Epstein-Barr virus nuclear antigen 1 evades direct immune recognition by CD4$^+$ T helper cells

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The Epstein-Barr virus (EBV) nuclear antigen 1 (EBNA1) is the only viral protein regularly expressed in EBV-associated malignancies. Immune recognition of EBNA1 by CD8$^+$ T cells is prevented by an internal glycine-alanine repeat (GAr) which blocks proteasomal degradation. To test whether EBV-infected cells could be recognized by T helper cells, human CD4$^+$ T cell clones specific for EBNA1 were isolated from latently EBV-infected individuals. These T cells, however, failed to recognize EBV-positive target cells. To investigate whether endogenous presentation of EBNA1 epitopes on MHC class II was prevented by the GAr domain, a mutant EBV strain with an EBNA1 lacking the GAr (EBNA1ΔGA) was generated and used to establish an Epstein-Barr virus-immortalized lymphoblastoid B cell line (LCL). The EBNA1ΔGA LCL were not recognized by the EBNA1-specific T cell clones either, indicating that the GAr domain does not mediate this effect. Immune recognition could be restored by overexpression of EBNA1, for which at least 60-fold higher levels of both EBNA1 or EBNA1ΔGAr protein were required. These results demonstrate that EBNA1 evades direct recognition by CD4$^+$ T helper cells, since its steady state level is below the threshold required for efficient presentation on MHC class II. These findings have important implications for the design of immunotherapeutic approaches to target EBV-positive malignancies.

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1 Introduction

Epstein-Barr virus (EBV), a ubiquitous human gamma herpesvirus, is associated with a number of human malignancies, including Hodgkin's disease, Burkitt's lymphoma, immunoblastic lymphoma, nasopharyngeal carcinoma, and several others [1, 2]. Common to all EBV-associated tumors is that they involve the latent cycle of the virus. EBV encodes eight antigenically distinct latent-phase proteins, the Epstein-Barr nuclear antigens (EBNA)1, -2, -3A, -3B, -3C, and -LP and latent membrane proteins LMP1 and -2, all of which are found expressed in EBV-immortalized lymphoblastoid B cell lines (LCL) [3]. The successful treatment of immunoblastic lymphoma by the adoptive transfer of EBV-specific T cell lines, generated *in vitro* by repeated stimulation with autologous LCL, has fueled efforts to target EBV latent antigens in other virus-positive malignancies, such as Hodgkin's lymphoma and nasopharyngeal carcinoma [4]. These tumors, however, express a more restricted set of EBV-encoded antigens and generally lack expression of the EBNA3 family of proteins, which tend to induce the strongest CD8$^+$ T cell response across a broad range of HLA class I alleles [5, 6].

EBNA1 is the only viral protein consistently expressed in all EBV-associated malignancies. It is essential for viral DNA replication and the extrachromosomal maintenance of viral episomes in infected cells ([7] and references therein). EBNA1 is protected from HLA class I presentation by virtue of an internal glycine-alanine repeat (GAr) domain, which inhibits antigen processing via the ubiquitin/proteasome pathway [8, 9]. Human CD8$^+$ responses to EBNA1 do exist, most likely as a result of stimulation by antigen-presenting cells (APC) that

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Abbreviations: EBNA1: Epstein-Barr nuclear antigen 1 **GAr:** Glycine-alanine repeat **Ii:** Invariant chain **IRES:** Internal ribosomal entry site **LCL:** Epstein-Barr virus-immortalized lymphoblastoid B cell line **NGF-R:** Nerve growth factor receptor

process exogenous protein and cross-present antigen on MHC class I to CTL [10]. Although such EBNA1-specific CTL have been isolated from healthy virus carriers, these T cells fail to recognize virus-infected cells expressing EBNA1 and are therefore considered to be biologically ineffective [11].

Accumulating evidence suggests that $CD4^+$ T helper cells play critical roles in initiating, regulating and sustaining immune responses against viruses [12] and tumors [13]. By providing help in the form of secreted cytokines and cell membrane ligands, $CD4^+$ T cells orchestrate adaptive immune responses. Furthermore, T helper cells can exert direct effector functions on MHC class II^+ target cells [14, 15]. The polyclonal LCL-stimulated T cell preparations used to treat EBV-positive lymphoproliferative lesions of immunosuppressed patients contain $CD4^+$ as well as $CD8^+$ components, and both components may be necessary for the clinical effectiveness of this adoptive T cell therapy [16, 17]. It is therefore important to determine which latent EBV antigens elicit $CD4^+$ responses, what is the frequency of such T cells in the memory pool, and how these T cells contribute to EBV immunity.

Because EBNA1 is expressed in all EBV-associated malignancies and the majority of these tumor cells express MHC class II molecules, EBNA1 might be a unique and direct target of the $CD4^+$ T helper response. T helper cells specific for EBNA1 have been detected in the peripheral blood of latently infected healthy individuals by several groups, and EBNA1-specific $CD4^+$ T cell clones have been isolated in some of these studies [18–23]. While all these T cell clones have been shown to recognize epitopes derived from EBNA1 in an MHC class II-restricted fashion, the recognition of endogenous EBNA1 in LCL and Burkitt's lymphoma cells by these clones has remained contentious. Here, we isolated EBNA1-specific $CD4^+$ T cell clones from peripheral blood of latently infected virus carriers and assessed presentation of endogenous EBNA1 on MHC class II.

2 Results

2.1 Generation of human $CD4^+$ T cells specific for EBNA1

In order to generate EBNA1-specific T helper cells, EBNA1 was expressed in Sf9 cells as histidine-tagged protein and purified from cell lysates over Nickel-NTA columns. Purified EBNA1 protein was used to generate EBNA1-specific $CD4^+$ T cell lines from three latently EBV-infected individuals by repeated stimulation of $CD4^+$ T cells with irradiated, EBNA1 protein-pulsed autologous PBMC. After five to seven rounds of stimulation, T cell lines with reactivity against EBNA1 but not against a control protein expressed in Sf9 cells were obtained from 3/3 latently infected donors (Fig. 1). This successful reactivation of EBNA1-specific T helper cell responses *in vitro* implies that EBNA1-specific $CD4^+$ T cells are present at significant numbers in the peripheral blood of latently infected individuals, as had already been demonstrated in two independent studies on a large number of latently EBV-infected donors [19, 20]. These T cell lines were cloned by limiting dilution and $CD4^+$ T cell clones specific for EBNA1 were expanded for further analysis.

2.2 Characterization of EBNA1-specific T cell clones

The EBNA1-specific T cell clones were characterized with regard to T helper subtype, restriction element usage, and fine specificity. FACS analysis of cell surface markers showed that all clones were $CD4^+$, $CD8^-$, TCRα/β^+, and TCRγ/δ^- (data not shown). After antigen recognition, all T cell clones secreted GM-CSF and IFN-γ, but not IL-4, IL-10, or TGF-β, a cytokine pattern characteristic of a T helper 1 subtype (Fig. 2A). From donor JM, the largest number (14) of individual EBNA1-specific T cell clones were established, which were therefore chosen for further analysis. To determine the restriction elements of these clones, PBMC from the three T cell donors and from HLA-typed lab volunteers were used. The PBMC were incubated with either EBNA1 or control protein for 24 h, irradiated, washed thoroughly and then used as APC in T cell assays. In addition, antibodies directed against HLA-DR and -DP were used

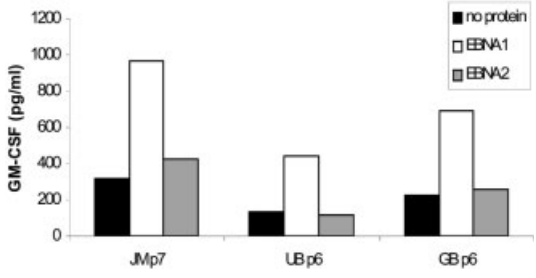

Fig. 1. Generation of EBNA1-specific T helper cell lines. Purified EBNA1 (500 ng/ml) was added to PBMC (1×10^6/ml) from three latently EBV-infected donors, which were then used to stimulate autologous $CD4^+$ T cells. The specificity of the T cell lines was assessed by measuring cytokine secretion of the T cells. After four to seven rounds of stimulation, the T cell lines showed reactivity against PBMC pulsed with EBNA1, but not with EBNA2 protein. One of three independent experiments is shown.

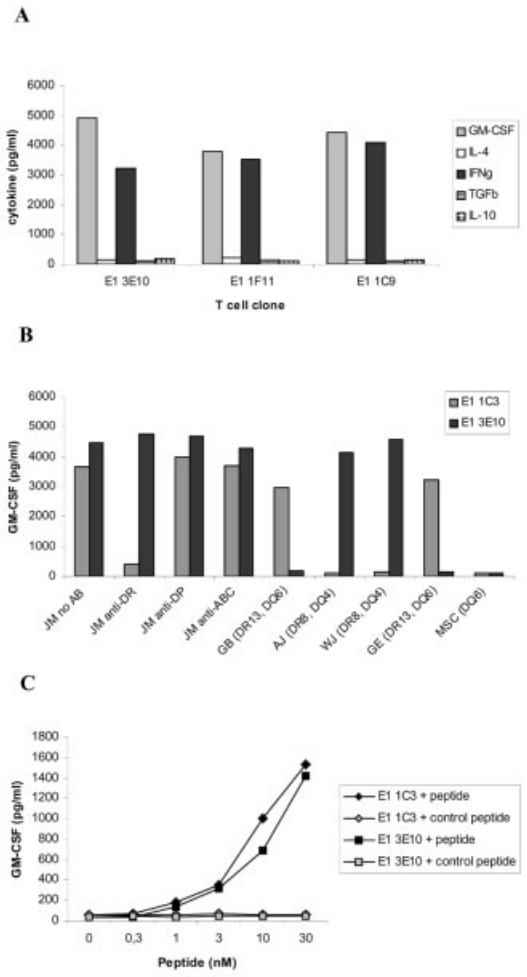

Fig. 2. Characterization of EBNA1-specific $CD4^+$ T cell clones. (A) The T helper subtype of the EBNA1-specific T cell clones was determined by assessing the pattern of cytokines secreted after co-culture with EBNA1 protein-pulsed autologous PBMC. Three representative T cell clones from the three donors are shown. (B) PBMC from various HLA-typed donors were pulsed with EBNA1 protein, then washed extensively, irradiated and used as stimulator cells for the T cell clones. The relevant HLA class II restriction elements shared by the allogeneic target cells and donor JM are given. In addition, monoclonal antibodies (50 μg/ml) directed against HLA-DR (clone L243), -DP (clone B7/21) and HLA-A, -B, -C (clone W6/32) were used to block T cell recognition. Specificity of the blocking antibodies was verified with T cell clones restricted by HLA-A2, HLA-DR1, and HLA-DP3 (data not shown). As shown for the T cell clones E1–1C3 and E1–3E10, the EBNA1-specific T cells derived from donor JM are restricted by HLA-DR13 or HLA-DQ4. (C) Peptides at various concentrations were pulsed onto autologous PBMC for 2 h at 37°C. Subsequently, unbound peptide was removed by repeated washing, and the cells were used as stimulators for the T cells. GM-CSF secretion was measured after 24 h by ELISA. One of three experiments is shown.

to determine the restriction elements (Fig. 2B). These experiments showed that nine of the fourteen T cell clones were restricted by HLA-DRB1*1301, while the remaining five clones were HLA-DQB1*0402 restricted.

In order to map the epitopes, deletion constructs of EBNA1 were expressed in *E. coli* as histidine-tagged fusion proteins and subsequently tested in T cell assays. Proteins recognized by the T cell clones were further truncated and tested likewise, until the epitopes were narrowed down to less than 20 amino acids. Ultimately, synthetic peptides spanning these regions were used to precisely map the epitopes. These experiments showed that all clones restricted by the same HLA allele recognized the same epitope. All DR13-restricted T cell clones recognized the amino acids 527–541 (AIPQCRLTPLSRLPF) in the B95.8 EBNA1 protein, while all DQ4-restricted T cells recognized the amino acids 481–495 (IAEGLRALLARSHVE). Interestingly, the latter peptide had recently been predicted to contain promiscuous binding motifs for HLA-DR [23]. To assess the sensitivity of the clones, autologous PBMC were pulsed with various concentrations of the peptides and, after unbound peptides were removed by repeated washing, probed with the T cell clones. As shown in Fig. 2C, both clones recognized their cognate peptides at concentrations as low as 1 nM, while no reactivity was observed against the control peptide.

2.3 EBNA1-specific $CD4^+$ T cell clones fail to recognize LCL and EBV-positive Burkitt's lymphoma cells

To test whether these clones are able to recognize endogenous EBNA1 synthesized within target cells, LCL were established from the donor by infection with supernatant from the marmoset B95.8 producer cell line. Despite the high avidity of the T cells for their cognate MHC-peptide complex, autologous LCL were not recognized by the T cell clones, neither were allogeneic LCL expressing the appropriate MHC class II alleles. Furthermore, the EBNA1-specific T cell clones isolated from donor UB and GB did not recognize their autologous LCL either (data not depicted). To investigate, whether this lack of recognition is specific for LCL or is a common feature of EBV-infected cells, the EBV-negative Burkitt's lymphoma cell lines BL30 (HLA-DR13), BL70 (HLA-DR13), and DG75 (HLA-DR13 and HLA-DQ4), and the respective EBV convertants BL30-B95.8, BL70-B95.8, and DG75–2089 were included in this analysis. Although all these cell lines were able to present exogenously added EBNA1, the EBV-positive convertants were not recognized by the T cells (Fig. 3). These

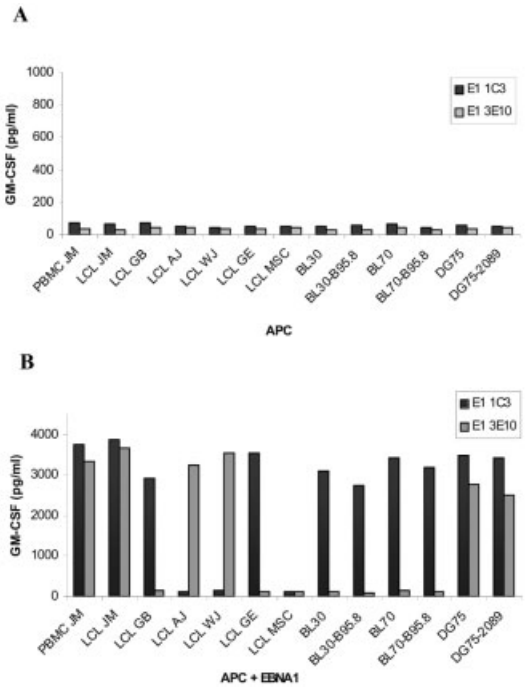

Fig. 3. EBNA1-specific CD4$^+$ T cell clones fail to recognize endogenous EBNA1. (A) T cell recognition was performed by co-culturing EBNA1-specific T cells with autologous or allogeneic target cells at a 1:1 ratio for 24 h. Target cells were either LCL established from the same HLA-typed donors as in Fig. 2B or from the EBV-negative Burkitt's lymphoma cell lines BL30 (HLA-DR13), BL70 (HLA-DR13), and DG75 (HLA-DR13, -DQ4) and the respective EBV-positive convertants BL30-B95.8, BL70-B95.8 and DG75–2089. GM-CSF secretion was determined 24 h later by ELISA. (B) T cell recognition of the same target cells as in (A), but pre-pulsed with EBNA1 protein. One of three experiments is shown.

EBNA1-specific T cell clones also failed to recognize newly EBV-infected autologous B cells and were unable to prevent the outgrowth of these EBV-immortalized B cells, or to inhibit the growth of established LCL (data not shown).

2.4 The GAr domain of EBNA1 does not block antigen presentation

The GAr domain of EBNA1 is known to inhibit proteasomal degradation of EBNA1 and thus presentation on MHC class I [8, 9]. To test whether the GAr is responsible for the lack of presentation of epitopes derived from EBNA1 on MHC class II, an EBV strain was generated expressing a mutant EBNA1 protein that lacks the GAr domain (EBNA1ΔGA). Viral stocks of this mutant EBV strain 2764 were used to infect B cells from donor JM. In parallel, B cells from the same donor were infected with the parental EBV strain 2089 [24], and several independent clones were established. The efficiency of LCL outgrowth was comparable with both viral strains, suggesting that the GAr of EBNA1 is dispensable for B cell immortalization. Identity and integrity of the EBV genomes in the LCL was verified by Southern blot analysis (data not shown), and expression of wild-type EBNA1 or EBNA1ΔGA protein in these cells was examined by Western blot (Fig. 4A). Three independent clones from both EBV strains were tested in T cell recognition assays with the two autologous CD4$^+$ T cell clones. Neither of these LCL were recognized by the T cells, demonstrating that the GAr domain does not confer this phenotype (Fig. 4B). Moreover, EBNA1 and EBNA1ΔGA proteins were recognized to the same extent when added exogenously to APC, demonstrating that the GAr domain does not interfere with antigen processing in the endosomal/lysosomal compartment (Fig. 4C). No differences were observed between LCL generated by infection with the EBV strains B95.8, 2089, or 2764 in terms of MHC class II expression and recognition of exogenous EBNA1. Furthermore, peptide titration experiments did not detect any differences between these three groups, especially in the low concentration range, indicating no detectable differences in cell surface antigen density (data not shown). To test whether recognition of a subset of LCL occurred below the detection limit of supernatant cytokine ELISA, ELISPOT assays were performed. While in T cell titration experiments using LCL JM pulsed with EBNA1 protein as stimulator cells the number of spots obtained was directly equivalent to the number of T cells probed, the same low background number of spots was obtained when using unpulsed LCL or PBMC as APC (data not shown). To exclude that LCL have inhibitory effects on the T cells generated by stimulation with protein-pulsed PBMC, proliferation of these T cell clones after stimulation with EBNA1-pulsed PBMC or LCL was compared. T cells stimulated with EBNA1-pulsed LCL proliferated even better than those grown on PBMC, excluding this possibility (data not shown).

2.5 Overexpression of EBNA1 restores T cell recognition

The lack of recognition of endogenous EBNA1 by the CD4$^+$ T cell clones could either be due to low EBNA1 protein levels or slow turnover, which both might contribute to insufficient presentation on MHC class II. Alternatively, a mechanism that actively prevents this presentation pathway and is mediated by EBNA1 domains different from the GAr could cause similar

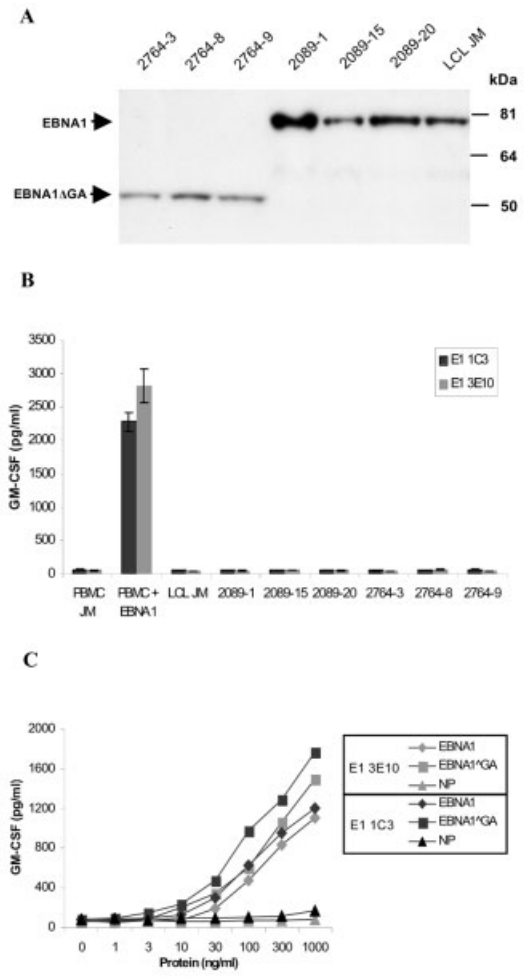

Fig. 4. The GAr domain of EBNA1 does not affect antigen presentation. (A) Western blot analysis of EBNA1 expression in LCL JM and in three independent lymphoblastoid B cell clones established after infection of B cells from donor JM with either EBV-2089 or the EBNA1ΔGA deletion mutant strain EBV-2764, followed by limiting dilution cloning. (B) T cell recognition assay of LCL expressing full-length EBNA1 (2089) or truncated EBNA1ΔGA (2764). (C) T cell recognition of exogenous EBNA1 and EBNA1ΔGA. Autologous PBMC were pulsed with purified EBNA1 or EBNA1ΔGA at various concentrations for 24 h. After extensive washing to remove residual protein, the cells were used as target cells for the EBNA1-specific T cell clones. GM-CSF secretion was measured 24 h later by ELISA. Purified influenza nuclear protein (NP) was used as control. One of four experiments is shown.

effects. To address these possibilities, the sequence encoding the first 80 amino acids of the invariant chain (Ii) was fused to the N terminus of full-length EBNA1. Upon transfection into cells, targeting motifs within the Ii sequence have been shown to direct the resulting chimeric Ii-antigen fusion protein into the endosomal/lysosomal compartment, where processing and loading of antigen-derived peptides onto MHC class II molecules takes place [25]. LCL transfected with this Ii-EBNA1 expression plasmid were recognized by the T cells, suggesting that processing and presentation of endogenous EBNA1 can occur. Constructs with or without the GAr domain performed identically, further indicating that this domain does not influence presentation on MHC class II (data not shown). LCL transiently transfected with CMV enhancer/promoter-driven EBNA1 expression constructs, however, were not recognized by the T cell clones. Because the transfection efficiency of these cells, as determined by GFP expression, is only 2–5%, the level of antigen expression may not be sufficient for T cell recognition in ELISA. ELISPOT experiments using these EBNA1-transfected LCL as APC revealed that a small percentage of the transfected cells were capable of stimulating the T cells, suggesting that endogenous presentation of EBNA1 on MHC class II may occur, provided that EBNA1 protein levels are sufficiently high (data not shown).

To increase the percentage of EBNA1-transfected cells and to quantitate the amount of endogenous EBNA1 required for T cell stimulation, LCL were transfected with a bi-cistronic vector encoding EBNA1 or EBNA1ΔGA and the extracellular and transmembrane part of the low-affinity nerve growth factor receptor (NGF-R) separated by an internal ribosomal entry site (IRES). This allows sorting of transfected cells with a monoclonal antibody directed against the extracellular domain of the NGF-R [26]. More than 90% of the MACS-sorted cells expressed NGF-R as determined by FACS analysis (data not shown). These cells were then tested in T cell recognition assays with the EBNA1-specific T cell clones, and the EBNA1 protein level in these cells was determined in parallel by Western blot analysis. By transfecting LCL expressing wild-type EBNA1 with an EBNA1ΔGA expression construct and vice versa, the amount of EBNA1 protein derived from the expression construct could be directly correlated with endogenous EBNA1. Endogenous EBNA1 is known to be expressed in a very narrow range at 25,000–44,000 molecules per cell in LCL [27]. These experiments showed that NGF-R-sorted cells expressed at least 60-fold more plasmid-encoded than endogenous EBNA1 protein (Fig. 5A, B). Transfected and NGF-R-sorted cells stimulated cytokine secretion by the T cell clones (Fig. 5C), but the cytokine levels were much lower in comparison with peptide-loaded LCL (Fig. 4C). Because only a fraction of these cells stimulated the T cell clones in ELISPOT experiments (data not shown), the level of endogenous EBNA1 protein required for efficient presentation on MHC class II is likely to be much higher.

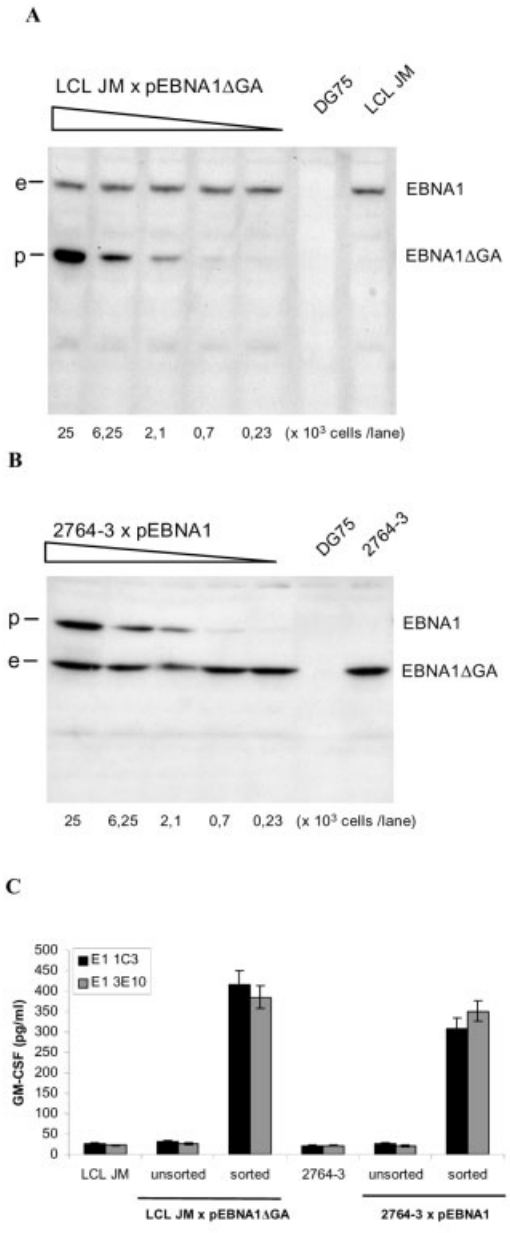

Fig. 5. Correlation of endogenous EBNA1 protein level and T cell recognition. LCL JM expressing wild-type EBNA1 (A) and LCL 2764–3 expressing GAr-deleted EBNA1 (B) were transfected with pEBNA1ΔGA-IRES-NGF-R (pEBNA1ΔGA) and pEBNA1-IRES-NGF-R (pEBNA1), respectively. After 48 h, the transfected cells were MACS-purified with an antibody directed against NGF-R and analyzed for EBNA1 expression by Western blot immunodetection. To correlate the amount of endogenous (e) versus plasmid-derived (p) EBNA1 protein, serial dilutions of transfected cells were prepared and directly compared to the amount of EBNA1 expressed in the parental LCL JM and 2764–3. As an internal standard, 5×10^5 cells of LCL JM expressing wild-type EBNA1 (A) or 5×10^5 cells of LCL 2764–3 expressing GAr-deleted EBNA1 (B) were lysed together with the given numbers of transiently transfected and sorted LCL as indicated. Lysate from 5×10^5 EBV-negative DG75 and from untransfected LCL JM and 2764–3 cells were included as control. In parallel, T cell recognition of transfected cells, before and after MACS-sorting, was assessed by ELISA (B). One of five experiments is shown.

2.6 Polyclonal EBV-specific T cell lines do not contain EBNA1-specific CD4$^+$ T cells

EBV-specific T cell lines generated by repeated *in vitro* stimulation with autologous LCL have been successfully used to treat post-transplant lymphoproliferative disease (PTLD), but the targets of the EBV-specific T helper cells present in these oligoclonal T cell populations have not been defined. Since EBNA1-specific T cells are unable to recognize EBNA1-expressing target cells directly, such EBV-specific T cell lines should be devoid of EBNA1-specific T helper cells. To test this hypothesis, EBV-specific T cell lines were generated from donor JM by repeated stimulation with autologous LCL, and the number of T cells specific for the EBNA1 peptides within these lines was assessed by ELISPOT. CD4$^+$ PBMC and CD4$^+$ T cell lines stimulated four, seven, or ten times with autologous LCL completely lacked T cells specific for the EBNA1 epitopes (Fig. 6). These results show that EBNA1-specific CD4$^+$ T cells are incapable of directly recognizing EBNA1-positive target cells.

3 Discussion

The successful treatment of immunoblastic lymphoma in bone marrow and solid organ transplant recipients by the infusion of EBV-specific T cell lines has spurred efforts to target EBV-encoded antigens in other virus-associated malignancies [28]. However, the array of EBV antigens expressed in these tumors is confined to a small number of proteins. EBNA1 is the only viral protein expressed in all virus-associated malignancies. T helper responses against EBNA1 have been described, and independent studies indicated that EBNA1-specific T helper responses may even prevail over those against other latent antigens [19, 20]. Whether these EBNA1-specific T helper cells recognize endogenous EBNA1 presented on EBV-infected cells and whether this recognition elicits antiviral effector functions has remained a matter of debate. In two independent studies, EBNA1-specific CD4$^+$ T cells have been shown to recognize endogenous EBNA1 in LCL, Burkitt's and Hodgkin's lymphoma cells [19, 22], while in two other studies, EBNA1-specific T cells failed to do so [18, 20]. Because latent infection of cells by EBV is dependent on the expression of EBNA1,

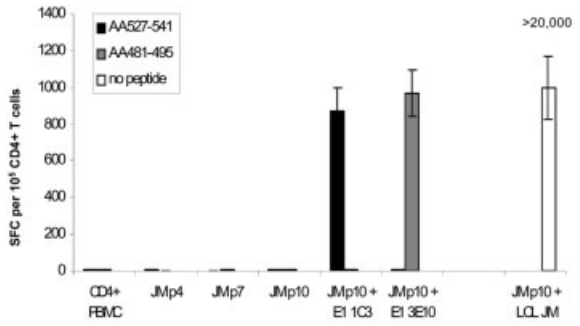

Fig. 6. LCL-stimulated EBV-specific T cell lines lack EBNA1 reactivity. From donor JM, freshly isolated CD8-depleted PBMC and $CD4^+$ T cell lines stimulated four, seven, or ten times with autologous LCL were tested for reactivity against the EBNA1 peptides in IFN-γ ELISPOT assays. Known numbers of responder T cells were co-cultured with target cells, consisting of EBNA1 peptide-pulsed or unpulsed, irradiated, autologous PBMC. Results are expressed as spot-forming cells (SFC) per 10^5 $CD4^+$ responder T cells. The number of SFC was similar when using peptide-pulsed or unpulsed target cells and was always <10. As control, the T cell lines were supplemented with 10^3 cells of the peptide-reactive T cell clones E1–1C3 or E1–3E10, which resulted in an almost equivalent number of spots. EBV reactivity of the $CD4^+$ T cell line stimulated ten times with autologous LCL (JM p10) was verified by using LCL JM as target cells. One of five experiments is shown.

this question has important implications for understanding and enhancing EBV immunity. In all cases, EBNA1-specific T cells were generated *in vitro* by stimulating $CD4^+$ T cells from latently EBV-infected individuals with APC pulsed with exogenous EBNA1 protein or peptide or transduced with a viral vector encoding EBNA1. Furthermore, the same types of APC were used in these studies, including dendritic cells [20, 21]. Thus, methodological differences in generating the T cells are unlikely to explain these discrepant results. Moreover, according to the ProPred HLA-DR binding site prediction program (http://www.imtech.res.in/raghava/propred), the various HLA-DR-associated EBNA1 epitopes are predicted to bind to their respective HLA-DR molecules equally well. T cells specific for these EBNA1 epitopes recognized [21] or failed to recognize ([18, 20] and this study) endogenous antigen, suggesting that differences in MHC class II binding do not account for these discrepancies.

Our results also show that differences in the avidity of the various T cells for their cognate MHC/antigen complexes are unlikely to reconcile these discrepant results. Although the EBNA1-specific T cell clones recognized PBMC pulsed with as little as 1 nM of their cognate peptides, these T cells still required more than 60-fold overexpression of endogenous EBNA1 protein for recognition. Why such high levels of endogenous EBNA1 are required for presentation on MHC class II is not clear. In a recent study, EBNA1-specific $CD4^+$ T cells have been shown to recognize LCL and HEK293 cells transiently transfected with an Ii-EBNA1 expression plasmid [22]. The same T cells failed to recognize HEK293 cells transiently transfected with an EBNA1 expression plasmid, implying that processing and presentation of EBNA1 may be cell type dependent. Although such cell type-specific differences have not been observed in our experiments with the panel of different LCL and Burkitt's lymphoma cell lines tested, this possibility warrants further investigation. We have shown previously that endogenous presentation of a cytosolic/nuclear antigen on MHC class II is mediated by autophagy and that this pathway is active in LCL [29]. Whether cell line-dependent differences in the activity of this pathway exist, or whether EBNA1 evades cytoplasmic autophagocytosis and hence antigen presentation by its exclusive nuclear localization, is currently under investigation.

Our results demonstrate that EBNA1-specific T helper cells are unable to recognize EBV-infected target cells directly, because much higher levels of EBNA1 protein are required for efficient detection of EBNA1 epitopes presented on MHC class II. By using a genetic approach we showed that the GAr domain in EBNA1, which prevents presentation on MHC class I, does not mediate this effect. Nevertheless, T helper responses against EBNA1 have been detected in the peripheral blood in almost all latently infected healthy individuals. A similar situation has been observed for the CTL response to EBNA1 [11]. $CD8^+$ T cells directed against EBNA1 have been described, but these T cells fail to recognize endogenous EBNA1 and are therefore considered biologically ineffective. Whether EBNA1-specific $CD4^+$ T cells share the same fate, or whether these T cells play an important indirect role in the immune response against EBV when cross-presented on MHC class II by professional APC, remains to be determined. The absence of $CD4^+$ T cells specific for EBNA1 in polyclonal LCL-stimulated T cell populations as used for the treatment of immunoblastic lymphoma in transplantation patients suggests that such T cells are not critical for immunotherapy of these tumors. Thus, the role of EBNA1-specific $CD4^+$ T cells in the control of EBV infection and virus-positive tumors *in vivo* still needs to be elucidated.

4 Materials and methods

4.1 Cell culture

The Burkitt's lymphoma cell lines and the various LCL were grown as suspension cultures in B cell medium consisting of

RPMI 1640, 10% FCS, 1% nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 μg/ml gentamicin. LCL were generated by culturing PBMC from latently EBV-infected donors with supernatant of the marmoset cell line B95–8 in B cell medium supplemented with 1 μg/ml cyclosporine A.

4.2 Recombinant EBV plasmids

To generate the GAr deletion in the open reading frame of EBNA1 in the context of the complete genome of the B95.8 strain of EBV, a conventional shuttle plasmid was constructed to modify the maxi-EBV p2089 by the chromosomal building technique [30, 31]. An Afl II/Xba I fragment treated with Klenow enzyme, spanning from nucleotide coordinate 106,717 to 110,764 of the B95.8 strain of EBV with the internal GAr deletion in EBNA1 derived from p2761, was cloned into the single Sma I site in p2423 to yield the final shuttle plasmid p2728.4. This shuttle plasmid was used for recombination with the maxi-EBV p2089 plasmid in the *E. coli* DH10B strain to yield the modified maxi-EBV plasmid p2764.1. DNA from several colonies were carefully analyzed with restriction enzymes and the genetic composition of one clone was confirmed by Southern blot hybridization and partial DNA sequencing.

4.3 Maxi-EBV-converted DG75 cells and growth-transformed LCL

For the generation of the 2764 maxi-EBV virus stock, large-scale plasmid DNA of the clone p2764.1 was prepared and transiently transfected into the EBV packaging cell line TR2$^-$ [32] together with the expression plasmids encoding BZLF1 and BALF4 [7, 33]. Similarly, the wild-type 2089 virus stock was prepared from a stable cell line as described [24]. The virus stocks were used to infect primary B cells derived from Ficoll gradient-purified PBMC. Infected B cells (1×10^4–1×10^5) were initially plated on Wi38 fibroblast feeder layers in a volume of 100 μl/well in 96-well cluster plates in the presence of 1 μg/ml cyclosporine A. Single-cell clones were expanded in suspension 4–6 weeks after infection. Since the maxi-EBV carry the hygromycin phosphotransferase gene as described [24], DG75 cells could be infected with 2089 virus stock and selected in 96-well cluster plates in B cell medium in the presence of 200 μg/ml hygromycin.

4.4 Generation of CD4$^+$ T cell lines and clones

Whole blood was obtained from healthy laboratory members, and HLA was typed using PCR-based DNA typing. The HLA class II type of donor JM is: DRB1*0801, DRB1*1301, DRB3*0101, DQB1*0402, DQB1*0603, DPB1*0401, DPB1*1301. EBV status was determined by serological staining for IgG antibodies for viral capsid antigen. PBMC were isolated from heparinized venous blood by density gradient centrifugation on Ficoll-Paque (Amersham Biosciences, Freiburg, Germany). Positive selection of CD4$^+$ PBMC was performed using α-CD4$^+$ microbeads, LS columns, and a MidiMACS separator (Miltenyi Biotec, Bergisch-Gladbach, Germany). To generate CD4$^+$ EBNA1-specific T cell lines, 1×10^6/ml PBMC were pulsed with EBNA1 protein (500 ng/ml) in AIM-V serum-free medium (Invitrogen, Karlsruhe, Germany) for 24 h, washed, irradiated (40 Gy) and used as stimulators for autologous CD4$^+$ PBMC (1×10^6/ml) at a 1:1 ratio in T cell medium (RPMI 1640 supplemented with 10% heat-inactivated human serum, 2 mM L-glutamine, 10 mM HEPES, and 50 μg/ml gentamicin). After 48 h, IL-2 (10 U/ml) was added to the cultures. The T cell lines were restimulated every 10 days in the same fashion and split when needed. T cell clones were generated by limiting-dilution cloning in 96-well round-bottom plates (Greiner Bio-One, Solingen-Wald, Germany) by seeding 3, 1, and 0.3 T cells/well in T cell medium supplemented with 10 U/ml IL-2, 250 ng/ml PHA, 1×10^4 irradiated EBNA1-pulsed autologous PBMC, and 5×10^4 irradiated (40 Gy) feeder cells consisting of a mix of allogeneic PBMC from three donors. After 3 days, most of the medium was removed and replenished with T cell medium supplemented with 10 U/ml IL-2. Restimulations were performed every 12 days, and outgrowing clones were transferred and expanded in 24-well plates under the same conditions.

4.5 ELISPOT assay and cytokine ELISA

ELISPOT assays for the detection of IFN-γ release were performed as described [34]. Cytokine ELISA were performed essentially as described [29]. Briefly, 1×10^5 target cells and 1×10^5 T cells were co-cultured in 96-well flat-bottom plates in 200 μl T cell medium for 24 h. Subsequently, 100 μl supernatant was collected and the amount of cytokine determined by ELISA (R&D Systems, Wiesbaden, Germany). Values shown are the means of duplicate determinations. In those cases where the values varied by >5%, the standard deviation is indicated with bars.

4.6 Construction of plasmids

For generating EBNA1-recombinant baculovirus, the coding sequence of EBNA1 was cloned into the pTrcHisB plasmid (Invitrogen), fusing the Anti-Xpress antibody epitope and a histidine-tag to the N terminus of EBNA1. This chimeric open reading frame was transferred into the baculovirus shuttle vector pVL1393 and inserted into baculovirus DNA by homologous recombination, according to the guidelines of the manufacturer (BaculoGold; BD PharMingen, Heidelberg, Germany). Baculovirus recombinant for EBNA2 was generated in the same way. After infection (4 days) of Sf9 cells with recombinant baculovirus, the cells were lysed, and recombinant proteins were purified over Nickel-NTA columns (Qiagen, Hilden, Germany).

To delete the GAr domain in EBNA1, the regions encoding amino acids 1–89 and 327–641 were amplified by PCR and joined via a Kpn I site that had been introduced without changing the amino acid sequence (plasmid p2761). For construction of the pEBNA1-IRES-NGF-R and pEBNA1ΔGA-IRES-NGF-R plasmids, the respective open reading frames were first inserted into the pIRES plasmid (BD Clontech, Heidelberg, Germany), then the whole expression cassettes were transferred into the pINCO plasmid [29]. Transfection of cells was performed by electroporation as described [29], and protein expression allowed for 48 h. Subsequently, live cells were incubated with the α-NGF-R mouse monoclonal antibody HB8737 (ATCC, Manassas, VA) for 15 min on ice. After removal of unbound antibody by washing, cells were incubated for 10 min with microbead-coupled goat anti-mouse antibody (Miltenyi Biotec) and MACS-purified.

4.7 Western blot analysis

Proteins were separated on 10% SDS-PAGE gels and, after blotting, stained with the EBNA1-specific rat monoclonal antibody 1H4. Horse radish peroxidase-coupled anti-rat IgG (Dianova, Hamburg, Germany) was used as secondary antibody. Western blots were developed using the ECLplus kit (Amersham Biosciences).

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