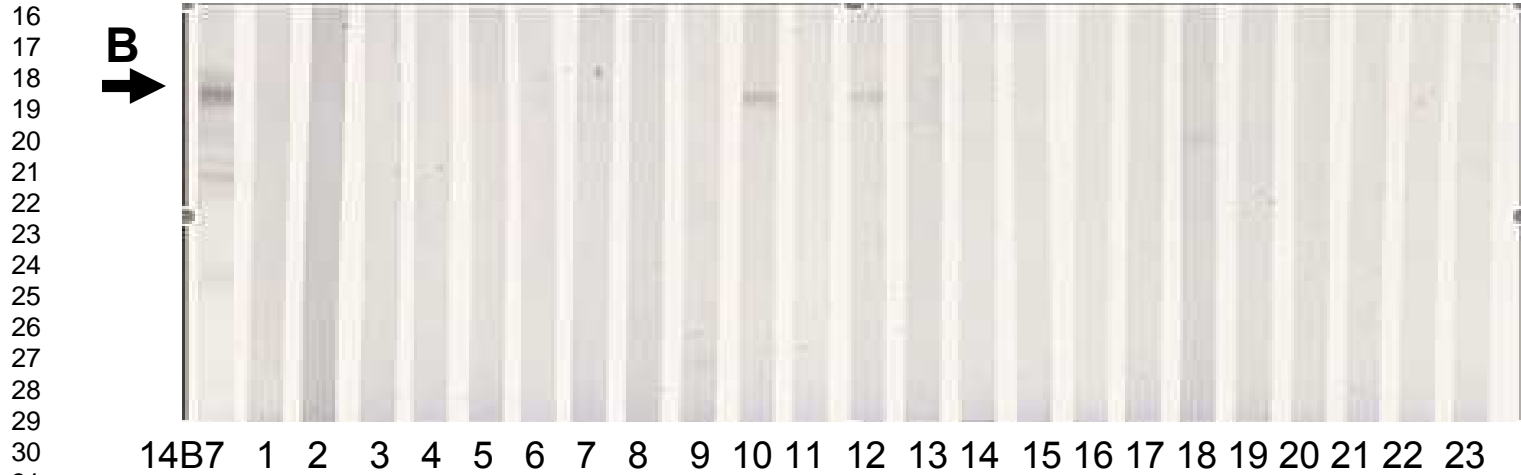
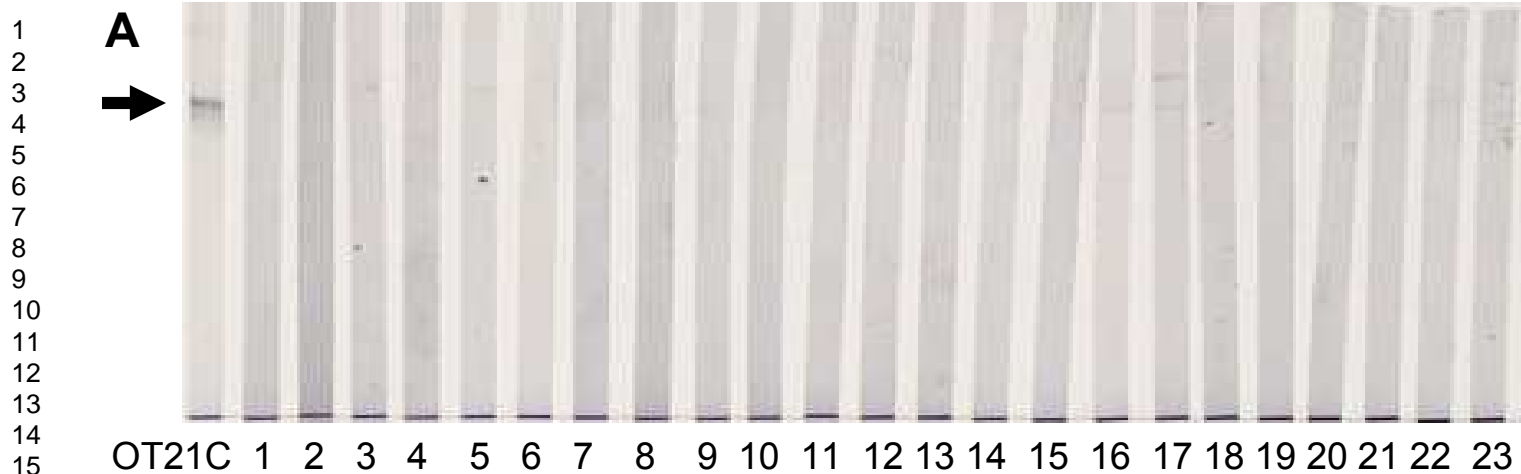


Figure 2 part 1

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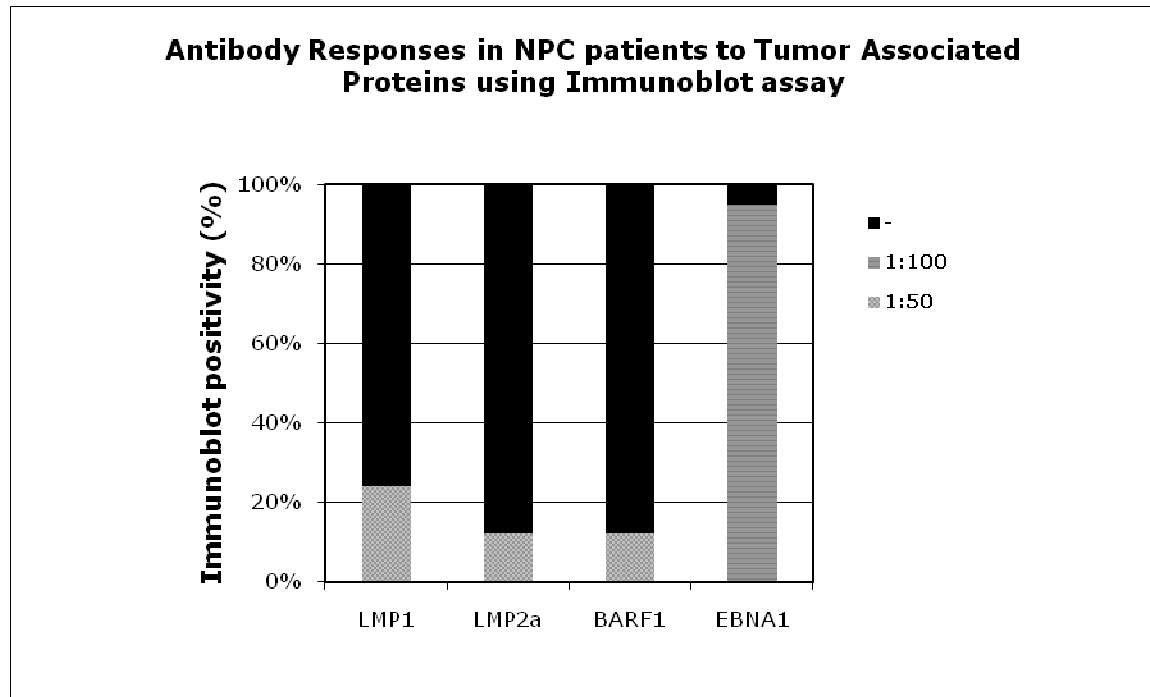
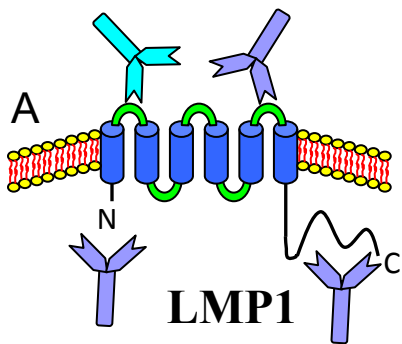
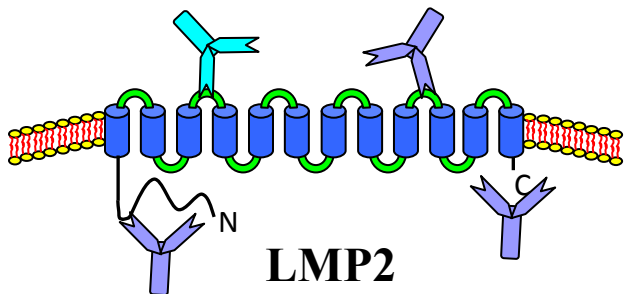


Figure 2 part 2

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IgG Response to LMP1



IgG Response to LMP2

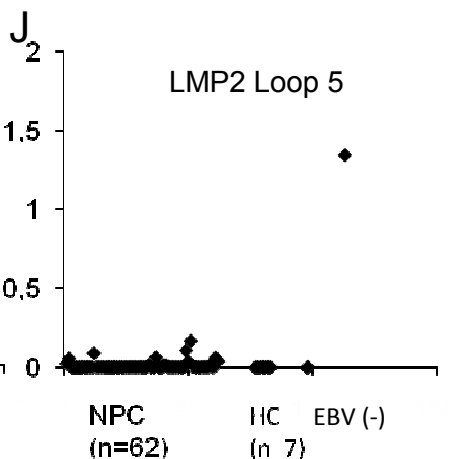
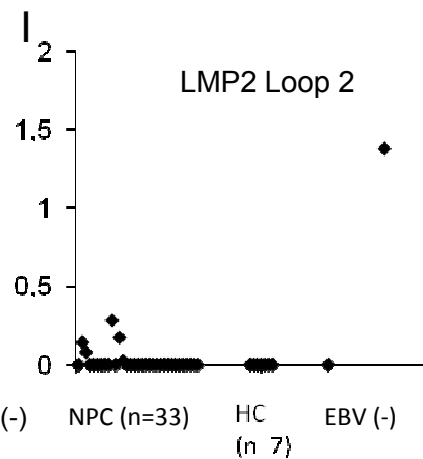
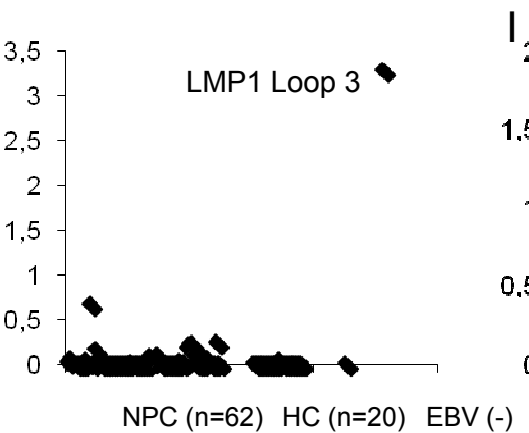
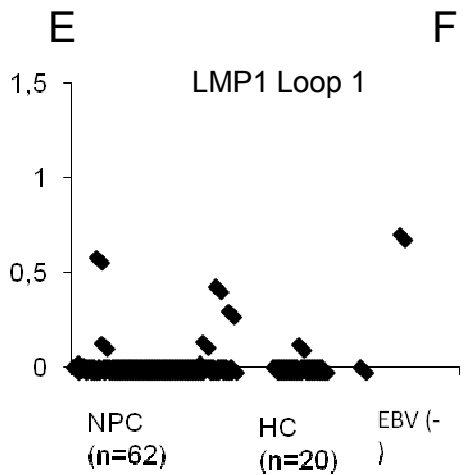
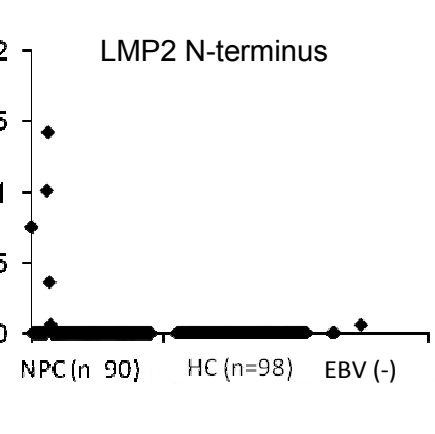
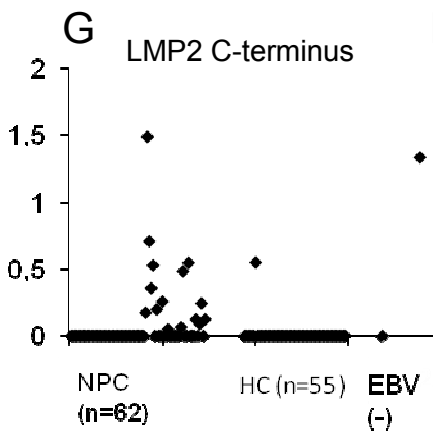
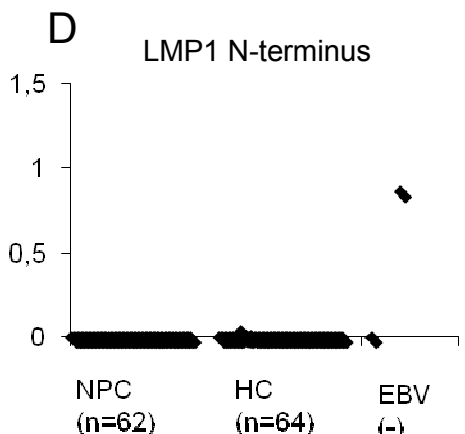
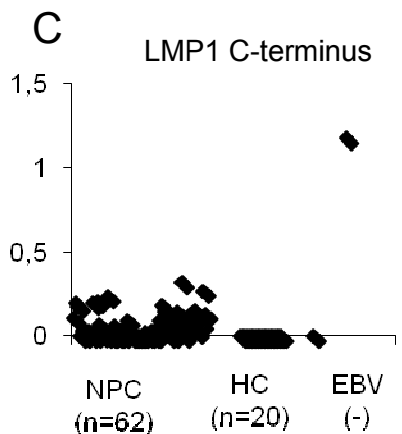


Figure 3
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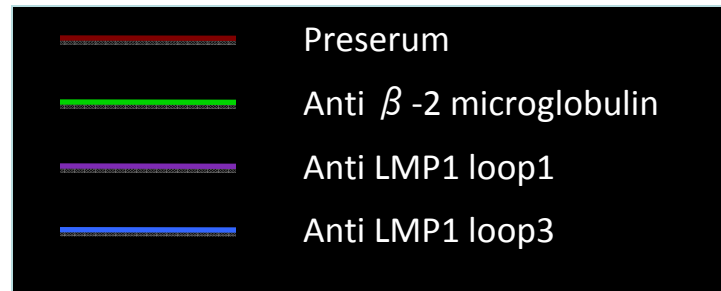
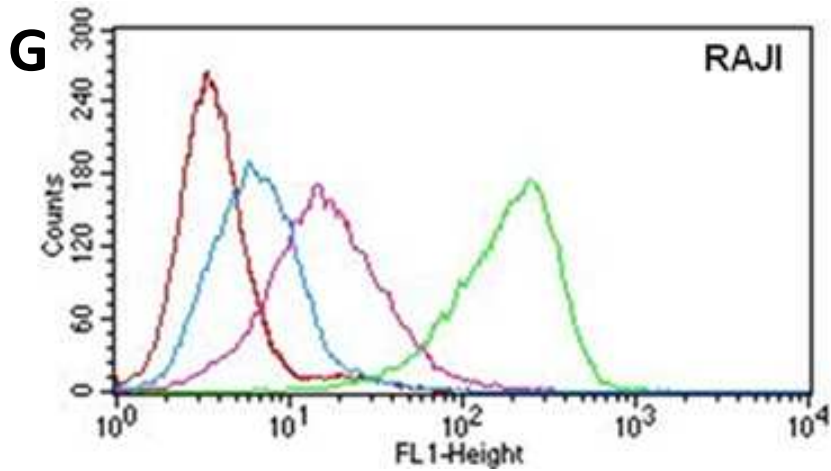
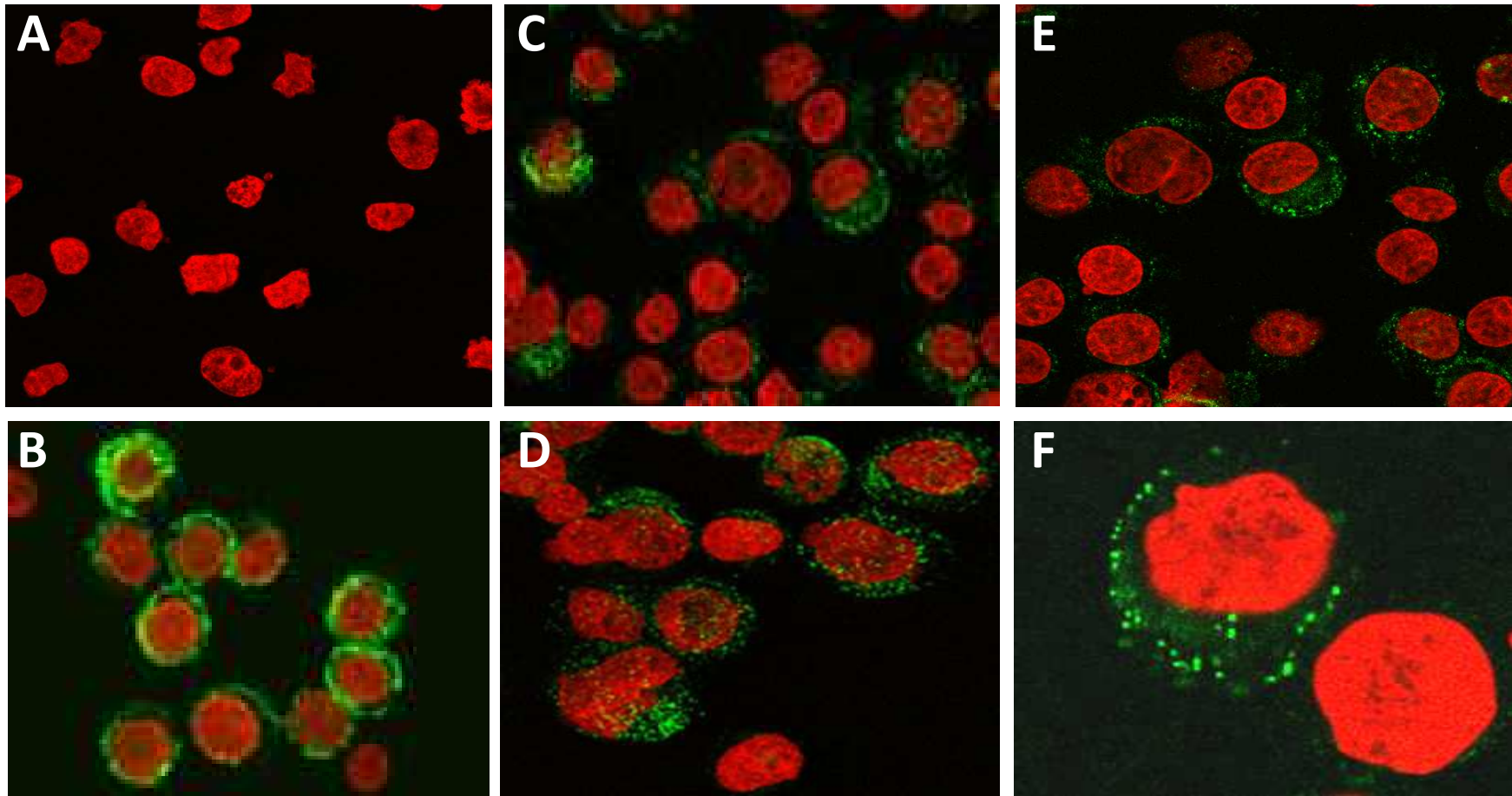


Figure 4

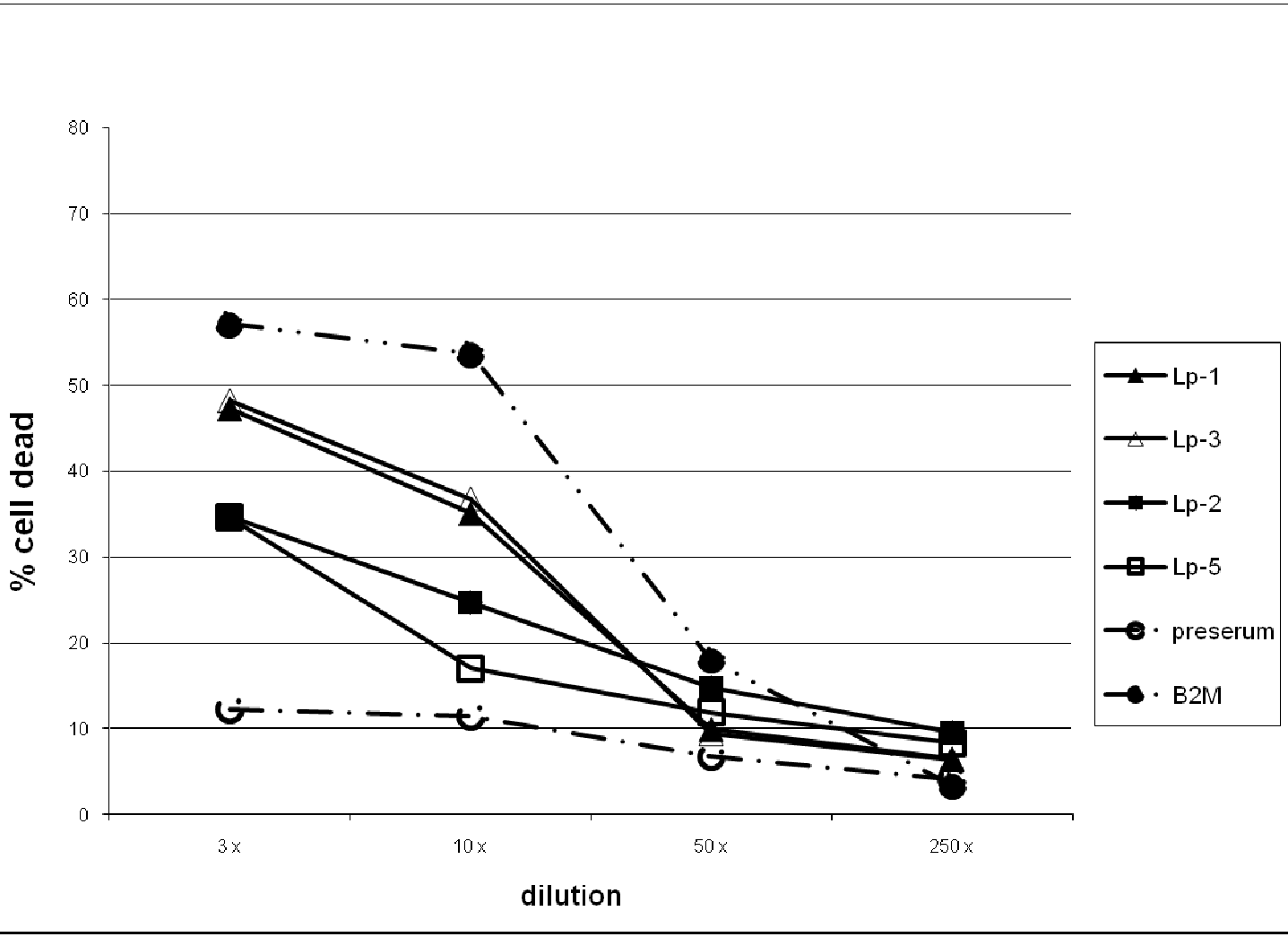


Figure 5

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6 **Humoral Immune Responses to Epstein-Barr virus encoded Tumor Associated**
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8 **Proteins and their Putative Extracellular Domains in Nasopharyngeal**
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10 **Carcinoma Patients and Regional Controls**
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13 **Dewi K. Paramita¹, Christien Fatmawati², Hedy Juwana³, Frank G. van Schaijk³,**
14 **Jajah Fachiroh¹, Sofia M. Haryana¹,**
15 **and Jaap M. Middeldorp³**
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18
19 1. *Molecular Biology Laboratory, Fac. Medicine, Gadjah Mada University,*
20 *Yogyakarta, Indonesia*
21 2. *Fac. Medicine, Gadjah Mada University, Yogyakarta, Indonesia*
22 3. *Dept. Pathology, Vrije Universiteit Medical Center, Amsterdam,*
23 *The Netherlands.*
24
25

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27 LMP2, BARF1
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32 **Corresponding Author:**
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34 Prof. dr. J.M. Middeldorp
35 Dept. Pathology
36 Vrije Universiteit Medical Center
37 De Boelelaan 1117
38 1081 HV Amsterdam
39 The Netherlands
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42 Tel.: +31-20-4444052
43 Fax.: +31-20-4442964
44 E mail: j.middeldorp@vumc.nl
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ABSTRACT

Epstein-Barr virus latency proteins EBNA1, LMP1, LMP2 and BARF1 are expressed in tumor cells of nasopharyngeal carcinoma (NPC). IgG and IgA antibody responses to these non-self tumor antigens were analysed in NPC patients (n=125) and regional controls (n=100) by three approaches, focusing on the putative LMP1, LMP2 extracellular domains.

Despite abundant IgG and IgA antibody responses to lytic antigens and EBNA1, patients had low titer (1:25 - 1:100) IgG to LMP1 (81.2%), LMP2 (95.6%) and BARF1 (84.8%), while immunoblot showed such reactivity in 24.2%, 12.5% and 12.5% at 1:50 dilution, respectively. Few IgA responses were detected, except for EBNA1. Controls only showed IgG to EBNA1. ELISA using peptides from different domains of LMP1, LMP2 and BARF1 also yielded mostly negative results. When existing, low level IgG to intracellular C-terminus of LMP1 (62.9%) prevailed.

Rabbit immunisation with peptides representing extracellular (loop) domains yielded loop-specific antibodies serving as positive control. Importantly, these rabbit antibodies stained specifically extracellular domains of LMP1 and LMP2 on viable cells and mediated complement-driven cytolysis. Rabbit anti-LMP1 loop-1 and -3 killed 50.4% and 59.4% of X50/7 and 35.0% and 35.9% of RAJI cells, respectively, and 22% of both lines were lysed by anti-LMP2 loop-2 or -5 antibodies.

This demonstrates that (extracellular domains of) EBV-encoded tumor antigens are marginally immunogenic for humoral immune responses. However, peptide specific immunization may generate such antibodies, which can mediate cell killing via complement activation. This opens options for peptide-based tumor vaccination in patients carrying EBV latency-II type tumors such as nasopharyngeal carcinoma.

INTRODUCTION

Epstein-Barr virus (EBV) is a human γ -herpesvirus, that infects more than 90% of the world population, and is associated with a spectrum of diseases, including infectious mononucleosis (IM) [Henle et al., 1974], Burkitt's lymphoma (BL) [Epstein et al., 1964], Hodgkin's disease [Kapatai and Murray, 2007; Wu et al., 1990], extranodal T/NK cell lymphoma [De Bruin et al., 1993; van Gorp et al., 1996], immunoblastic B-cell lymphomas in immunocompromised individuals [Snow and Martinez, 2007], gastric carcinoma [van Beek et al., 2004] and nasopharyngeal carcinoma [zur Hausen et al., 1970].

EBV persists for life in its human host after the primary infection and is well controlled by the host's immune system. Life-long immunosurveillance is reflected by the persistence of antiviral antibodies and virus reactive (cytotoxic) T cells [Rickinson and Kieff, 2007]. Different sets of proteins expressed during EBV's lytic and latent life cycle induce qualitatively and quantitatively different immune responses [Fachiroh et al., 2004,2006; Hislop et al., 2007]. Similar to other herpes viruses, EBV reactivation can occur in patients with immune defects or immune suppression reflected by aberrant IgG/M/A antibody responses [Meij et al., 1999]. Importantly, EBV may cause a number of malignancies of lymphoid and epithelial origin in both immunosuppressed and immunocompetent individuals, which are also reflected by aberrant antibody responses to EBV.

In the neoplastic cells of these malignancies, several EBV latent gene products are expressed corresponding to the latency type. Nasopharyngeal carcinoma is one of the latency type II tumors and is characterized by expression of EBNA1, LMP1, LMP-2A/ -2B proteins [Brooks et al., 1992; Heussinger et al., 2004; Khabir et al., 2005] with co-expression of the epithelial oncogene BARF1 [Brink et al., 1998; Decaussin et al., 2000; Seto et al., 2005]. In view of potential immunogenicity of virus-encoded "nonself" proteins, it is surprising that LMP1, LMP2 expressing tumors occur in immunocompetent individuals, who are considered to have the capacity of mounting an effective immune response to these "non-self" proteins.

CD8⁺ T cell responses to EBV latent antigens are skewed towards immunodominant epitopes derived from the EBNA3A, 3B, and 3C protein family. Accompanying subdominant responses map to additional epitopes from the same EBNA3 family or from LMP2, and much less often to epitopes from EBNA2, EBNA-LP, or LMP1 [Hislop et al., 2007; Khanna et al., 1992; Murray et al., 1992]. Only

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3 limited data are available for T cell responses to BARP1 [Martorelli et al., 2008].
4 Early work on EBNA1 as CD8⁺T cell target showed that the internal 250 amino acid
5 glycine-alanine repeat (GAR) protects the endogenously expressed EBNA1 from
6 CD8⁺T recognition [Levitskaya et al., 1995], as consequence from GAR-mediated
7 interference with proteasomal degradation [Dantuma et al., 2002]. EBV has multiple
8 evasion strategies in establishing and maintaining latency in the face of a CD8⁺T cell
9 response by switching-off antigen expression in those cells constituting the latent
10 reservoir [Thorley-Lawson, 2001], by inducing T-cell anergy [Dukers et al., 2000] or
11 Treg's [Marshall et al., 2003] or by active interference with antigen processing and
12 presentation during lytic replication [Wiertz et al., 2007; Zuo et al., 2008]. In addition
13 to the EBV-driven immune evasion, nasopharyngeal carcinoma cells can release
14 HLA-class II positive exosomes containing galectin 9, which can trigger apoptosis of
15 mature Th1 cells [Keryer-Bibens et al., 2006; Klibi et al., 2009].
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26 EBNA1 is well recognized as a major target for humoral immune responses.
27 However, only few studies addressed the role of LMP1 and LMP2 proteins as targets
28 for humoral immune responses in detail. Antibody reactivity to LMP1 has been
29 described in different EBV-related patient populations, including nasopharyngeal
30 carcinoma, Hodgkin Disease, mononucleosis, and Burkitt Lymphoma patients, using
31 different techniques, such as ELISA, immunoblot, and migration inhibition assays
32 [Chen et al., 1992; Lennette et al., 1995; Meij et al., 1999; Meij et al., 2002; Modrow
33 and Wolf, 1986; Rowe et al., 1988; Sulitzeanu et al., 1988]. Previous studies
34 indicated that LMP1 is a protein with a low immunogenicity for the humoral immune
35 response in humans. In nasopharyngeal carcinoma only 7.5% (3/40) patients had
36 low serum levels of antibodies directed to LMP1, whereas antibodies to LMP2A/2B
37 were detected at low titer in about 40-60% of nasopharyngeal carcinoma sera from
38 different ethnicity [Lennette et al., 1995; Meij et al., 1999]. Structurally, LMP1 and
39 LMP2A/B are suggested to protrude from the cell surface via several conserved
40 small loop domains connecting the transmembrane helices [Modrow and Wolf, 1986].
41 However these loop-domains have not been studied as target for humoral immune
42 response to date. Importantly, such anti-loop antibodies may have potentially
43 important function in targeting complement and/or FcR-bearing killer cells to LMP1, 2
44 expressing tumor cells. A prior study of antibody to BARP1 in sera with EBV-
45 associated diseases including nasopharyngeal carcinoma suggested that the BARP1
46 protein may serve as target on EBV-infected cells for antibody dependent cytotoxicity
47 [Tanner et al., 1997]. However, this study has not been confirmed and recent data
48 indicate that BARP1 is rapidly and completely secreted from the EBV positive cells,
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3 making it a disputable target for antibody dependent cytotoxicity [de Turenne-Tessier
4 et al., 2005; Seto et al., 2005].
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8 In this study, antibody responses to EBV-tumor associated antigens LMP1,
9 LMP2 and BARF1 in nasopharyngeal carcinoma patients were evaluated in details
10 compared to healthy EBV carriers. Specific antibodies to the putative LMP1 and
11 LMP2 extracellular loop domains were further developed and evaluated whether
12 such antibodies can mediate complement killing of the LMP1 and LMP2 expressed
13 cell lines, e.g. RAJI and X50/7. The results may provide a basis for understanding
14 EBV tumor immune escape and indicate options for a novel approach to target
15 extracellular domains of LMP1 and LMP2 expressing tumor cells.
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MATERIAL AND METHODS

Sera from nasopharyngeal carcinoma patients and Healthy EBV Carriers.

Serum panels from histologically confirmed nasopharyngeal carcinoma patients (overall n=125) were collected from department of Ear, Nose and Throat (ENT), Dr. Sardjito General Hospital, Yogyakarta. Nasopharyngeal carcinoma sera were taken on the first visit of patients to the clinic, prior to treatment. Nasopharyngeal carcinoma staging was done by ENT examination and CT-scan and classified according to the 1996 criteria established by UICC (Union International Cancer Control). Sera from healthy EBV carriers (overall n=100) were obtained from the local red-cross blood bank. All sera were extensively analysed for reactivity to multiple EBV-encoded lytic cycle proteins in prior studies [Fachiroh et al., 2004, 2006; Paramita et al., 2007, 2008]. Nasopharyngeal carcinoma tissues from available formalin fixed paraffin embedded nasopharyngeal carcinoma tumor biopsies were examined the EBV status by EBER in situ staining (DAKO, PNA) and analysed the expression of LMP1 using S12 or OT21C moAbs base immunohistochemistry [Meij et al., 2002].

Cell culture. The EBV positive RAJI Burkitt Lymphoma cell line, the *in vitro* EBV transformed B cell line X50/7, BJAB-LMP1 (kind gift of M. Rowe) and Daudi-LMP1 (kind gift of P Busson) were cultured in RPMI-1640 medium comprising 25mM Hepes and glutamin (Sigma, St.Louis, USA), 10% fetal calf serum (FCS, Hyclone, Etten-Leur, The Netherlands), 100 IU/ml penicillin and 50 µg/ml streptomycin (p/s) at 37°C in a humidified 5% CO₂ atmosphere. Both cell lines express relatively high levels of LMP1 and LMP2 [Bernasconi et al., 2006; Meij et al., 2000b]. Insect cells were cultured as described below.

The BJAB-LMP1 cell is originally from EBV negative cell line BJAB transfected with LMP1 expression vectors. The LMP1-transfected clones of BJAB were established using a tetracycline-regulated vector system and were maintained in culture medium containing 1.5 mg/ml G418, 0.5 mg/ml hygromycin B, and 1 µg/ml tetracycline. Tetracycline withdrawal induced LMP1 expression as previously described [Floettmann et al., 1996].

Recombinant proteins. The Baculovirus constructs expressing full-length LMP1, LMP2A, BARF1 and EBNA1 without the GAr domain were made under control of the polyhedrin promoter [Meij et al., 2000a; Meij et al., 2000b]. Sf9 cells were cultured to the log phase (1×10^6 cells/mL) and infected with one of the Baculovirus constructs.

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3 A high dose of 1-5 PFU/cell was used for recombinant protein production and cells
4 were harvested at 48 hours post infection (pi). For immunofluorescence experiments
5 infection at 1 PFU/cell for 48 hours was used leaving about 50% uninfected cells in
6 the preparation, which were used as specificity control. Insect cells were cultured in
7 serum-free SF900-II medium at 28°C.
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12 **EBV synthetic peptides.** Immunodominant epitopes on EBV proteins were derived
13 by computer prediction techniques, as described by Modrow and Wolf [Modrow and
14 Wolf, 1986], using high scores for hydrophilicity, flexibility, and β -turn probability.
15 Peptides mimicking different domains of LMP1, LMP2 and BARP1 proteins were
16 synthesized with a peptide synthesizer (433A; Applied Biosystem, Foster City, CA).
17 Peptides representing putative extracellular loop domains of LMP1 and LMP2 were
18 also synthesized as circular peptides by inserting two cysteine residues at the ends
19 forming a S-S bridges upon oxidation [Timmerman et al., 2005]. Most peptides were
20 extended at the N-terminus with additional lysine residues for improving solubility and
21 coupling options. All peptides were purified in reverse phase high performance liquid
22 chromatography (Beckman System Gold, Mijdrecht, The Netherlands). Peptide
23 coupling to carrier proteins KLH or TTd was performed by standard techniques using
24 commercial reagents (Sigma, St.Louis, USA). Peptide denomination and amino acids
25 sequences are listed in table 1.
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36 **Monoclonal and polyclonal antibodies.** Monoclonal (MoAb) and polyclonal (PoAb)
37 antibodies were obtained by immunization of mice and rabbits with synthetic peptides
38 or purified recombinant EBNA1, LMP1, LMP2 and BARP1 proteins expressed in
39 insect cells. Female Chinchilla rabbits were immunized with either keyhole limpet
40 hemocyanine (KLH) or tetanus toxoid (TTd) conjugated synthetic peptides or
41 isotachopheresis isolated recombinant proteins [Meij et al., 1999]. Before
42 immunization pre-serum of each rabbit was drained from the ear. For primary
43 immunization 1 mg antigen was mixed well with 1 ml Freund's Complete Adjuvant
44 (FCA) and injected subcutaneously and intramuscularly. Each rabbit was coded as k
45 followed with numbers (xx). Approximately 30 days (+/-1 day) after primary
46 immunization 5 ml blood was drawn and coded as kxx/-1. First, second and third
47 immunizations with Freund's incomplete adjuvant (FIA) were given with an interval of
48 approximately 1 month. Booster blood samples (kxx/ -2, -3, 4, or -5) were taken 10
49 days after booster injection [Aarbiou & Middeldorp, unpublished]. Production of
50 monoclonal antibodies to various intracellular domains of LMP1 and LMP2 was
51 described before [Fruehling et al., 1996; Meij et al., 1999; Meij et al., 2000b], MoAbs
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3 to N- and C-terminal domains of BARF1 were made in-house by standard
4 procedures [Klarenbeek and Middeldorp, unpublished].
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8 **Immunofluorescent staining on fixed recombinant antigen-expressing cells.**

9 Cytospins were made with Sf9 cells either infected with wild type (wt) baculovirus or
10 recombinant baculovirus. Slides were fixed in cold (-20°C) acetone and pre-
11 incubated in PBS containing 2% fetal calf serum (PBS/2%FCS) for 10 min. All
12 washings were done three times in PBS/0.05% Tween-20 (PBSt). Antibody dilutions
13 were made in PBS/2% FCS and incubated at RT. MoAbs were diluted in 100 – 1000
14 times and human sera were used in a 1:25, 1:50, 1:100 and 1:200 and incubated for
15 1 h unless stated otherwise. After washing, the slides were incubated for 30 min with
16 FITC-labeled rabbit anti-mouse Ig or anti human IgG secondary antibodies (DAKO,
17 Glostrup, Denmark). Finally slides were counterstained for 5 min. with a 1:1 mix of
18 DAPI and Evans blue or 1:500 ToPro3 (Partec, Heerhugowaard, The Netherlands)
19 washed, dipped with mounting fluid Vectashield, sealed with a coverslip and
20 evaluated with a Leica DMRB fluorescence microscope (Leica, Cambridge,
21 England).
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31 **SDS-PAGE and western blot analysis.** Recombinant proteins were solubilized in
32 standard Laemmli sample and boiled for 5 min. and separated in 10% acrylamide
33 gels using the Mini Protean II system (BioRad, Hercules, USA) under reducing
34 condition. Polypeptides were transferred from the gel onto 0.2 µm nitrocellulose
35 (Schleicher & Schuell, s'Hertogenbosch, the Netherlands) by Western blotting (Mini-
36 Trans blot cells, BioRad). After transfer, nitrocellulose sheets were washed with H₂O
37 and dried between filter paper and stored at 4°C until use. Marker proteins (Bio-Rad
38 Low MW marker) were run on the side to indicate the molecular weight of
39 polypeptides. Non-specific binding sites were saturated with blocking buffer (5%
40 horse serum and 5% non-fat dry milk (Campina, Eindhoven, the Netherlands) in PBS
41 pH 7.2) followed by incubation with Moab or PoAb at appropriate dilutions or sera at
42 different dilutions made in blocking buffer. After washing with PBSt, specific bound
43 IgG and IgA were detected with horseradish-peroxidase (HRP)-conjugated
44 secondary antibody (Dako) in blocking buffer and HRP-activity was visualized by
45 using 4-chloro-1-naphthol [Fachiroh et al., 2004].
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57 **Synthetic Peptide ELISA.** Standard microtiter plates (Biobasic, Toronto, Canada)
58 were coated overnight at 4°C with 135 µl of one of the peptides in a concentration of
59 1 µg/ml in 0.05M carbonate buffer, pH 9.6. Excess coating fluid was removed and
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3 non-specific binding sites were blocked subsequently for 1 h with 200ul/well of
4 PBS/3% BSA at 37°C. Further incubations were performed for 1 h at 37°C followed
5 by four washes with PBSt. Human sera were diluted 1:50 in ELISA sample buffer
6 (PBSt; 0.1% Triton-X100, 1% BSA), followed by washing and incubation with HRP-
7 labeled rabbit anti-human IgG (1:3000) and IgA (1:2000) (DAKO) diluted in conjugate
8 buffer (PBSt; 0.1% (v/v) Triton-X100, 1% BSA and 2% normal rabbit serum).
9 Peptide-specific Moab or PoAb were diluted in ELISA sample buffer and detected
10 with rabbit anti-mouse or swine anti-rabbit HRP conjugates (Dako) (both at 1:1000),
11 respectively. HRP activity was detected using 3,3',5,5'-tetramethylbenzidine (TMB)
12 (BioMerieux, Boxtel, The Netherlands) and the reaction was stopped by adding 1M
13 H₂SO₄. The optical density was determined at 450 nm (Anthos 2001 reader, Anthos
14 Labtec, Wals, Austria).
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24 **Membrane immunofluorescence on viable cells.** Log-phase grown RAJI, X50/7,
25 Daudi-LMP1, BJAB-LMP1 and BJAB cell suspensions were used and all incubations
26 were performed on ice with pre-cooled solution unless mentioned otherwise. Prior to
27 immunofluorescence, lymphoprep purification was performed to remove dead cells
28 from the suspension. Cells were transferred to FACS tubes at 0.5×10^6 cells/ 100 μ L
29 staining buffer [Hank balanced salt solution (HBSS); 0.1% (w/v) NaN₃; 1.0% (w/v)
30 BSA, fraction V]. Subsequently, appropriate dilutions of PoAb anti-LMP1 loop-1 and
31 -3 and LMP2 loop-2, and -5 were added and incubated for 20 minutes. Following
32 two washes with staining buffer, fluorescein isothiocyanate-labeled swine anti-rabbit
33 Ig (1:100) in FACS buffer was added and incubated for 20 minutes. For confocal
34 microscopy cells were washed in HBSS, cyto-centrifuged onto glass slides and
35 counterstained for 5 min. Microscopic analysis was done using a Leica TCS confocal
36 microscope (Leica, Cambridge, England) and results were digitally stored. For FACS
37 analysis, the cells were washed three times with staining buffer and resuspended in
38 100 μ L propidium iodide solution. Data acquisition was performed on FACSCalibur
39 flow cytometer (BD Biosciences, Franklin Lakes, NJ). Cell staining with anti- β_2 M
40 antibody (Dako) served as positive control.
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53 **MTT assay.** To evaluate the cytolytic capacity of anti-LMP1 and -LMP2 loop-specific
54 antibodies, complement cytotoxicity studies were performed with MTT read-out (Cell
55 Proliferation Kit I, Roche, Mannheim, Germany) using EBV, LMP1, 2 positive RAJI
56 and X50/7 cell lines and appropriate controls. All incubations were performed at
57 37°C and 5% CO₂. Prior to the experiment, lymphoprep purification was performed
58 to remove dead cells. Cells were placed on a 96 well plate at 10^4 cells /25 μ l per
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3 well. Antibody anti-loop-1 and -3 LMP1 and anti-loop-2 and -5 LMP2 (1:3, 1:10, 1:50
4 and 1:250) were added, followed by the addition of 50 μ l 30 times diluted rabbit
5 complement (Innovative Research, Novi, Michigan, USA) and incubated for 2 hrs. As
6 controls, cells were incubated with rabbit pre-serum or beta-2 microglobuline.
7 Subsequently 5 μ l MTT labeling reagent was added. After 4 hrs, 50 μ L solubilization
8 reagent was added and after overnight incubation, the optical density was
9 determined at 550-600 nm. Percentage of dead cell was calculated by using the
10 formula below.
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18 **Percentage (%) cell death:**

19 OD of untreated cells (blank) – OD of treated cells
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21 _____ X 100%
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23 OD of untreated cells (blank)
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27 **Declaration on human and animal studies.** From all nasopharyngeal carcinoma
28 patients in this study informed consent was obtained on the use of their
29 serum/plasma and tumor samples for research purposes and all procedures were
30 approved by the medical ethical committee of the Sardjito University Hospital,
31 Gadjah Mada University, Indonesia. Sera from healthy controls were obtained from
32 the archives and used with permission as detailed in previous studies (Fachiroh et
33 al., 2006).
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38 All animal experiments were performed under approval of specific animal
39 handling and immunization protocols at Organon Teknika, Boxtel, and VU University
40 medical center, Amsterdam, The Netherlands.
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43 All experiments were conducted in compliance with local laws and
44 institutional guidelines, and are in concordance with ethical standards of the
45 Declaration of Helsinki.
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RESULTS

Humoral immune responses in nasopharyngeal carcinoma patients and healthy EBV carriers to recombinant EBV-encoded Tumor Associated Protein.

In this study, we explore the antibody responses of nasopharyngeal carcinoma patients to individual recombinant proteins LMP1, LMP2 and BARF1. Antibody responses to the individual proteins were analyzed by indirect immunofluorescence and immunoblot techniques. Sf9 insect cells infected with recombinant Baculovirus expressing full-length LMP1, LMP2A and BARF1 were used as antigen (rLMP1, rLMP2A, rBARF1, respectively), mainly as described previously [Meij et al., 1999; Meij et al., 2000a; Meij et al., 2000b]. A low MOI was chosen to leave 40-60% Sf9 cells uninfected, serving as internal specificity control in each experiment. Recombinant EBNA1 deleted of the GAr (rEBNA1) was used as positive control and all sera and MoAbs were analysed in parallel on Sf9-cells infected with wild type Baculovirus (wtBac). Expression of LMP1, LMP2A, BARF1 and EBNA1 in the infected Sf9 cells was confirmed by staining with specific MoAbs to the individual EBV proteins (Figure 1). Human antibody staining was interpreted with the MoAb staining pattern as reference.

Overall, immunofluorescence results with nasopharyngeal carcinoma sera showed rather low (most 1:25-1:100) IgG reactivity to acetone-fixed rLMP1, rLMP2A and rBARF1 being detectable in 81.2%, 95.6% and 84.8% of tested sera, respectively, whereas IgG to rEBNA1 was present at higher titers (> 1:200) in 100% of the sera (n=32) (Figure 1J). In general, observed background reactivity with uninfected Sf9 cells and Sf9-wtBac was minimal and, when present, wt-Bac staining pattern could be discriminated from EBV antigen-specific staining. In simultaneous immunofluorescence analysis, IgA reactivity to rLMP1, LMP2A and BARF1 was observed at even lower titer (< 1:25) and at lower frequency in 40.9%, 54.5%, and 59.0% of nasopharyngeal carcinoma sera, respectively. IgA to rEBNA1 was observed at slightly higher titer (1:100) in 81.8% of the sera (n=22) (data not shown).

Subsequently, to reveal potential immune responses to possible linear epitopes in fully denatured EBV tumor proteins, a set of nasopharyngeal carcinoma sera (n=123) was tested for IgG and IgA reactivity by immunoblot analysis at dilutions of 1:50 using lysates of Sf9 cells expressing either rLMP1, rLMP2A, rBARF1 or rEBNA1. Figure 2A-C show that control MoAbs OT21C, 14B7 and 4A6 recognize clear bands at 63kD (LMP1), 54kD (LMP2a) and 30kD (BARF1). In contrast to

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3 immunofluorescence, immunoblot analysis revealed very low IgG responses to
4 LMP1, LMP2A and BARP1 indicated by weak intensity of the specific protein band in
5 24.2%, 12.5% and 12.5% nasopharyngeal carcinoma patients, respectively. In
6 general EBV-protein specific staining by immunoblot was only detectable using the
7 lowest dilution (1:50), if detectable at all. IgG reactivity to rEBNA1 was observed at
8 94.9% of nasopharyngeal carcinoma patients (Figure 2D), and showed similar clear
9 band at 55kD as revealed by MoAb OT1X (figure not shown). None of
10 nasopharyngeal carcinoma patients had detectable IgA response to the LMP1,
11 LMP2A and BARP1 by immunoblot analysis, but a weak IgA response to EBNA1 was
12 observed at 56.5% nasopharyngeal carcinoma patients. These data indicated that
13 nasopharyngeal carcinoma patients, who have high-level antibody reactivity to
14 multiple lytic cycle antigens and EBNA1 [Fachiroh et al., 2006; Fachiroh et al., 2004],
15 are largely lacking potent antibody responses to tumor associated membrane
16 antigens LMP1 and LMP2A, as well as BARP1, as examined with intact full length
17 recombinant proteins.
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28 **LMP1 expression and antibody reactivity in nasopharyngeal carcinoma cases.**

29 No relation was found between LMP1, LMP2 and BARP1 responses (when present)
30 with TNM stage of the tumor. In cases analyzed for serological responses to LMP1
31 by immunofluorescence assay (n=32) or immunoblot (n=125), the presence of LMP1
32 was detected at the tumor level using MoAb base immunohistochemistry. Results
33 are shown in table 3A and 3B. Overall 80% of the nasopharyngeal carcinoma were
34 found to LMP1 expression using immunohistochemistry. In cases having antibody
35 reactive with LMP1 by immunofluorescence assay has positive correlation with LMP
36 expression on the tumor (68.8% concordance), but by IB has negative correlation
37 (33.6% concordance) (table 3).
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46 Immunofluorescence and immunoblot may detect different epitopes, which is related
47 to the level of denaturation of the antigen used, being minimal in
48 immunofluorescence assay using acetone fixation, and maximal in immunoblot using
49 SDS boiling. Therefore, it was decided to analyse this option in more detail. The
50 functional importance of detecting antibody responses to LMP1 and LMP2
51 conformational domain will be of particular interest when expressed on the tumor cell
52 surface.
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58 **Antibody Responses to defined extracellular peptide-epitopes of and LMP1,**
59 **LMP2 and BARP1.** To more precisely study the epitope specificity in the sera of
60 nasopharyngeal carcinoma patients, defined synthetic peptides representing putative

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3 extracellular domains of LMP1, LMP2 and BARF1 were created and used as antigen
4 in ELISA. Cytoplasmic peptide epitopes of LMP1 and LMP2 and extracellular
5 domain of BARF1 were selected for having high scores for hydrophilicity, flexibility
6 and β -turn probability as described before [Meij et al., 1999; Middeldorp and Meloen,
7 1988; Modrow and Wolf, 1986]. In addition, for LMP1 and LMP2 synthetic peptides
8 were also created representing the extracellular loop 1 and 3 (connecting the 1st to
9 2nd and 5th to 6th transmembrane helix, respectively) and loop 2 and 5 (connecting the
10 3rd to 4th and 9th to 10th helix respectively), respectively (Figure 3A & 3B). Synthesis
11 of cytoplasmic peptide domains of LMP1 have been described previously [Meij et al.,
12 1999]. For LMP1, peptide domain in circular conformation to more closely mimick
13 the in vivo structure was used. Circular peptides were created by oxidation of the
14 sulfide bridge in peptides OTP 405 and OTP 407 (Table 1) [Timmerman et al., 2005].
15 These peptides were used as antigens in indirect ELISA. Epitope-specific antibodies
16 were generated by rabbit immunization using carrier proteins conjugated to the
17 peptides. These newly developed antibodies were used as positive control in the
18 ELISA (Figure 3). All human sera used were strongly responsive to VCA-p18 and
19 EBNA1 synthetic peptides as described before [Fachiroh et al., 2006].
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32 Analysis of LMP1, 2 and BARF1 peptide-epitope specific antibody response by
33 ELISA did not show major differences between nasopharyngeal carcinoma patients
34 and healthy EBV carriers. When detectable, positive responses were marginal in
35 most cases and the most significant response (62.9% positive) in nasopharyngeal
36 carcinoma patients is confined to IgG against the intracellular C-terminus of LMP1
37 (Figure 3C). Overall analysis is depicted in table 2. Table 2A shows the number of
38 donors and patients having IgG responses to the individual peptides of tumor-
39 associated EBV proteins. IgG responses to LMP2 in healthy EBV carriers were
40 lower compared to responses to LMP1 and BARF1. There was no difference in
41 LMP1 loop-peptide responses when using circular (created by S-S bridge oxidation)
42 or linear peptides (data not shown). None of healthy EBV carriers had IgG responses
43 to LMP2 loop peptides. Responses to C-terminus and N-terminus LMP2 are found
44 only in 2.0% and 1.8% of healthy EBV carriers, respectively. About 5% of healthy
45 EBV carriers had IgG response to subfragments of LMP1 and BARF1. Table 2B
46 shows the number of nasopharyngeal carcinoma patients and healthy EBV carriers
47 with IgA responses to peptides of LMP1, LMP2 and BARF1. IgA responses are
48 lower as compared to IgG responses, and most of the IgA responses in
49 nasopharyngeal carcinoma patients can also be addressed the C-terminus of LMP1
50 (27.4%).
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Accessibility of LMP1 and LMP2 loop domains on viable EBV transformed cells. LMP1 and LMP2 are transmembrane proteins, with six or twelve membrane-spanning domains, respectively, connected by intracellular and extracellular loops. The extracellular loop domains are potential targets for functional immune responses, and may mediate killing of EBV transformed cells via complement dependent cytotoxicity or killer cell dependent cytotoxic pathways known as antibody dependent cytotoxicity. To study the accessibility of the extracellular loops of LMP1 and LMP2 on viable cells, evaluation of specific antibody recognition of these proteins expressed on viable RAJI, X50/7, Daudi-LMP1, BJAB-LMP1 and BJAB cell lines was done by FACS analysis and confocal microscopy. All cell lines except BJAB cells were positive for LMP1 and LMP2A mRNA as determined by reverse transcription PCR and by intracellular protein staining. For the latter, permeabilized cells were treated with monoclonal antibodies OT21C and 14B7, recognizing the intracellular epitopes of LMP1 and LMP2A respectively (data not shown). Both LMP1 and LMP2A revealed a heterogeneous intracellular staining pattern between individual cells of a cell population as described before [Lennette et al., 1995; Rowe et al., 1988]. The presence of LMP1 and LMP2A loop domains on the surface of those cell lines were detected by indirect fluorescence and FACS analysis using anti-loop specific antibodies for LMP1 loop 1 and 3 and LMP2 loop2 and 5 (Figure 4 A-G). LMP1 clearly expressed on RAJI and X50/7 (5-15% of the cells), but clearly negative with Namalwa and BJAB. Figure 4 shows a representative fine patch-like staining observed on 15-20% of RAJI cells for loop1 and 3 LMP1 (Figure 4C,D) and BJAB as negative control (figure 4A). FACS analysis of RAJI cells using similar antibodies confirmed the low-level staining in a restricted number of Raji cells (Figure 4G). On cells artificially expressing LMP1 (Daudi-LMP1 and BJAB-LMP1) by vector transfection much higher staining was seen (20-50%; Figure 4E). Best LMP2 expression was seen for loop2 on X50/7 cells (Figure 4F). Rabbit antibody against β 2M served as positive control and strongly reacted with >88% of all cell lines (Figure 4B and 4G). Staining pattern of individual viable cells was determined by confocal microscopy, revealing a heterogenous staining pattern similar to the cytoplasmic staining patterns, with some cells being negative, and others being positive and showing a patch-wise distribution of LMP1 and LMP2 related epitopes. This is the first demonstration that extracellular LMP1 and LMP2 related loop domains, can potentially function as targets for antibody-based therapy.

Complement lysis by anti-LMP1 and -LMP2 loop-specific antibodies. Since LMP1 and LMP2 are expressed in multiple EBV tumors, including nasopharyngeal

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3 carcinoma, targeting of the extracellular domains may have therapeutic potential.
4 This study demonstrated that immunization of rabbits using synthetic peptides
5 mimicking the extracellular loop domains of LMP1 and LMP2 could generate specific
6 anti-loop antibodies. This approach might be applicable to humans as well, aiming for
7 therapeutic vaccination. Considering this option, the functional activity of the anti-loop
8 antibodies was evaluated and complement mediated lysis was analysed using RAJI
9 and X50/7 cell lines. Figure 5 shows that by 4 hr complement lysis 35%, 35.3%,
10 22.4% and 22.3% of RAJI cells and 50.4%, 59.4%, 22% and 22.7% of X50/7 cells
11 were killed by anti-LMP1 loop 1 and 3 and anti-LMP2 loop 2 and loop 5 antibodies
12 respectively, as measured by MTT assay. Killing potential of each antibody clearly
13 was dose dependent as reflected in the decrease with higher dilution. No cell lysis
14 was observed by pre-serum obtained from these rabbits (Figure 5, bottom line) and
15 no lysis was observed with Namalwa or EBV negative Ramos or BJAB cell lines
16 (data not shown), whereas using β 2M as target closely to 80% cells were lysed in
17 this assay. These results demonstrate that newly developed anti-loop antibodies can
18 target specifically and functionally extracellular domains of LMP1 and LMP2, which
19 may have important therapeutic implications.
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DISCUSSION

Individuals with EBV infection develop antiviral immune responses to a wide variety of EBV proteins and epitopes. Monitoring of anti-EBV antibody responses has yielded useful applications for diagnosis in various EBV-associated diseases, such as nasopharyngeal carcinoma. Elevated antibody titers to EBV antigen e.g. early antigens (EA), viral capsid antigens (VCA) and the EBNA1 protein are frequently found in nasopharyngeal carcinoma patients and are relevant as diagnostic and prognostic markers [Cheng et al., 2002; Fachiroh et al., 2006; Fachiroh et al., 2004; Karray et al., 2005; Ng et al., 2005; Paramita et al., 2007]. However most of the EBV antigens used for diagnosis are not expressed in tumor cells, but are derived from sporadic cells entering the lytic stages infection accompanying the malignant process.

Besides EBNA1, which is expressed universally in all EBV tumor cells, latent EBV proteins such as LMP1 and LMP2 are regularly detected in nasopharyngeal carcinoma [Heussinger et al., 2004; Khabir et al., 2005]. This study could detect LMP1 expression about 80% of the cases analysed (n=125), but we did not have access to the LMP2-reactive antibodies used by Heussinger. In addition, recent studies revealed the expression and secretion of BARF1 protein in nasopharyngeal carcinoma and gastric cancer in absence of lytic gene expression (Seto et al., 2005). Due to the expression of non-self viral proteins in the nasopharyngeal carcinoma tumor cells, the possibility appears that these proteins might become targets of immune response, aiding in protection. Previous studies demonstrated that EBNA1, LMP2A and to a lesser extend LMP1 can elicit virus-specific cellular immunity and are proposed as antigen for immunotherapy [Comoli et al., 2004; Comoli et al., 2005; Hislop et al., 2007; Swanson-Mungerson et al., 2003]. However, information on humoral immune responses to LMP1, LMP2A and BARF1 antibodies is rather limited [Frech et al., 1993; Lennette et al., 1995; Meij et al., 1999; Tanner et al., 1997]. In the present study using immunofluorescence-analysis on acetone-fixed recombinant proteins expressed in insect cells, IgG antibodies to LMP1 and LMP2 were found in a significant number of nasopharyngeal carcinoma patients (81.2 % and 95.6%, respectively), albeit in low titers, but hardly in controls. This confirms and extends previous studies that used smaller numbers of patients and controls [Frech et al., 1993; Lennette et al., 1995; Meij et al., 1999]. In all samples tested, the LMP1,2 responses were much lower compared to IgG-EBNA1. By using a similar method this study found that 84.8% nasopharyngeal carcinoma patients have a detectable but

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3 low titered IgG response to BARF1. Antibody responses against BARF1 protein
4 have been studied before using sera from chronic and acute infectious
5 mononucleosis and nasopharyngeal carcinoma patients [Tanner et al., 1997]. Using
6 transduced RAJI cells they demonstrated significant antibody dependent cytotoxicity
7 reactivity to BARF1-expressing RAJI cells in sera from nasopharyngeal carcinoma
8 patients. However no study has yet confirmed antibody responses to BARF1 to
9 strengthen these findings. In fact, BARF1 seems to be rapidly and completely
10 secreted by BARF1 expressing cells, leaving little protein in or on the cells for
11 detection [de Turenne-Tessier et al., 2005; Seto et al., 2005]. The role of anti-BARF1
12 immune responses remains to be further established.
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20 Immunoblotting confirmed the low-level antibody responses, being detectable
21 at 24.2%, 12.5%, and 12.5% of nasopharyngeal carcinoma patients for LMP1, LMP2
22 and BARF1, respectively. The lower response rates compared to
23 immunofluorescence-analysis may be due to the fact that antigens prepared by SDS-
24 PAGE may have lost certain conformational epitopes. Again anti-EBNA1 antibodies
25 were clearly detected, confirming the immunodominance of EBNA1. IgA-specific
26 analysis showed similar low responses to LMP1, LMP2A and BARF1, but again
27 clearly detectable responses to EBNA1. This demonstrates a lack of local mucosa-
28 specific responses to the tumor-associated latent EBV membrane antigens, hinting at
29 specific defects in their presentation to the immune system. These observations are
30 clearly in contrast to the responses to the marginally expressed but highly
31 immunogenic lytic antigens, to which abundant IgG and IgA antibody responses are
32 detectable in the same nasopharyngeal carcinoma patients [Fachiroh et al., 2006;
33 Fachiroh et al., 2004; Paramita et al., 2007]. Importantly, most (80%)
34 nasopharyngeal carcinoma cases analysed showed LMP1 expression. This study
35 found a positive correlation between LMP1 expression and Ab-responses using
36 immunofluorescence analysis, but a negative correlation when using immunoblot
37 (table 3). This may suggest that conformational epitopes, which are more reactive by
38 immunofluorescence assay may be triggered in LMP1 positive tumor cases, whereas
39 antibodies to linear (denatured) LMP1 are triggered differently (i.e. by cross
40 presentation). The finding in nasopharyngeal carcinoma differs from previous
41 observation in Hodgkin Disease, where LMP1 antibodies were most prevalent in EBV
42 seropositive but tumor negative cases [Meij et al., 2002].
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59 The data from this study using EBV-recombinant proteins showed that
60 nasopharyngeal carcinoma patients only have weak humoral immune responses to

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3 LMP1, LMP2 and BARF1. However the potential importance of LMP1 and LMP2 as
4 targets for immunotherapy, prompted us to further analyze the presence of
5 antibodies in nasopharyngeal carcinoma patients directed to defined extracellular
6 epitopes of LMP1, LMP2 and BARF1 in the form of synthetic peptides. No such
7 information was available yet, and, in fact, the extracellular accessibility of domains
8 of LMP1 and LMP2 has not been clearly demonstrated before. Therefore we
9 extended our previous studies and explored responses to defined peptide epitopes
10 mimicking these domain [Meij et al., 1999]. In rabbits, polyclonal epitope-specific
11 antibodies were developed directed against distinct domains of LMP1, LMP2 and
12 BARF1. These antibodies, having a high affinity for their epitopes in denaturated as
13 well as in the native conformation on viable cells, were used as positive controls.
14 Using these anti-loop antibody reagents, demonstration of the presence and
15 functional accessibility of extracellular loop domains of LMP1 and LMP2 was done,
16 opening option as targets for therapeutic applications [Middeldorp, 2002]. However,
17 in naturally EBV infected nasopharyngeal carcinoma patients and healthy EBV
18 carriers these LMP1 and LMP2 loop domains seem to evade from immune
19 recognition, as anti-loop antibody responses are mostly negative (Figure 3 I,F,I,J;
20 Table 2). The results of peptide-specific analysis confirm the presence of some
21 antibody responses to the intracellular C- and N-terminal domains of LMP1 and
22 LMP2, although only at a low levels (Figure 3C,D). Intrinsic properties of LMP1 and
23 LMP2 and their limited expression in the plasma membrane may be responsible for
24 the low immunogenicity. On the other hand, this study shows that LMP1 and LMP2
25 antibodies specifically directed against the extracellular loop domains can be
26 generated by immunization of rabbits using related peptides and these antibodies
27 can activate the complement system to kill LMP1 and LMP2 expressing cells. X50/7
28 cells can be killed by complement (50.4% and 59.4%) in higher percentage
29 compared to RAJI cells (35% and 35.9%) most likely reflecting different level of
30 LMP1 and LMP2 expression or differences in loop-accessibility. This requires further
31 analysis but is in line with known LMP1 expression levels in different cell lines [Meij
32 et al., 2000b]. Detection of extracellular domains requires viable cells and low
33 temperature incubation to inhibit aggregation and internalization activity. A
34 heterogeneous staining pattern of small patches of FITC-labeled anti-loop antibodies
35 was demonstrated in the cell membrane. Also individual cells among the cell
36 population showed a clear distribution (Figure 5). This corresponds with the known
37 heterogeneous intracellular expression of LMP1, being abundant in some cells and
38 barely detectable in others in the same culture [Rowe et al., 1988]. The relation
39 between intracellular situated and membrane-associated LMP1 and LMP2 remains
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3 to be analysed in detail (studies in progress). Conclusion from this study suggests
4 that limited humoral immune responses to EBV-encoded tumor antigens LMP1,
5 LMP2 and BARP1 allow malignant cells to escape from control. Augmentation of
6 immune reactivity to EBV-tumor associated antigens especially LMP1 and LMP2, by
7 active or passive immunization, may be important to the prevention and treatment of
8 nasopharyngeal carcinoma as a member of latency type II tumors. The finding that
9 immunization of rabbits using these peptides can generate highly reactive epitope-
10 specific antibodies opens new prospects for immunotherapy and vaccination of
11 patients suffering from EBV associated tumors [Middeldorp, 2002].
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For Peer Review

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For Peer Review

Legends to the figures:**Figure 1.****Antibody detection by indirect immunofluorescence of acetone-fixed Sf9 cells infected with Baculo-EBNA1 (A, B), Baculo-LMP1 (C, D), Baculo- LMP2A (E, F), Baculo-BARF1 (G, H) and WT-baculovirus (I).**

Cells were incubated with OT1X (1:200, mouse anti-EBNA1) (A), OT21C (1:100, mouse anti-LMP1) (C), 14B7 (1:100, rat anti-LMP2A) (E), K150-3 (1:100, rabbit anti-BARF1) (G), and serum of NPC patients containing antibodies anti-EBNA1, LMP1, LMP2A and BARF1 (B, D, F, H respectively). Sf9-WT-Bac served as negative control incubated with either Mo/PoAbs or NPC serum (I). Similar pattern was observed as Baculo-BARF1 incubated using K150-3, when the cells were incubated with mouse MoAb 4A6 (mouse anti-BARF1). (J) Bar chart shows the summary of antibody responses of NPC sera to Sf9 cells infected with Baculo- EBNA1, -LMP1, -LMP2A and -BARF1 respectively, as detected by indirect immunofluorescence. For an indication of the level of antibody response, NPC sera were examined at various dilutions, as Indicated.

Figure 2.**Immunoblot analysis of SF9-baculo expressed recombinant proteins stained with monoclonal, mono-reactive polyclonal antibodies and NPC sera.**

(A) Baculo-LMP1 strips, (B) Baculo-LMP2A strips, (C) Baculo-BARF1 strips, stained with Mo/PoAbs specific to the protein (strip 1 for each immunoblot series) and stained with NPC sera (line 2-end for each series). (A-OT21C) Baculo-LMP1 strip stained with OT21C MoAb showing band on 63kD, (B-14B7) Baculo-LMP2A strip stained with 14B7 PoAb showing band on 54kD, (C-K150-3) Baculo-BARF1 strip stained with K150-3 PoAb showing band on 30kD. (A1 - A23) Baculo-LMP1 strip stained with NPC serum (1:50), (B1-B23) Baculo-LMP2A strip stained with NPC serum (1:50), (C1-C19) Baculo-BARF1 strip stained with NPC serum (1:50). (D) Bar charts summary of antibody responses of NPC sera to Sf9 cells infected with Baculo-EBNA1, -LMP1, -LMP2A and -BARF1 using IB. For an indication of the level of antibody response, NPC sera were examined at 1:50 dilutions.

Figure 3.**Structural representation of LMP1 and LMP2A molecule in the plasma membrane and IgG responses of NPC patients and healthy EBV carriers to peptide epitopes of LMP1 and LMP2A.**

Both proteins contain an intracellular N and C-terminus. (A) LMP1 is characterized by three short extracellular loops connecting the six membrane-spanning segment. (B) LMP2 has 6 short extracellular loops, connecting 12 membrane spanning domains. (A) Anti-C terminus, -N terminus, -loop-1 and -loop-3 of LMP1 and (B) anti-C terminus, -N terminus, -loop-2 and -loop-5 of LMP2 specific antibodies were generated by rabbit immunization. Polyclonal antibodies generated from rabbit immunization with the LMP1 peptide showed strong specific reactivity to each epitope as shown at the upper right of each graph as positive control: (C) K49-3 to the C-terminus LMP1, (D) K48-3 to the N-terminus LMP1, (E) K31-3 to the Loop1 and (F) K56-3 to the Loop-3. Polyclonal antibodies generated from rabbit immunization with the LMP2A peptides showed strong specific reactivity to each epitope as shown at the upper right of each graph as positive control: (G) K41-3 to C-terminus, (H) K42-3 to N terminus, (I) K47-3 to Loop-2 and (J) K43-3 to Loop-5. NPC: nasopharyngeal carcinoma, HC: Healthy EBV carrier.

Figure 4.**Accessibility of extracellular loops of LMP1 on viable RAJI and BJAB cells as determined by specific anti-loop antibodies.**

(A) In all experiments EBV negative BJAB cells produced a complete negative membrane staining with the LMP1 and LMP2 loop-specific sera. (B) As positive control, RAJI and BJAB cells showed more than 88% positive staining using anti- β -2microglobulin. (C, D) A fine patch-like staining was observed on the surface of RAJI cells with anti-LMP1 loop-1 and loop-3 specific antisera (C, D respectively). Note that some cells in the culture were negative for Loop-1-3 expression, which were generally having small nuclei, representing non-cycling cells. (E) Anti-LMP1 loop-1, -3 specific antisera produced a similar, but more abundant patch-like staining on stably transfected BJAB cells induced for LMP1 expression from a tetracyclin regulated promoter during 24 hours, similar as observed with LMP1 transfected Daudi cells (data not shown). (F) Antisera to LMP2 loop-2 and -5, produced a similar patched staining pattern on X50-7 cells, with somewhat larger patches than observed for LMP1. (G) Flow cytometry histogram comparing the levels of accessibility of anti loops LMP1 on Raji cells. Cells were gated for viability by 7-AAD exclusion. Staining was obtained with the indicated rabbit anti-LMP1 loop-1 (purple line), rabbit anti-

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3 LMP1 loop-3 (blue line) and using rabbit anti-b2M as positive control (green line).
4 Background staining with rabbit pre-serum shows low signal as indicated by the red
5 line, which is similar to anti-loop antiserum on control BJAB cells (data not shown).
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11 **Figure 5.**

12 **Complement mediated cell lysis.** RAJI cells were incubated with a dilution series of
13 anti-loop antibodies (3x, 10x, 50x and 250x) and 30 times diluted complement
14 solution. Percentage cell death is plotted against antibody dose. In 3x dilution of anti-
15 loop antibodies, approximately 49% RAJI cells were killed by anti-loop-1 and -3
16 LMP1 and 35% by anti-loop-2 and loop-5 LMP2A. Similar results or even higher
17 killed-cells were obtained in other EBV carrying LCL lines but not in Namalwa, an
18 EBV positive cell lines with LMP1 and LMP2 negative. Anti-B2M antibody was taken
19 as a positive control, and used at larger dilution (1:5000) and therefore is not
20 reaching >80% lysis. Preserum was used as negative control and to distinguish with
21 specific binding of specific antibodies.
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Table 1.
Amino acid sequence of LMP1, LMP2 and BARP1-derived synthetic peptides

Peptide	Amino acid sequence	AA position
LMP1:		
OTP 415 (Loop1)	H-KKKCYIVMSDWTGGALLVLYC-NH ₂	41-56
OTP 417 (Loop3)	H-KKKCALYLQQNWWTLLVDLLC-NH ₂	157-172
OTP 81 (N-term/LMP1-A)	H-MEHDLERGPPGPRRPPRGPLSS-OH	1-23
OTP 75 (C-term/LMP1-D)	H-GSSGSGGDDDDPHGPVQLSYYD-OH	365-386
LMP2:		
OTP 307 (Loop2)	H-AICLTWRIEDPPFNSLLFAL-OH	183-202
OTP 308 (Loop5)	H-GSILQTNFKSLSTEFIPNLFGM-OH	363-385
OTP 309 (C-term)	H-RCCRYCCYYCLTLESEERPPTPYRNTV-OH	461-497
OTP 310 (N-term)	H-SGSSGNTPTPPNDEERESNEEPPPPYEDPY-OH	35-64
BARF1:		
OTP 539 (C term)	H-LGPEIEVSWFKLGPGEQVLIGRMHHDVIFIEWP-FRGFFD-OH	40-80
OTP 541 (N term)	H-DLSLPKPWHLPVTCVKGNDKEEAHGVYVSGYL-SQ-OH	187-231

Table 2A.
Positive IgG responses (%) in NPC and healthy EBV carriers to LMP1, LMP2 and BARP1 peptides.

	LMP1 (%)				LMP2A (%)				BARF1 (%)	
	Loop1	Loop3	C-term	N-term	Loop2	Loop5	C-term	N-term	C-term	N-term
HC	5.0	5.0	5.0	6.3	0	0	2.0	1.8	5.0	5.0
NPC	14.5	38.7	62.9	11.3	21.2	24.2	8.9	21.9	3.2	21

Table 2B.
Positive IgA responses (%) of NPC and healthy EBV carriers to LMP1, LMP2 and BARP1 peptides.

	LMP1 (%)				LMP2A (%)				BARF1 (%)	
	Loop1	Loop3	C-term	N-term	Loop2	Loop5	C-term	N-term	C-term	N-term
HC	5.0	5.0	10.0	3.0	0	0	1.0	5.5	5.0	5.0
NPC	1.6	0	27.4	3.2	18.2	3.2	20.0	17.8	6.5	11.3

Table 3A.
Correlation between LMP1 expression using IHC and IgG reactivity to LMP1 recombinant proteins by IFA in NPC patients

		IHC		Concordance
		+	-	
IFA	+	21	5	68.8%
	-	5	1	

Table 3B.
Correlation between LMP1 expression using IHC and IgG reactivity to LMP1 recombinant proteins by IB in NPC patients

		IHC		Concordance
		+	-	
IB	+	27	5	33.6%
	-	78	15	