Published in final edited form as:

Cytotherapy. 2019 February ; 21(2): 212–223. doi:10.1016/j.jcyt.2018.08.001.

Epstein-Barr Virus (EBV)-derived BART1 encodes CD4- and CD8-restricted epitopes as targets for T-cell Immunotherapy

Mamta Kalra^{1,2,3}, Ulrike Gerdemann^{1,2,3}, Jessica D Luu^{1,2,3}, Minthran C Ngo^{1,2,3}, Ann M Leen^{1,2,3,4}, Chrystal U Louis^{1,2,3}, Cliona M Rooney^{1,2,3,4,5}, and Stephen Gottschalk^{1,2,3,4}

¹Center for Cell and Gene Therapy, Texas Children's Hospital, Houston Methodist, Baylor College of Medicine, Houston, Texas 77030

²Texas Children's Cancer Center, Texas Children's Hospital, Baylor College of Medicine, Houston, Texas 77030

³Department of Pediatrics, Baylor College of Medicine, Houston, Texas 77030

⁴Department of Pathology and Immunology, Baylor College of Medicine, Houston, Texas 77030

⁵Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, Texas 77030

Abstract

Background: EBV type II latency tumors such as Hodgkin lymphoma (HL), Non-Hodgkin lymphoma (NHL) and nasopharyngeal carcinoma (NPC) express a limited array of EBV antigens including Epstein-Barr nuclear antigen (EBNA)1, latent membrane protein (LMP)1, LMP2, and BamH1-A right frame 1 (BART1). Adoptive immunotherapy for these malignancies have focused on EBNA1, LMP1, and LMP2 since little is known about the cellular immune response to BART1.

Methods: To investigate if BART1 is a potential T-cell immunotherapy target, we determined the frequency of BART1-specific T-cell responses in the peripheral blood of EBV-seropositive healthy donor and patients with EBV-positive malignancies, mapped epitopes, and evaluated the effector function of *ex vivo* generated BART1-specific T-cell lines.

Results: BART1-specific T cells were present in the peripheral blood of 12/16 (75%) EBV-positive healthy donors and 13/20 (65%) patients with EBV-positive malignancies. *Ex vivo* expanded BART1-specific T-cell lines contained CD4- and CD8-positive T-cell subpopulations, and we identified 23 BART1 peptides, which encoded MHC class I- and/or II-restricted epitopes. Epitope mapping identified one HLA-A*02-restricted epitope that was recognized by 50% of HLA-A*02, EBV-seropositive donors, and one HLA-B*15(62)-restricted epitope. *Ex vivo*

Corresponding author: Stephen Gottschalk, Department of Bone Marrow Transplant and Cellular Therapy, St Jude Children's Research Hospital, 262 Danny Thomas Place, MS321, Memphis, TN 38105, Phone: 901-595-2166, stephen.gottschalk@stjude.org.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

DISCLOSURE OF INTEREST

AML and CMR are cofounders of ViraCyt. UG, MCN, AML, CUL, CMR, SG have patent applications in the field of T-cell and gene-modified T-cell therapy for cancer.

expanded BART1-specific T cells recognized and killed autologous, EBV-transformed lymphoblastoid cell lines and partially HLA-matched EBV-positive lymphoma cell lines.

Discussion: BART1 should be considered as an immunotherapy target for EBV type II (and III) latency. Targeting BART1, in addition to EBNA1, LMP1, and LMP2, has the potential to improve the efficacy of current T-cell immunotherapy approaches for these malignancies.

Keywords

EBV; BART1; Immunotherapy; EBV-positive malignancies

INTRODUCTION

Immunotherapy with Epstein-Barr virus (EBV)-specific T cells is actively being pursued for a broad range of EBV-associated malignancies outside of the transplant setting including Hodgkin lymphoma (HL), Non-Hodgkin lymphoma (NHL), and nasopharyngeal carcinoma (NPC).[1–6] These malignancies express a limited array of EBV type II latency proteins that include Epstein-Barr nuclear antigen (EBNA)1, latent membrane protein (LMP)1, LMP2, and BamH1-A right frame 1 (BART1).[7–11] Several groups have shown that the adoptive transfer of EBV-specific T cells that recognize EBNA1-, LMP1-, and/or LMP2 have antitumor activity in early phase clinical studies for NL, NHL, and NPC,[1–6, 12] and later phase clinical studies are in progress. However, there is a continued need to explore the potential immunotherapeutic benefit of extending the spectrum of targeted EBV antigens, both to enhance antitumor activity and prevent immune escape.

While the cellular immune response to EBNA1, LMP1, and LMP2 is well characterized, BART1-specific T-cell responses have been largely ignored.[13–16] BART1, a secretory protein, is a homolog to the human proto-oncogene *c-fms*, and plays a role in the malignant transformation of EBV-positive cells.[17, 18] In addition, it is part of EBV's immune evasion strategies since it binds to human macrophage colony stimulating factor (M-CSF). [19, 20] Here, to investigate the immunogenicity of BART1, we used an unbiased, peptide library approach to assess the frequency of BART1-specific T cells in EBV-seropositive healthy donors and patients with EBV-associated malignancies. We demonstrate that BART1-specific T cells are present in ~70% of donors and patients, and recognize MHC class I as well as class II-restricted epitopes. We performed detailed epitope mapping for two MHC class I restricted epitopes. One of these was HLA-A*02-restricted and T-cell responses against this epitope could be detected in ~50% of HLA-A*02, EBV-seropositive donors. Moreover, *ex vivo* expanded BART1-specific T cells were able to kill autologous EBV-transformed lymphoblastoid cell lines (LCLs) and/or partially matched EBV-positive lymphoma cell lines.

MATERIALS AND METHODS

Cell lines and primary cells

Raji (Burkitt lymphoma) and 293T (human embryonic kidney) cell lines were purchased from American Type Culture Collection (ATCC; CCL-86, CRL-3216, respectively) and were maintained in RPMI (Thermo Scientific HyClone, Waltham, MA; Raji) and DMEM

(Thermo Scientific HyClone, Waltham, MA; 293T) media supplemented with 10% fetal bovine serum (FBS) (Thermo Scientific HyClone, Waltham, MA) and 2 mmol/l GlutaMAX-I (Invitrogen, Carlsbad, CA). The SNK6 (NK/T-cell lymphoma) cell line and SNT16 cell line (clonal T-cell line, which is used as model for EBV-positive T-cell lymphoma, from patients with chronic active EBV infection (CAEBV)) were kindly provided Dr. Norio Shimizu (Tokyo Medical and Dental University, Japan), [21, 22] and maintained in complete T-cell medium (TCM; 50% RPMI plus 50% Click's (EHAA) medium supplemented with 5% Human AB serum (Valley Biomedical, Winchester, VA), 2 mmol/l GlutaMAX-I containing 700 IU/ml of IL2 (Biological Resources Branch, National Cancer Institute, Frederick, MD). LCLs overexpressing BARTF1 were generated by transducing LCLs with the lentiviral vector pCDH.CMV.BARTF1.EF1.GFP/puro. This vector was generated by cloning the PCR amplified BARTF1 gene of EBV B95-8 into pCDH.CMV.EF1.GFP/puro (Systems Biosciences, Mountain View, CA).

Blood was obtained from EBV-seropositive healthy volunteers or patients on Baylor College of Medicine Institutional Review Board approved protocols, after informed consent was obtained in accordance to the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Lymphoprep (Axis Shield, Oslo, Norway) and cryopreserved. PBMCs were used to generate LCLs, activated T cells (ATCs), dendritic cells (DCs), and effector T-cell lines. The HLA-type of the healthy donors and SNK6 and SNT16 is listed in Supplementary Table 1.

LCL generation: PBMCs were infected with the B95-8 strain of EBV in the presence of cyclosporin A (Novartis, Morris Plains, NJ) as described previously.[23] *ATC generation:* T cells were stimulated by culturing PBMCs (2×10^6 /ml) on plates coated with human CD3- (CRL-8001, ATCC) and CD28- (Becton Dickinson, Mountain View, CA) specific antibodies. Cells were maintained in RPMI supplemented with 2 mmol/l GlutaMAX-I, and 10% FBS. IL2 (100 U/ml) was added 24 hours after initial activation and every 3 days thereafter. *Dendritic cell (DC) generation:* Monocytes were isolated from PBMCs by CD14 selection using MACS Beads (Miltenyi, Bergisch Gladbach, Germany), and cultured in DC media (CellGenix USA, Antioch, IL) supplemented with 2 mmol/l GlutaMAX-I, 800 U/ml GM-CSF (Sargramostim Leukine; Immunex, Seattle, WA) and 1,000U/ml IL4 (R&D Systems, Minneapolis, MN) for 5 days. IL4 and GM-CSF were replenished on day 3. On day 5, immature DCs were matured using a cytokine cocktail containing 10 ng/ml IL6, 10 ng/ml IL1 β , 10 ng/ml TNF- α (R&D Systems, Minneapolis, MN), 1 μ g/ml PGE2 (Sigma, St Louis, MO), 800 U/ml GM-CSF and 1,000 U/ml IL4 for 48 hours. Pelleted, mature DCs were pulsed with peptide libraries for 30–60 minutes and resuspended in TCM prior to use, as previously described.[24]

Peptides and peptide libraries

Peptide libraries (pepmixes) consisting of 15 amino acid long peptides with an 11 amino acid overlap that covered the entire coding region of BZLF1 or the N-terminal portion of EBNA1 were purchased from JPT (Berlin, Germany). Individual 15 amino acid peptides with an 11 amino acid overlap that covered the entire coding region of BARTF1 were synthesized by GeneMed (San Antonio, TX). The purity of the used pepmixes was >70%.

From these, a peptide library consisting of all peptides (pepmix) or selected peptide pools were prepared. For epitope mapping, additional BARF1 peptides were synthesized by GeneMed. Pepmixes and individual peptides were dissolved in tissue culture grade dimethyl sulfoxide (DMSO; Sigma). Stock concentrations of pepmixes and individual peptides were 400 to 1,000-fold higher than in the performed experiments, leading to a final DMSO concentration of 0.25%.

Antigen-specific T-cell line generation

PBMC method: PBMC ($2-4 \times 10^6$) were pulsed with EBNA1 and BZLF1 or BARF1 pepmixes (0.5–2 $\mu\text{g/ml}$), and cultured in TCM supplemented with IL4 (1,000 U/ml) and IL7 (10 ng/ml) for 8 to 10 days before analysis. During this culture period, T cells expanded 2- to 3-fold. **DC method:** CD14-negative PBMCs ($10-20 \times 10^6$) were used as responder cells and stimulated with pepmix- or individual pulsed (25 $\mu\text{g/ml}$) DCs at a stimulator:responder (S:R) ratio of 1:10. Cells were cultured in TCM supplemented with IL7 (10 ng/ml), IL12 (10 ng/ml), IL15 (5 ng/ml) and IL6 (100 ng/ml) (R&D Systems).[25] On day 10, T cells were harvested and restimulated with pepmix-pulsed DCs at a S:R ratio of 1:10 in the presence of IL7 (10 ng/ml). Cells were cultured in TCM for another 7 days fed with IL2 (50 U/ml, Biological Resources Branch, National Cancer Institute, Frederick, MD) or IL15 (5 ng/ml) twice weekly from day 14. During this culture period, cells expanded 5- to 10-fold. On day 17 after initial stimulation, cells were harvested, counted and analyzed for their specificity and functional capacity. To further expand the cells, T cells were restimulated weekly at a S:R ratio of 1:10 with pepmix-pulsed DCs in TCM, supplemented with IL2 (50 U/ml) or IL15 (5 ng/ml) twice weekly.

Real-time RT PCR assay

RNA was isolated from cell lines using RNeasy columns (Qiagen, Hilden, Germany) following manufacturer's protocol. The purity and yield of RNA was determined by Nanodrop (Nanodrop products, Wilmington, DE). One hundred nanograms of RNA from each sample was reverse transcribed using a high capacity cDNA synthesis kit (Applied Biosystems Inc., Waltham, MA). BARF1 expression was determined using a two-step SYBR green kit (Qiagen, Germany) with BARF1-specific primers (5'-primer: TGTCATCTTTATAGAGTGGCCTTTCA; 3'-primer: TTGCCGTCATGGGAGATGTT).[26] GAPDH gene expression was used as an endogenous control. Amplification was carried out for 40 cycles on iQ5 iCycler (Biorad Laboratories, Hercules, CA) followed by melt curve analysis to ensure the specificity of the product.

Elispot assay

Enzyme-linked Immunospot (Elispot) assays were performed as previously described.[27] Briefly, cells were plated in triplicates at a seeding density of 1×10^5 - 2×10^5 cells/well, and antigen-specific T-cell reactivity was measured after direct stimulation with pepmixes (0.5–2 $\mu\text{g/ml}$), peptide pools (0.5–2 $\mu\text{g/ml}$) or individual peptides (2 $\mu\text{g/ml}$). After overnight incubation, plates were developed, dried at room temperature and sent to Zellnet Consulting (New York, NY) for quantification. The frequency of T cells specific to each antigen was expressed as specific spot forming cells (SFC) per input cell numbers. SFC 2-fold above background were considered positive.

Cytotoxicity assay

Cytotoxicity assays were performed as previously described.[28] Briefly, target cells were labeled with 0.05 mCi ^{51}Cr (Cr), and incubated with T cells at various effector to target (E:T) ratios for 4 hours. In assay where ATCs served as targets, ATCs were pulsed with peptides during ^{51}Cr -labeling. Targets in media alone or 1% Triton X-100 were used for spontaneous and maximum ^{51}Cr release, respectively. Supernatants were collected and radioactivity measured on a gamma counter. Mean percentage of specific lysis of triplicate samples was calculated as $[(\text{experimental release} - \text{spontaneous release})/(\text{maximum release} - \text{spontaneous release})] \times 100$.

Statistical analysis

GraphPad Prism 5 software (GraphPad Software, Inc. La Jolla, CA) was used for parametric and non-parametric analyses as appropriate. A p value of 0.05 was considered significant.

RESULTS

BARF1-specific T cells are present in the peripheral blood of EBV-seropositive healthy donors and patients with EBV-positive malignancies

To determine the circulating frequency of BARF1-specific T cells, PBMCs from EBV-seropositive healthy donors (n=16), or patients with EBV-positive NPC (n=15) or NK/T-cell lymphoma (n=5) were stimulated with BARF1 pepmix. After 8–10 days of culture IFN- γ Elispot assays were performed. PBMCs were stimulated in parallel with pepmixes encoding the immunodominant EBV antigens EBNA1 and BZLF1 (E/B). After stimulation with BARF1 pepmix, BARF1-specific T-cell responses were detected in 12/16 screened healthy donors with a median SFC for all donors of 64 per 2×10^5 cells in comparison to a median SFC of 15 per 2×10^5 cells for controls ($p < 0.001$; Fig. 1A). BARF1-specific T cells were detected at a similar frequency in T-cell cultures from NPC (10/15) and NK/T-cell lymphoma (3/5) patients (median SFC for BARF1: 65; median SFC for controls: 19; $p < 0.0001$; Fig. 1A). In contrast, no significant BARF1-specific T-cell responses ($p > 0.05$) were detected in T-cell cultures stimulated with E/B pepmixes (Fig. 1B) confirming specificity. Overall, the median frequency of BARF1-specific T cells in T-cell cultures was 5 to 8-fold lower than the frequency of EBNA1/BZLF1-specific T cells (Fig. 1C).

MHC class I- and II-restricted T-cell epitopes are clustered in the N-terminal region of BARF1

To map immunogenic T-cell epitopes, we used 5 pools of BARF1 peptides each containing 10 (Pool (P)1 – 4) or 13 peptides (P5). BARF1-specific T cells were reactivated from the 12 healthy donors who had BARF1-specific T-cell responses, and IFN- γ Elispot assays were performed using individual pools. BARF1-specific T-cells were detected in 9/12 donors (Fig. 2A,B), and while all pools elicited IFN- γ responses, the majority (55%) of strong responses (defined as $10 \times$ SFC over background) were located in the N-terminal pools (P1 and P2).

To identify MHC class I- and II-restricted T-cell epitopes, BARF1-specific T cells were expanded from donors D3, D4, D5, D10 and D16 using repeated stimulations with pepmix-loaded autologous DCs. CD4- and CD8-positive T cells were separated by magnetic beads

prior to performing IFN- γ Elispot assays with BARF1 peptide pools as stimulators. In general, MHC class II-restricted responses dominated, however MHC class I-restricted responses were also detected in all five pools tested (Fig. 2C). Individual, immunogenic peptides in each pool were identified by performing IFN- γ Elispot assays (except for P2 for D16 and P5 for D3 and D16). Twenty three peptides were identified that either induced IFN- γ secretion of CD4- and/or CD8-positive T cells, indicating that BARF1 elicits both helper (CD4-positive) and cytotoxic (CD8-positive) T cells (Fig. 3; Supplementary Table 2).

Identification of two MHC-class I restricted BARF1-specific T-cell epitopes

Having demonstrated that MHC class I- and II-restricted epitopes are present in BARF1 using peptides that encode 15 amino acids, we next characterized two MHC class I-restricted epitopes. We focused on D3, whose CD8-positive T cells selectively recognized peptides 33 and 34, and D16, whose CD8-positive T cells recognized peptides 7 and 8. BARF1-specific T-cell lines generated with peptides 33/34 or 7/8 induced antigen specific IFN- γ responses (Supplementary Fig. 1A,B) and demonstrated cytotoxicity against peptide-loaded autologous ATCs in an antigen dependent manner (Fig. 4A,B).

To determine the minimal peptide binding sequence of these epitopes, we generated a panel of peptides that consisted of 8 to 11 amino acids corresponding to the overlapping region of peptides 33/34 or 7/8. Peptide-specific T-cell lines were then screened using IFN- γ Elispot assay (Fig. 4C,D). For D3, the minimal peptide that induced IFN- γ secretion consisted of 9 amino acids (SQFPDFSVL; SQF), and for D16 consisted of 8 amino acids (TLTSYWRR; TLT).

In order to determine the HLA-restriction of the identified epitopes, we performed standard cytotoxicity assays with peptide-specific T cells as effectors and autologous or partially HLA-matched, peptide-loaded ATCs as targets. For D3 (A*01,03; B*15(62),57), we observed significant killing of autologous peptide-loaded and of HLA-B*15(62)-matched ATCs (Fig. 5A,B), indicating that SQF is a HLA-B*15(62)-restricted epitope. For D16 (A*02,32; B*35,40) killing was restricted to HLA-A*02 (Fig. 5C), identifying TLT as a HLA-A*02-restricted epitope. To evaluate the frequency of TLT-specific T-cell responses in HLA-A*02-positive individuals, we screened 13 HLA-A*02-positive, EBV-seropositive healthy donors and found TLT-specific T-cell activity in 7 (55%; Fig. 5D). In contrast only 3 and 4 donors, respectively, responded to two HLA-A*02-restricted BARF1 epitopes (KLGPGEQV (KLG); RFIAQLLL (RFI)), that had been identified by others using SYPEITHI and BIMAS prediction software programs.[14] These results identify TLT as an immunodominant, HLA A*02-restricted BARF1 epitope.

SQF- and TLT-specific T cells recognize target cells that endogenously express BARF1

Finally, to assess the biological relevance of BARF1-specific T cells, we performed cytotoxicity assays to measure the cytolytic activity of SQF- and TLT-specific T cells against BARF1-positive target cells. For TLT-specific T cells we used EBV- and BARF1-positive targets autologous (Auto)-LCL, mismatched (MM)-LCL, HLA-A*02-matched SNK6 (NK/T-cell lymphoma) and SNT16 (CAEBV) cells (Supplemental Fig. 2), while HLA-negative K562 cells served as controls. TLT-specific T cells significantly killed Auto-LCL as

well as HLA-A*02-matched SNK6 and SNT16 cell lines, with no recognition of K562 or MM-LCLs (Fig. 6A). Auto-LCL were also readily killed by SQF-specific T cells in contrast to MM-LCL (Fig. 6B). In addition, we observed a further increase in killing of Auto-LCLs that were genetically modified to express BARF1 and GFP/puro (Auto-LCL-BARF1) in contrast to Auto-LCLs modified with a control vector only encoding GFP/puro (Auto-LCL-GFP). Together, these results demonstrate that BARF1-specific T cells kill target cells that endogenously express BARF1.

DISCUSSION

In this study we characterized T-cell responses against BARF1, an EBV type II and III latency-associated antigen, which is expressed in a broad range of EBV-associated malignancies. We show that BARF1 contains MHC class I- and II-epitopes, and that T cells specific for these epitopes can be expanded *ex vivo* and kill HLA-matched EBV-positive lymphoma cells.

EBV type II latency tumors express four EBV proteins, EBNA1, LMP1, LMP2, and BARF1. To date only EBNA1, LMP1, and LMP2 have been interrogated in significant depth with respect to their ability to induce T-cell responses. All three have been found to induce subdominant CD8-positive T-cell responses when compared with lytic (BZLF1, BRLF1) or immunogenic EBV type III latency proteins (EBNA 3A, 3B, 3C). EBNA1 has also been found to induce strong CD4-positive T-cell responses, whereas only few MHC class II-restricted epitopes have been identified for LMP1 and LMP2.[16] Adoptive transfer of LMP1- and LMP2-specific T cells has shown promising antitumor activity in patients with EBV-positive lymphoma.[3] Broadening the specificity of the infused T-cell product to not only include EBNA1-specific T cells but also BARF1-specific T cells has the potential to reduce the risk of antigen loss variants. In addition, EBNA1- and BARF1-specific T cells have the potential to boost the antitumor activity of patient-derived T-cell products with a low frequency of LMP1- and LMP2-specific T cells.

Up to date, BARF1-specific T-cell responses have only been identified in HLA*A2-positive, EBV-seropositive healthy donors and NPC patients using five peptides that were selected based on prediction algorithm.[14] Using an unbiased 'pepmix approach' with overlapping 15^{mer} peptides spanning the entire coding region of BARF1, we demonstrate here that BARF1-specific T-cell responses can be detected in the majority (~70%) of healthy, EBV-seropositive donors and patients with EBV-positive malignancies. We used Elispot assays as a readout, which readily detects 1/10,000 antigen-specific T cells; it is possible that with more sensitive techniques, BARF1-specific T cells could be detected in all EBV-seropositive individuals. The frequency of BARF1-specific T cells in T-cell cultures was 4- to 8-fold lower in comparison to EBV antigens that induce dominant CD4 (EBNA1)- and CD8 (BZLF1)-positive T-cell responses, identifying BARF1 as a subdominant EBV antigens. However, this could also be due to differences in the proliferation of BARF1- and EBNA1-/BZLF1-specific T cells during the culture period. This issue should be resolved in future studies; in addition, the frequency of BARF1-specific T-cell responses should be directly compared to other EBV latent type II antigens. BARF1 induced CD4- as well as CD8-positive T-cell responses similar to other EBV antigens expressed in EBV type II latency.

CD4-positive T-cell responses dominated, but CD8-positive T-cell responses were also present in 4 out of 5 donors tested. Inducing not only CD8-, but also CD4-positive T cells might be advantageous given the promising results of adoptively transferring CD4-positive, EBNA1-specific T cells in patient with PTLD.[29]

We identified one HLA-A*02-restricted peptide (TLT) in our screen. The minimal amino acid sequence of this epitope consisted of 8 amino acids (TLTSYWRR). Interestingly, a 9mer (TLTSYWRR↓V) including the 8mer had no activity whereas a 11^{mer} (VTLSYWRR↓VS) and 15^{mer} (LGERVTLTSYWRR↓VS) did. While processing of long peptides has been studied in detail in APCs,[30] it is not well understood how long peptides are processed in regular cells. Cytosolic as well as endosomal peptidase have been implicated, and we speculate that TLTSYWRR↓VS is a substrate for one of these peptidases whereas TLTSYWRR↓V is not due to the close proximity of the cleavage site to the peptide's c-terminus. While we have confirmed our finding in at least five independent experiments, it is advisable to perform additional experiments in the future to confirm our findings. Martonelli *et al.* had detected TLT-specific T-cell responses in peripheral blood at low frequency using the TLT 9^{mer} in IFN- γ Elispot assays with peptide-loaded monocytes as APCs.[14] However, TLT-specific T cells were not expanded to confirm their specificity as done by us.

To demonstrate that T cells specific of the HLA-A*02-restricted BARF1 epitope TLT or HLA B*15(62)-restricted SQF epitope recognize cell lines that endogenous express BARF1, we performed cytotoxicity assays with Auto-LCL, Auto-LCL overexpressing BARF1 (only for SQF-specific T cells), MM-LCL, and/or partially HLA-matched EBV-positive NK/T-cell lymphoma cell lines (only for TLT-specific T cells). BARF1-specific T cells killed EBV-positive target cells in a MHC-class I restricted fashion, establishing that the generated T cells are of high enough avidity to recognize cell lines in which BARF1 is expressed from its physiological promoter including autologous LCL, which were not pulsed with exogenous peptides. It would have been ideal, to evaluate the activity of BARF1-specific T cells against autologous, primary EBV type II latency lymphoma or NPC tumor cells. However, we did not have access to primary tumor samples for this study. BARF1-specific T cells could also be used to elucidate the role of BARF1 in tumorigenesis. For example, if antigen loss variants would not emerge in the presence of BARF1-specific T cells, this would suggest that expression of BARF1 is critical for the malignant phenotype of EBV-positive tumors.

While LCLs express BARF1, EBV-specific T-cell lines generated with LCLs are dominated by T-cell specific for immunodominant EBV type III latency antigens such as EBNA3A, 3B, 3C, or lytic antigens such a BZLF1.[13] To generate T-cell lines with an increased frequency of T cells specific for EBV type II latency proteins we have developed a peptide-based approach, which does not rely on the use of LCLs as APCs.[24] This approach has potential advantages, foremost it does not rely on LCL generation, which requires a live virus and is time consuming. Others have demonstrated that pretreating LCLs with doxycycline increases BARF1 expression, and that T-cell lines generated with doxycycline-treated LCLs have a higher frequency of BARF1-specific T cells.[15] In addition, recombinant adenoviral vectors encoding full length EBV type II latency antigens or corresponding epitopes have

been used to generate T-cell lines for the adoptive immunotherapy of EBV-positive HL, NHL, and NPC.[1, 3, 6]

In conclusion, our study demonstrates that BARF1 is a target for CD4- and CD8-positive T cells. BARF1-specific T cells can be reactivated and expanded from peripheral blood of EBV-seropositive donors and recognize and kill EBV-positive cancer cells. Based on these results we have included BARF1 pepmix in addition to EBNA1, LMP1 and LMP2 pepmixes in our current clinical grade manufacturing protocol of EBV-specific T cells. Two clinical studies to evaluate the safety and efficacy of adoptively transferred BARF1-, EBNA1-, LMP1- and LMP2-specific T cells in patients with EBV-positive lymphoma (NCT01555892) or NPC (NCT02065362) are currently in progress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This work was supported by NIH grants P50 CA126752 and P01 CA094237, CPRIT grant RP110172, and the Sidney Kimmel Foundation.

ABBREVIATIONS

ATC	Activated T cell
ATCC	American type culture collection
BARF	BamH1 A right frame
BZLF	BamHI Z leftward reading frame
CAEBV	Chronic active EBV infection
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr Virus
EHAA	Eagle's Hank's amino acids
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HL	Hodgkin lymphoma
HLA	Human leukocyte antigen

IL	Interleukin
LCL	Lymphoblastoid cell lines
LMP	Latent membrane protein
M-CSF	Macrophage colony stimulating factor
MHC	Major histocompatibility complex
NHL	Non-Hodgkin lymphoma
NPC	Nasopharyngeal carcinoma
RPMI	Roswell Park Memorial Institute medium
TCM	T cell medium
TNF	Tumor necrosis factor

REFERENCES

- [1]. Bollard CM, Gottschalk S, Leen AM, Weiss H, Straathof KC, Carrum G, Khalil M, Wu MF, Huls MH, Chang CC, Gresik MV, Gee AP, Brenner MK, Rooney CM, Heslop HE, Complete responses of relapsed lymphoma following genetic modification of tumor-antigen presenting cells and T-lymphocyte transfer, *Blood* 110(8) (2007) 2838–2845. [PubMed: 17609424]
- [2]. Louis CU, Straathof K, Bollard CM, Ennamuri S, Gerken C, Lopez TT, Huls MH, Sheehan A, Wu MF, Liu H, Gee A, Brenner MK, Rooney CM, Heslop HE, Gottschalk S, Adoptive transfer of EBV-specific T cells results in sustained clinical responses in patients with locoregional nasopharyngeal carcinoma, *J.Immunother* 33(9) (2010) 983–990. [PubMed: 20948438]
- [3]. Bollard CM, Gottschalk S, Torrano V, Diouf O, Ku S, Hazrat Y, Carrum G, Ramos C, Fayad L, Shpall EJ, Pro B, Liu H, Wu MF, Lee D, Sheehan AM, Zu Y, Gee AP, Brenner MK, Heslop HE, Rooney CM, Sustained complete responses in patients with lymphoma receiving autologous cytotoxic T lymphocytes targeting Epstein-Barr virus latent membrane proteins, *J.Clin.Oncol* 32(8) (2014) 798–808. [PubMed: 24344220]
- [4]. Comoli P, Pedrazzoli P, Maccario R, Basso S, Carminati O, Labirio M, Schiavo R, Secondino S, Frasson C, Perotti C, Moroni M, Locatelli F, Siena S, Cell therapy of stage IV nasopharyngeal carcinoma with autologous Epstein-Barr virus-targeted cytotoxic T lymphocytes, *J.Clin.Oncol* 23(35) (2005) 8942–8949. [PubMed: 16204009]
- [5]. Chia WK, Teo M, Wang WW, Lee B, Ang SF, Tai WM, Chee CL, Ng J, Kan R, Lim WT, Tan SH, Ong WS, Cheung YB, Tan EH, Connolly JE, Gottschalk S, Toh HC, Adoptive T-cell transfer and chemotherapy in the first-line treatment of metastatic and/or locally recurrent nasopharyngeal carcinoma, *Mol.Ther* 22(1) (2014) 132–139. [PubMed: 24297049]
- [6]. Smith C, Tsang J, Beagley L, Chua D, Lee V, Li V, Moss DJ, Coman W, Chan KH, Nicholls J, Kwong D, Khanna R, Effective treatment of metastatic forms of Epstein-Barr virus-associated nasopharyngeal carcinoma with a novel adenovirus-based adoptive immunotherapy, *Cancer Res.* 72(5) (2012) 1116–1125. [PubMed: 22282657]
- [7]. Cohen JI, Epstein-Barr virus infection *N. Engl.J.Med* 343(7) (2000) 481–492.
- [8]. Longnecker RM, Kieff E, Cohen JI, Epstein-Barr Virus, in: Knipe DM, Howley PM (Eds.), *Fields Virology*, Lippincott Williams & Wilkins, Philadelphia, 2013, pp. 1898–1959.
- [9]. Decaussin G, Sbih-Lammali F, de Turenne-Tessier M, Bouguermouh A, Ooka T, Expression of BARF1 gene encoded by Epstein-Barr virus in nasopharyngeal carcinoma biopsies, *Cancer Res.* 60(19) (2000) 5584–5588. [PubMed: 11034107]
- [10]. Jang BG, Jung EJ, Kim WH, Expression of BamHI-A Rightward Transcripts in Epstein-Barr Virus-Associated Gastric Cancers, *Cancer Res Treat* 43(4) (2011) 250–4. [PubMed: 22247711]

- [11]. Fiorini S, Ooka T, Secretion of Epstein-Barr virus-encoded BARF1 oncoprotein from latently infected B cells, *Viol J* 5 (2008) 70. [PubMed: 18533018]
- [12]. Straathof KC, Bollard CM, Popat U, Huls MH, Lopez T, Morris MC, Gresik MV, Gee AP, Russell HV, Brenner MK, Rooney CM, Heslop HE, Treatment of Nasopharyngeal Carcinoma with Epstein-Barr Virus-specific T Lymphocytes, *Blood* 105 (2005) 1898–1904. [PubMed: 15542583]
- [13]. Hislop AD, Taylor GS, T-Cell Responses to EBV, *Current topics in microbiology and immunology* 391 (2015) 325–53. [PubMed: 26428380]
- [14]. Martorelli D, Houali K, Caggiari L, Vaccher E, Barzan L, Franchin G, Gloghini A, Pavan A, Da PA, Tedeschi RM, De R, Carbone VA, T. Ooka, De PP, Dolcetti R, Spontaneous T cell responses to Epstein-Barr virus-encoded BARF1 protein and derived peptides in patients with nasopharyngeal carcinoma: bases for improved immunotherapy, *Int.J.Cancer* 123(5) (2008) 1100–1107. [PubMed: 18546263]
- [15]. Fae DA, Martorelli D, Mastorci K, Muraro E, Dal Col J, Franchin G, Barzan L, Comaro E, Vaccher E, Rosato A, Dolcetti R, Broadening Specificity and Enhancing Cytotoxicity of Adoptive T Cells for Nasopharyngeal Carcinoma Immunotherapy, *Cancer immunology research* 4(5) (2016) 431–40. [PubMed: 27009165]
- [16]. Taylor GS, Long HM, Brooks JM, Rickinson AB, Hislop AD, The immunology of Epstein-Barr virus-induced disease, *Annual review of immunology* 33 (2015) 787–821.
- [17]. Takada K, Role of EBER and BARF1 in nasopharyngeal carcinoma (NPC) tumorigenesis, *Seminars in cancer biology* 22(2) (2012) 162–5. [PubMed: 22210180]
- [18]. Mohidin TB, Ng CC, BARF1 gene silencing triggers caspase-dependent mitochondrial apoptosis in Epstein-Barr virus-positive malignant cells, *J Biosci* 40(1) (2015) 41–51. [PubMed: 25740140]
- [19]. Ohashi M, Fogg MH, Orlova N, Quink C, Wang F, An Epstein-Barr virus encoded inhibitor of Colony Stimulating Factor-1 signaling is an important determinant for acute and persistent EBV infection, *PLoS pathogens* 8(12) (2012) e1003095. [PubMed: 23300447]
- [20]. Hoebe EK, Le Large TY, Greijer AE, Middeldorp JM, BamHI-A rightward frame 1, an Epstein-Barr virus-encoded oncogene and immune modulator, *Reviews in medical virology* 23(6) (2013) 367–83. [PubMed: 23996634]
- [21]. Nagata H, Konno A, Kimura N, Zhang Y, Kimura M, Demachi A, Sekine T, Yamamoto K, Shimizu N, Characterization of novel natural killer (NK)-cell and gammadelta T-cell lines established from primary lesions of nasal T/NK-cell lymphomas associated with the Epstein-Barr virus, *Blood* 97(3) (2001) 708–713. [PubMed: 11157488]
- [22]. Zhang Y, Nagata H, Ikeuchi T, Mukai H, Oyoshi MK, Demachi A, Morio T, Wakiguchi H, Kimura N, Shimizu N, Yamamoto K, Common cytological and cytogenetic features of Epstein-Barr virus (EBV)-positive natural killer (NK) cells and cell lines derived from patients with nasal T/NK-cell lymphomas, chronic active EBV infection and hydroa vacciniforme-like eruptions, *British journal of haematology* 121(5) (2003) 805–14. [PubMed: 12780797]
- [23]. Rooney CM, Smith CA, Ng CYC, Loftin SK, Sixbey JW, Gan Y-J, Srivastava D-K, Bowman LC, Krance RA, Brenner MK, Heslop HE, Infusion of cytotoxic T cells for the prevention and treatment of Epstein-Barr virus-induced lymphoma in allogeneic transplant recipients, *Blood* 92(5) (1998) 1549–1555. [PubMed: 9716582]
- [24]. Ngo MC, Ando J, Leen AM, Ennamuri S, Lapteva N, Vera JF, Min-Venditti A, Mims MP, Heslop HE, Bollard CM, Gottschalk S, Rooney CM, Complementation of Antigen-presenting Cells to Generate T Lymphocytes With Broad Target Specificity, *J.Immunother* 37(4) (2014) 193–203. [PubMed: 24714353]
- [25]. Gerdemann U, Katari U, Christin AS, Cruz CR, Tripic T, Rousseau A, Gottschalk SM, Savoldo B, Vera JF, Heslop HE, Brenner MK, Bollard CM, Rooney CM, Leen AM, Cytotoxic T Lymphocytes Simultaneously Targeting Multiple Tumor-associated Antigens to Treat EBV Negative Lymphoma, *Mol.Ther* 19(12) (2011) 2258–2268. [PubMed: 21915103]
- [26]. Seto E, Yang L, Middeldorp J, Sheen TS, Chen JY, Fukayama M, Eizuru Y, Ooka T, Takada K, Epstein-Barr virus (EBV)-encoded BARF1 gene is expressed in nasopharyngeal carcinoma and EBV-associated gastric carcinoma tissues in the absence of lytic gene expression, *Journal of medical virology* 76(1) (2005) 82–8. [PubMed: 15778977]

- [27]. Louis CU, Straathof K, Bollard CM, Gerken C, Huls MH, Gresik MV, Wu MF, Weiss HL, Gee AP, Brenner MK, Rooney CM, Heslop HE, Gottschalk S, Enhancing the in vivo expansion of adoptively transferred EBV-specific CTL with lymphodepleting CD45 monoclonal antibodies in NPC patients, *Blood* 113(11) (2009) 2442–2450. [PubMed: 18971421]
- [28]. Shaffer DR, Savoldo B, Yi Z, Chow KK, Kakarla S, Spencer DM, Dotti G, Wu MF, Liu H, Kenney S, Gottschalk S, T cells redirected against CD70 for the immunotherapy of CD70-positive malignancies, *Blood* 117(16) (2011) 4304–4314. [PubMed: 21304103]
- [29]. Icheva V, Kayser S, Wolff D, Tuve S, Kyzirakos C, Bethge W, Greil J, Albert MH, Schwinger W, Nathrath M, Schumm M, Stevanovic S, Handgretinger R, Lang P, Feuchtinger T, Adoptive transfer of epstein-barr virus (EBV) nuclear antigen 1-specific t cells as treatment for EBV reactivation and lymphoproliferative disorders after allogeneic stem-cell transplantation, *J.Clin.Oncol* 31(1) (2013) 39–48. [PubMed: 23169501]
- [30]. Rosalia RA, Quakkelaar ED, Redeker A, Khan S, Camps M, Drijfhout JW, Silva AL, Jiskoot W, van Hall T, van Veelen PA, Janssen G, Franken K, Cruz LJ, Tromp A, Oostendorp J, van der Burg SH, Ossendorp F, Melief CJ, Dendritic cells process synthetic long peptides better than whole protein, improving antigen presentation and T-cell activation, *European journal of immunology* 43(10) (2013) 2554–65. [PubMed: 23836147]

Highlights

- BARF1-specific T cells are present in the majority of EBV-seropositive individuals
- Class I- or II-restricted T-cell epitopes are recognized by BARF1-specific T cells
- EBV+ tumor cells are recognized and killed by BARF1-specific T cells
- BARF1 presents an attractive antigen for EBV-targeted T-cell therapies

Figure 1: BARF1-specific T cells are present in peripheral blood of healthy donors and patients with EBV-positive malignancies.

PBMCs of healthy donors (n=16), NPC patients (n=15) and NK-T lymphoma patients (n=5) were stimulated with BARF1 (A) or EBNA1 and BZLF1 (B,C) pepmixes. IFN- γ Elispot assays were used to determine the frequency of antigen-specific T cells. Data is presented as spot forming cells (SFC) per 200,000 cells. (A,B) Only BARF1-stimulated PBMCs showed significant IFN- γ production in the presence of BARF1 pepmix as compared to media control in both healthy donors ($p < 0.001$) and patients (black-NPC, white-NK-T lymphoma, $p < 0.001$). (C) PBMCs stimulated with EBNA1 and BZLF1 pepmixes elicited significantly high antigen-specific IFN- γ response as compared to media ($p < 0.001$).

Figure 2: T-cell epitopes are clustered in the N-terminal region of BARF1.

PBMC of healthy donors ($n=12$) were stimulated with BARF1 pepmixes. On day 10, T-cell reactivity towards 5 BARF1-peptide pools (P1–5) was determined by IFN- γ Elispot assays. (A) Representative responses towards peptide pools are shown for 5 donors. The results are expressed as SFC/200,000 cells. (B) Summary of pool recognition by 9/12 donors who showed reactivity towards one or more peptide pools. Data is presented as SFC-fold increase in comparison to control wells. (C) For epitope mapping, BARF1-specific T cells were generated from 5 donors by repeated stimulations with BARF1 pepmix-loaded DCs. CD4- and CD8-positive T cells were separated using magnetic beads and analyzed for reactivity towards peptide pools in IFN- γ Elispot assays. Results are expressed as percentage of total number of SFC observed with CD4- and CD8-positive T cells.

Figure 3: Distribution of MHC class I- and II-restricted epitopes in BARF1.

15^{mer} peptides are presented as solid horizontal bars. Yellow: MHC class I epitope; blue: MHC class II epitope; green MHC class I and II epitope.

Figure 4: Identification of two CD8-restricted BARF1 T-cell epitopes.

(A,B) Cytotoxicity assays were performed with p33/34- or p7/8-specific T cells as effectors and autologous ATCs pulsed with specific 15^{mer} or 11^{mer} peptides as effectors. DMSO-, or control peptide-pulsed targets were included as negative controls. Peptide-specific T cells showed significantly higher lysis of p33/34, p8 or 11^{mer} pulsed ATCs as compared to DMSO or control peptide (Co-Pep)-pulsed ATCs ($p < 0.0001$ at all E:T ratios; Co-Pep: BARF1 peptide P1). (C,D) IFN- γ Elispot assays with p33/34- or p7/8-specific T cells as effectors and peptides as stimulators. p33/34-specific T cells recognized a minimal epitope consisting of 9 amino acids (8^{mer} vs. all other peptides: $p < 0.0001$). p7/8-specific T cells recognize a minimal epitope starting with amino acid T and consisting of 8 amino acids. Significantly

less IFN- γ production was observed with a 9^{mer} ($p<0.001$) and a 8^{mer} starting with amino acid L ($p<0.0001$) compared to all other peptides.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 5: BARF1 p33/34-specific T cells are restricted through HLA-B*15(62) and p7/8-specific T cells through HLA-A*02.

(A) SQF-specific T cells killed peptide-pulsed autologous and HLA-B*15(62)-matched ATCs in contrast to DMSO-pulsed ATCs (***) ($p < 0.0001$). (B) SQF-specific T cells killed SQF-pulsed ATCs in context of HLA-B*15(62) at different effector: target ratios (E:T). DMSO- and control peptide (p1 BARF1 peptide)-pulsed targets served as controls ($p < 0.0001$ at all E:T ratios). (C) p7/8-specific T cells killed p8-pulsed autologous and HLA-A*02 matched ATCs in contrast to DMSO-pulsed ATCs (DMSO- vs. peptide-pulsed ATCs for each condition: ** $p < 0.001$, *** $p < 0.0001$). (D) TLT-, KLG-, RFI-specific T cells were reactivated from PBMCs of HLA-A*02-positive, EBV-seropositive donors ($n=13$). Significant epitope-specific reactivity was only observed in response to TLT peptide.

Figure 6: BARF1-specific T cells kill BARF1-expressing targets in a HLA-dependent manner.

(A) In a 4 hour ^{51}Cr release assay, TLt-specific T cells killed Auto-LCL, and HLA*A02-positive SNK6 and SNT16 cell lines. MM-LCLs (HLA*A01,03, B*15,57) and K562 were only killed at background levels (Auto-LCL vs. K562: $p < 0.05$, SNT16 vs. K562: $p < 0.05$, Auto-LCL vs. MM-LCL: $p < 0.05$, SNT16 vs. MM-LCL: $p < 0.05$ at all E:T ratios; SNK6 vs. MMLCL: $p < 0.05$ at 20:1 and 10:1). (B) In a 4 hour ^{51}Cr release assay SQF-specific T cells killed Auto-LCL, Auto-LCL-BARF1, Auto-LCL-GFP. MM-LCLs (HLA*A02,31, B*35,40) were not killed (Auto-LCL vs. MM-LCL: $p < 0.0001$ at all E:T ratios).