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# Standardized and Highly Efficient Expansion of Epstein-Barr Virus-Specific CD4$^+$ T Cells by Using Virus-Like Particles$^\nabla$

Dinesh Adhikary,[1] Uta Behrends,[1] Regina Feederle,[2] Henri-Jacques Delecluse,[2] and Josef Mautner[1]*

*Clinical Cooperation Group, Department of Pediatrics, Munich University of Technology and GSF-Research Centre for Environment and Health, Munich, Germany,[1] and German Cancer Research Center, Department of Virus Associated Tumours, Heidelberg, Germany[2]*

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**Epstein-Barr virus (EBV)-specific T-cell lines generated by repeated stimulation with EBV-immortalized lymphoblastoid B-cell lines (LCL) have been successfully used to treat EBV-associated posttransplant lymphoproliferative disease (PTLD) in hematopoietic stem cell transplant recipients. However, PTLD in solid-organ transplant recipients and other EBV-associated malignancies respond less efficiently to this adoptive T-cell therapy. LCL-stimulated T-cell preparations are polyclonal and contain CD4$^+$ and CD8$^+$ T cells, but the composition varies greatly between lines. Because T-cell lines with higher CD4$^+$ T-cell proportions show improved clinical efficacy, we assessed which factors might compromise the expansion of this T-cell population. Here we show that spontaneous virus production by LCL and, hence, the presentation of viral antigens varies intra- and interindividually and is further impaired by acyclovir treatment of LCL. Moreover, the stimulation of T cells with LCL grown in medium supplemented with fetal calf serum (FCS) caused the expansion of FCS-reactive CD4$^+$ T cells, whereas human serum from EBV-seropositive donors diminished viral antigen presentation. To overcome these limitations, we used peripheral blood mononuclear cells pulsed with non-transforming virus-like particles as antigen-presenting cells. This strategy facilitated the specific and rapid expansion of EBV-specific CD4$^+$ T cells and, thus, might contribute to the development of standardized protocols for the generation of T-cell lines with improved clinical efficacy.**

The oncogenic Epstein-Barr virus (EBV) belongs to the family of gammaherpesviruses and establishes lifelong persistent B-cell infections in more than 90% of the human population (18, 19). In healthy individuals, the majority of EBV-infected B cells show limited viral gene expression and a resting phenotype. The terminal differentiation of latently infected cells into plasma cells leads to virus reactivation, production, and reinfection of B cells (20). The expression of all viral latency genes causes growth transformation and the proliferation of infected B cells, which is reflected by the outgrowth of EBV-transformed lymphoblastoid B-cell lines (LCL) in vitro and by the association of EBV with a variety of B-cell lymphoproliferative diseases, including different types of lymphoma, in vivo (31). EBV infection is controlled by T cells, as indicated by an increased incidence of EBV-associated malignancies in patients with congenital or iatrogenically induced T-cell dysfunction (31) and by the successful treatment of EBV-associated posttransplant lymphoproliferative disease (PTLD) in hematopoietic stem cell transplant (HSCT) recipients by the infusion of polyclonal EBV-specific T-cell lines (33, 34). Such lines are prepared by the repeated stimulation of peripheral blood T cells with autologous LCL and contain CD4$^+$ and CD8$^+$ components. The antigens recognized by the EBV-specific CD8$^+$ T-cell component are derived mostly from latent as well as immediate-early and early lytic cycle proteins (16). Although CD4$^+$ T-cell responses to these antigens have been detected in peripheral blood of EBV-positive donors, CD4$^+$ T cells in LCL-stimulated preparations are directed almost exclusively against late-lytic-cycle antigens derived from structural proteins of the virus, which are efficiently presented on major histocompatibility complex class II (MHC-II) after the CD21-mediated uptake of EBV particles by B cells (1, 2, 9, 23). Such T cells are cytolytic and are able to prevent the proliferation of EBV-infected B cells and to inhibit the outgrowth of LCL from freshly infected B cells (2, 15). Targeting mostly nonoverlapping sets of viral proteins and different phases of the virus life cycle implies that CD4$^+$ and CD8$^+$ T cells complement each other in establishing protective immunity against EBV.

The successful treatment of immanent and manifest PTLD in HSCT recipients by the infusion of EBV-specific T-cell preparations has provided an important proof of principle for this form of immunotherapy, but owing to the considerable technical requirements and financial implications of extensive in vitro T-cell culture, adoptive T-cell therapy still has a limited role in the management of virus-associated complications in HSCT patients (27). To implement this treatment modality as a conventional therapeutic option, generic and more direct approaches for the generation of EBV-specific T-cell lines enriched in disease-relevant specificities need to be developed.

Two recent reports imply an important role of CD4$^+$ T cells in establishing antiviral immunity. First, low numbers of endogenous CD4$^+$ T cells has been identified as an important risk factor for the development of EBV-associated diseases in immunosuppressed patients (35). Second, patients with PTLD showed better clinical responses in a recent phase II trial when the infused T-cell lines contained higher proportions of CD4$^+$ T cells (14). For unknown reasons, the CD4/CD8 ratio in

* Corresponding author. Mailing address: Children's Hospital, University of Technology, Kölner Platz 1, D-80804 Munich, Germany. Phone: 49-89-7099518. Fax: 49-89-7099500. E-mail: mautner@gsf.de.
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LCL-stimulated T-cell preparations can vary from 2:98 to 98:3 (36).

Here, we investigated which factors compromise the expansion of EBV-specific $CD4^+$ T cells in LCL-stimulated T-cell preparations and developed a stimulation protocol that facilitates the standardized, highly efficient, rapid, and safe expansion of EBV-specific $CD4^+$ T cells ex vivo.

## MATERIALS AND METHODS

**Materials of human origin.** The use of materials of human origin for this study was approved by the ethics committee of the Munich University of Technology. Blood samples were obtained from healthy adult volunteers by venipuncture after receiving informed consent.

**Cell culture.** Peripheral blood mononuclear cells (PBMC) were purified by Ficoll-Paque (GE Healthcare) density centrifugation. All LCL and mini-LCL were established by infection of primary B cells with wild-type (WT) EBV produced by the B95.8 cell line or with mini-EBV, a genetically engineered virus mutant incapable of lytic virus replication, as described previously (2, 26). Mini-LCL are identical to LCL in terms of latent cycle protein expression, antigen presentation, and T-cell costimulation, but they do not express lytic cycle proteins of EBV and do not release viral particles (2, 26). The virus-like particle (VLP)-producer cell line TR− has been established by the stable transfection of HEK293 cells with an EBV mutant lacking the terminal repeats (8). As a control, HEK293 was transfected with WT EBV DNA, resulting in the cell line 293/2089. LCL and mini-LCL were grown as suspension cultures in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 1% nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 μg/ml gentamicin. In some experiments, FCS was replaced by individual or pooled human serum as indicated. HEK293 transfectants were cultivated as adherent cultures in RPMI 1640 medium supplemented with 10% FCS, 1% nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 100 μg/ml hygromycin. T cells were grown in 24-well plates in T-cell medium consisting of AIM-V lymphocyte medium (Invitrogen) supplemented with 10% pooled human serum, 2 mM L-glutamine, 10 mM HEPES, and 50 μg/ml gentamicin. Peripheral blood $CD4^+$ T cells were separated from PBMC by using α-CD4-MicroBeads and MACS columns (Miltenyi Biotec). The gp350/BLLF1- and BNRF1-specific $CD4^+$ T-cell clones 1D6 and 1H7 had been generated by the repeated stimulation of peripheral $CD4^+$ T cells with protein-pulsed PBMC as described previously (2, 22).

**T-cell recognition assays.** If not stated otherwise, T-cell recognition assays were performed by coculturing $1 \times 10^5$ target cells and $1 \times 10^5$ T cells for 20 h in 200 μl T-cell medium in 96-well flat-bottom plates as described previously (2). Cytokine release by the T cells was measured by enzyme-linked immunosorbent assay by following the protocol of the manufacturer (R&D Systems). In cell-mixing experiments, LCL were preincubated with mini-LCL at a 1:1 ratio for 24 h prior to the addition of T cells. In some experiments, PBMC or mini-LCL were pulsed for 24 h with recombinant proteins, WT EBV, or VLP. Unless otherwise stated, all displayed experiments were performed at least thrice, with similar results.

**T-cell receptor analysis.** The analyses of the T-cell receptor Vβ-chain variable region were performed by Vβ-chain-specific PCR followed by Southern blot hybridization of the PCR products using a Vβ common region-specific radioactive probe (13).

**Purification, titration, and concentration of viral particles.** Viral supernatants were obtained from densely grown cultures of the marmoset cell line B95.8 (WT EBV) or the BZLF1-transfected HEK293-derived cell lines 293/2089 (WT EBV) and 293/TR− (VLP) (7, 10). The concentration of WT EBV in B95.8 and 293/2089 in the filtered (0.8 μm) supernatants was determined by quantitative real-time PCR using primers specific for the viral *BALF5* gene. To determine the concentration of VLP in TR− supernatants, viral particles were pelleted from 5 ml supernatant by ultracentrifugation (2 h at 30,000 × *g*) and denatured in Laemmli buffer, and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electroblotted onto a nitrocellulose membrane (Hybond ECL; GE Healthcare), and hybridized with a rabbit polyclonal antibody specific for the viral tegument protein BNRF1. 293/2089 supernatant for which the titer had been determined was used as the standard.

**Flow cytometry.** For flow cytometry, cells were washed in ice-cold fluorescence-activated cell sorter (FACS) buffer (phosphate-buffered saline with 1% bovine serum albumin and 0.05% sodium azide), incubated with fluorescence-labeled antibodies for 20 min on ice, washed twice with FACS buffer, resuspended in 500 μl ice-cold FACS buffer containing 0.5 mg/ml propidium iodide, and analyzed in a BD FACScan using CellQuest software.

**Recombinant protein expression, purification, and quantification.** Recombinant EBV proteins were expressed as C-terminally histidine-tagged proteins in HEK293 cells using calcium phosphate or polyethylenimine transfection methods (28). The extraction, purification, and quantification of recombinantly expressed proteins have been described previously (1).

**Generation of VLP-reactive $CD4^+$ T-cell lines.** The VLP stimulation of $CD4^+$ T cells was performed by incubating PBMC with a 10-fold excess of VLP prepared in serum-free medium for 24 h. Subsequently, the cells were irradiated (40 Gy), washed, and cocultured with an equal number of $CD4^+$ T cells. After 24 h, 50 U/ml of interleukin-2 was added, and expanding cultures were split as needed. The lines were restimulated every 2 weeks in the same fashion.

## RESULTS

**The rate of spontaneous virus production in LCL cultures varies inter- and intraindividually.** Earlier results of our group had indicated that structural antigens of EBV are the immunodominant targets of LCL-stimulated $CD4^+$ T cells (1). Structural antigens are efficiently presented to $CD4^+$ T cells following the CD21-mediated uptake of released virus particles (2). To assess whether differences in the CD4/CD8 composition of LCL-stimulated T-cell lines are a reflection of differences in spontaneous virus production among different stimulator LCL, the concentrations of virus particles in the supernatants of several LCL were measured by two methods. The concentration of packaged virus DNA in supernatants was determined by quantitative PCR using primers specific for the viral gene *BALF5* (10, 17) (Fig. 1A), and the amount of transferable antigen in the supernatant was assessed in coculture experiments using T cells specific for the structural antigen BLLF1 (Fig. 1B). Both assays consistently detected significant fluctuations in virus production. As quantified by PCR, the amount of viral particles in the supernatant of different LCL varied more than 40-fold. Moreover, spontaneous virus production also varied intraindividually over time. When different passages of the same LCL were tested, striking differences in the amount of transferable antigen were detected in the supernatants (Fig. 1C). These results were paralleled by similar differences in the recognition of these LCL by lytic cycle antigen-specific $CD8^+$ T cells (data not shown). These results showed that spontaneous virus production in LCL cultures may vary greatly inter- and intraindividually. Since low levels of virus production remained undetected by T cells, such variation may affect the reactivation and expansion of EBV-specific $CD4^+$ T cells by LCL stimulation.

**Acyclovir treatment impairs the presentation of virion antigens by LCL.** To preclude the transfer of infectious virus into patients, T-cell lines for clinical use usually are prepared by stimulation with acyclovir-treated LCL. Because acyclovir limits virus production by interfering with late-lytic-cycle protein expression (6, 18), we assessed virus production in LCL cultures by coculturing mini-LCL with allogeneic LCL that had been left untreated or had been treated with acyclovir for 2 weeks. The recognition of acyclovir-treated allogeneic LCL by T cells specific for the late-lytic-cycle antigen BNRF1 was severely impaired compared to that for untreated LCL, demonstrating that the treatment of LCL with this drug selectively diminishes the presentation of late-lytic-cycle antigens (Fig. 2). T-cell recognition was reduced to background levels in acyclovir-treated LCL that produce low levels of EBV and was severely reduced, but still detectable, in EBV high-producer LCL (data

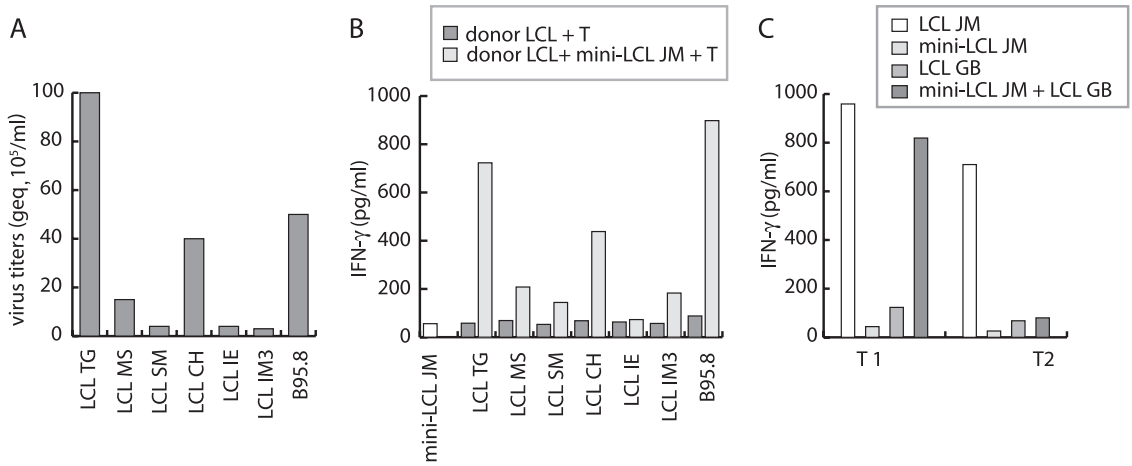


FIG. 1. Virus production by LCL varies inter- and intraindividually. (A) The number of viral particles in the supernatants of LCL from five healthy donors (TG, MS, SM, CH, and IE) and from a patient with acute EBV-associated infectious mononucleosis (IM3) and from the supernatant of the EBV-producer cell line B95.8 was determined by quantitative PCR using *BALF5*-specific primers. Viral titers are given as EBV genome equivalents (geq)/milliliter. (B) The same EBV-positive target cells were tested for recognition by the BLLF1-specific $CD4^+$ T-cell clone 1D6. Because none of the target cells expresses the restricting MHC-II molecule, the T cells recognized the target cells only after being cocultured with mini-LCL from donor JM (mini-LCL JM), which express the restricting MHC allele but are incapable of producing viral particles. T-cell recognition of the cell mixtures was target cell dependent but correlated with the amount of EBV genome equivalents detected in the culture supernatant as quantified by PCR. (C) Mini-LCL JM were cocultured for 24 h with MHC-mismatched LCL from donor GB (LCL GB) that had been cultured for different periods of time in vitro (T1 and T2). Subsequently, the cell mixtures were probed for recognition by the BLLF1-specific $CD4^+$ T cells from donor JM (clone 1D6). As a control, the T-cell recognition of autologous LCL JM, autologous mini-LCL JM, and allogeneic LCL GB is shown. IFN-γ, gamma interferon.

not shown). Thus, acyclovir treatment may impede the ex vivo expansion of EBV structure antigen-specific $CD4^+$ T cells and thereby contribute to the fluctuations in the CD4/CD8 composition of LCL-stimulated T-cell lines.

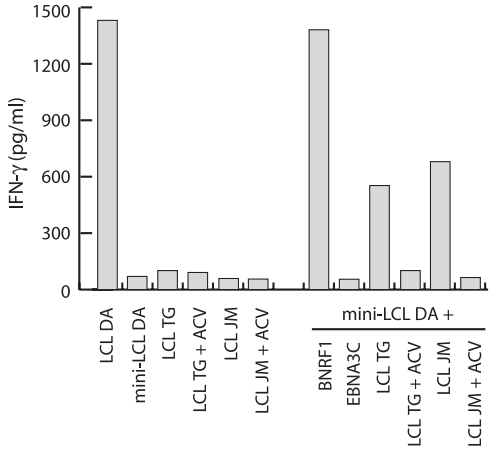


FIG. 2. Presentation of structural antigens of EBV is impaired after acyclovir treatment of LCL. Autologous LCL and mini-LCL from donor DA (mini-LCL DA), the MHC-mismatched LCL from donor JM (LCL JM), and LCL from donor TG (LCL TG), which had been left untreated or had been treated with 200 μM acyclovir (ACV) for 2 weeks, were tested for recognition by $CD4^+$ T cells specific for the virion antigen BNRF1. In addition, the allogeneic lines were cocultured for 24 h with autologous mini-LCL DA and then tested for T-cell recognition. Except for LCL DA, none of the LCL was recognized by the T cells directly. However, the cell mix of autologous mini-LCL DA and allogeneic LCL was recognized, but the acyclovir treatment of the LCL abolished recognition. For a specificity control, the T cells also were tested for the recognition of autologous mini-LCL pulsed with the relevant EBV protein (BNRF1) or an irrelevant EBV protein (EBNA3C). IFN-γ, gamma interferon.

**Preferential expansion of FCS-reactive $CD4^+$ T cells upon stimulation with LCL cultured in FCS-supplemented media.** Because antigens presented on MHC-II molecules are derived mostly from exogenous proteins taken up by endocytosis, serum supplements in culture media might add to the variable expansion of EBV-specific $CD4^+$ T cells after LCL stimulation. $CD4^+$ T cells reacting to FCS-derived antigens have been described in numerous studies (11, 24, 25, 29), but it remained unknown whether FCS-specific $CD4^+$ T cells prevail in LCL-stimulated $CD4^+$ T-cell cultures from virus-carrying individuals. To address this question, LCL continuously grown in medium supplemented with either 10% FCS (LCL-FCS) or 10% human serum (LCL-HS) were used to stimulate autologous T cells. When tested against LCL-FCS and LCL-HS, all of the $CD4^+$ T-cell lines established from 10 EBV-seropositive donors and 5 patients with infectious mononucleosis by LCL-FCS stimulation responded preferentially against LCL-FCS. In contrast, T-cell lines stimulated with LCL-HS recognized both types of target cells, indicating that they target viral or cellular antigens rather than serum supplements (Fig. 3). These results suggested that FCS-specific T cells dominate LCL-stimulated $CD4^+$ T-cell cultures and that, in order to obtain T-cell lines enriched in $CD4^+$ T cells specific for EBV antigens, FCS has to be omitted from culture media.

**Sera from EBV-seropositive donors impair antigen transfer by virions.** The results described above implied that the individual $CD4^+$ T-cell response to FCS affects the expansion of the $CD4^+$ component in LCL-stimulated T-cell preparations and that the proportion of EBV-specific $CD4^+$ T cells is increased when human serum instead of FCS was used as the medium supplement. However, when LCL-HS and LCL-FCS were tested for recognition by T-cell clones specific for structural antigens of EBV, LCL-HS often was recognized to a

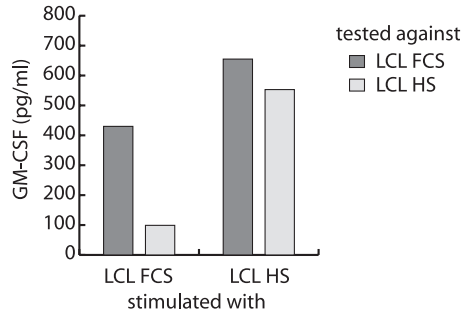


FIG. 3. In LCL-stimulated CD4$^+$ T-cell preparations, responses to FCS dominate virus-specific responses. CD4$^+$ T-cell lines were established from EBV-positive donors by repeated stimulation with autologous LCL cultured in medium supplemented with either FCS or HS and tested against both types of stimulator cells in cytokine secretion assays. CD4$^+$ T-cell lines that had been stimulated with LCL-FCS failed to recognize LCL-HS, suggesting that the lines predominantly recognized antigens derived from FCS. In contrast, CD4$^+$ T-cell lines that had been stimulated with LCL-HS recognized both types of target cells, indicating that this line recognized viral or cellular antigen(s). GM-CSF, granulocyte-macrophage colony-stimulating factor.

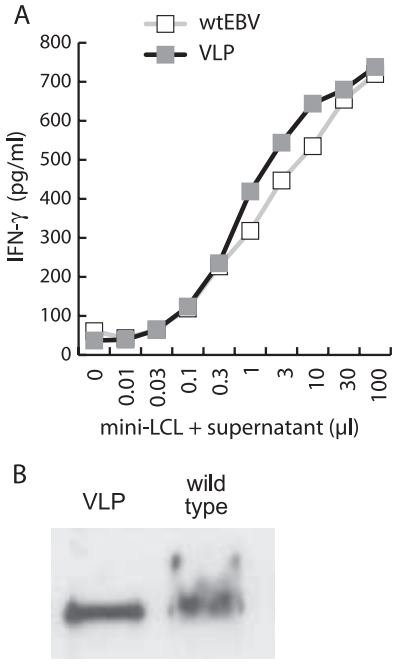


FIG. 5. Mini-LCL pulsed with either VLP or WT EBV are recognized by virion antigen-specific CD4$^+$ T cells with similar efficiencies. (A) Mini-LCL from donor JM (mini-LCL JM) were pulsed with supernatants from 293/TR− and 293/2089 cells containing $1 \times 10^7$/ml VLP or WT EBV particles, respectively, and then were tested for recognition by autologous BLLF1-specific CD4$^+$ T cells. Across the entire concentration range analyzed, WT EBV and VLP-pulsed mini-LCL were recognized by the T cells to the same extent. (B) The concentration of VLP and WT EBV in the supernatants used for the experiments shown in panel A was determined by Western blot analysis using an antibody against the tegument protein BNRF1. IFN-γ, gamma interferon.

lesser extent (Fig. 4A). Antibodies against structural antigens are known to circulate in the sera of EBV-infected subjects and might interfere with CD21-mediated virus uptake and hence the presentation of virion antigens by LCL (37). To test this hypothesis, virus supernatant of the B95.8 cell line either was left untreated or was incubated with human serum either derived from an EBV-seropositive (EBV$^+$) or EBV-seronegative (EBV$^-$) donor at a final concentration of 10% for 2 h and subsequently pulsed at increasing concentrations onto mini-LCL. After 24 h of incubation, virus-pulsed mini-LCL were tested for recognition by virion-specific T cells. Compared to the T-cell recognition of untreated controls, sera from EBV-positive donors reduced T-cell recognition, while serum from an EBV-negative donor had no effect (Fig. 4B). These results suggested that sera from EBV-positive donors contains components that specifically affect virion antigen presentation, most likely antibodies that block the receptor-mediated uptake of viral particles. This inhibitory effect was overcome with higher amounts of EBV particles, probably because antiviral antibodies become limiting (data not shown).

**Efficient presentation of structural antigens derived from VLP.** The results described above indicated that virus-neutralizing activity in human serum compromises the expansion of

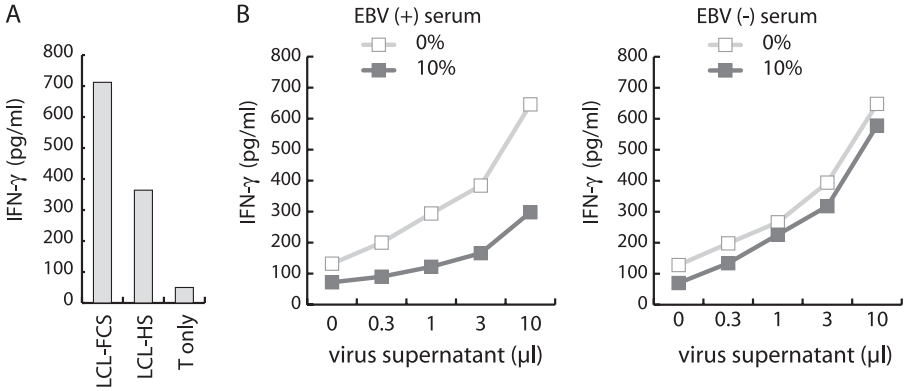


FIG. 4. Presentation of exogenous viral structure antigens is impaired by human serum components. (A) LCL grown in medium supplemented with either FCS or HS were tested for recognition by BLLF1-specific CD4$^+$ T cells (clone 1D6). T-cell recognition was reduced when LCL were cultured in human serum. (B) Mini-LCL grown in FCS-containing media were pulsed with increasing amounts of virus supernatant that had been left untreated or had been incubated at a final concentration of 10% human serum from an EBV$^+$ or EBV$^-$ donor for 2 h. The recognition of the cells by BLLF1-specific CD4$^+$ T cells (clone 1D6) was assayed 24 h later. IFN-γ, gamma interferon.

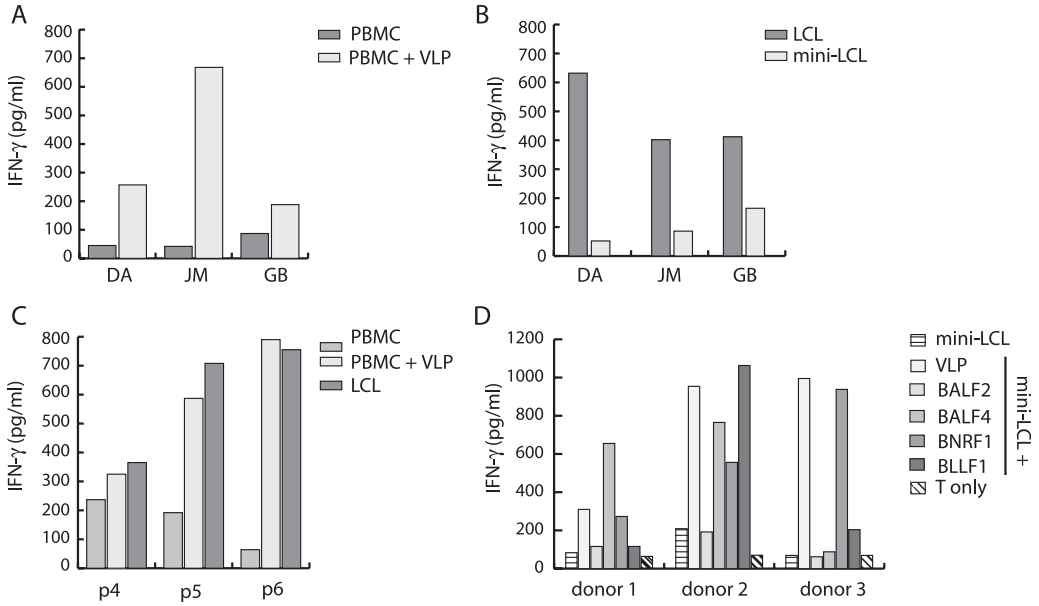


FIG. 6. EBV structural antigen-specific CD4+ T cells are efficiently expanded from peripheral blood of healthy EBV carriers by repeated stimulation with VLP-pulsed PBMC. (A) Autologous PBMC pulsed with VLP for 24 h were used for the repeated stimulation of peripheral CD4+ T cells of donors DA, JM, and GB. After five stimulations, all CD4+ T-cell lines recognized VLP-pulsed, but not barely unpulsed, PBMC, suggesting that these T cells recognized virion antigens. (B) When tested against autologous LCL and mini-LCL, these CD4+ T-cell lines showed much stronger responses against LCL, indicating that the VLP-stimulated T cells recognized antigens that also were presented by LCL. (C) After four to six rounds of VLP stimulation, CD4+ T cells became increasingly EBV specific, as indicated by increasing reactivity against VLP-pulsed autologous PBMC and autologous LCL and decreasing reactivity against unpulsed autologous PBMC. Results for donor JM are shown as an example. (D) To define the antigens recognized by the VLP-stimulated CD4+ T-cell lines, mini-LCL were pulsed separately with three structural proteins of EBV (BALF4, BNRF1, and BLLF1) and a nonstructural lytic cycle protein of EBV (BALF2). While none of the lines responded to BALF2, each line responded to at least one structural protein. IFN-γ, gamma interferon.

EBV-specific CD4+ T cells by LCL-HS stimulation and that these limitations can be overcome by pulsing LCL with excess virus particles. Such a protocol also would compensate for the differences in virus production by LCL and would be conducive to standardized stimulation conditions. Because safety concerns preclude the addition of WT virus supernatant to stimulator LCL, we assessed whether EBV virions devoid of viral DNA, i.e., VLP, are able to transfer virion antigens. Large amounts of VLP are produced upon the induction of the lytic cycle in the 293/TR− cell line, which carries an EBV genome that lacks the terminal repeats required for packaging viral DNA into virions (18). Thus, VLP are incapable of transforming primary human B cells in vitro (10). Mini-LCL were pulsed with increasing amounts of VLP or WT EBV and subsequently were tested for recognition by BLLF1-specific CD4+ T cells (Fig. 5). VLP- and WT EBV-pulsed mini-LCL were recognized with similar efficiency, demonstrating that VLP are able to transfer antigen. Similar results were obtained with a CD4+ T-cell clone specific for the tegument protein BNRF1 (data not shown).

**Virion-specific CD4+ T cells are efficiently expanded by repeated stimulation with VLP-pulsed PBMC in vitro.** Primary B cells express CD21 and are capable of presenting virion antigens following receptor-mediated virus uptake (2), implying that VLP-pulsed PBMC could be used as antigen-presenting cells to expand the number of virion-specific CD4+ T cells. Such protocols would obviate the lengthy procedure of establishing LCL and thereby shorten the T-cell preparation procedure significantly. To address this possibility, CD4+ T cells from peripheral blood of three EBV+ healthy donors were stimulated with autologous PBMC that had been pulsed with VLP from TR− cells cultured in serum-free medium. After five to six rounds of stimulation, the majority of cells within the VLP-stimulated T-cell lines reacted against VLP-pulsed but not unpulsed PBMC and against autologous LCL but not mini-LCL, indicating that they were specific for EBV structural antigens (Fig. 6A and B). This selective reactivity against VLP-pulsed PBMC and LCL was observed after four to six restimulations (Fig. 6C), which is much faster than that with LCL stimulation (1). The structural antigen specificities of these T-cell lines were verified by pulsing mini-LCL with single recombinant lytic cycle proteins and testing the cells for recognition by the T cells (Fig. 6D). Each line recognized at least one of the structural antigens tested, demonstrating that VLP-pulsed PBMC are able to reactivate and expand virion-antigen-specific CD4+ T cells.

**Highly efficient ex vivo expansion of EBV-specific CD4+ T cells using VLP-pulsed PBMC.** In a final set of experiments, we sought to compare the efficiency of expansion of EBV-specific CD4+ T cells following stimulation with either VLP-pulsed PBMC or LCL that had been cultured in medium supplemented with human serum. After six rounds of stimulation, the breadth of the T-cell response was assessed by analyzing T-cell receptor Vβ-chain expression. As shown in Fig. 7A, CD4+ T-cell lines from donor GB at passage six were oligoclonal when stimulated with VLP-pulsed PBMC but polyclonal when

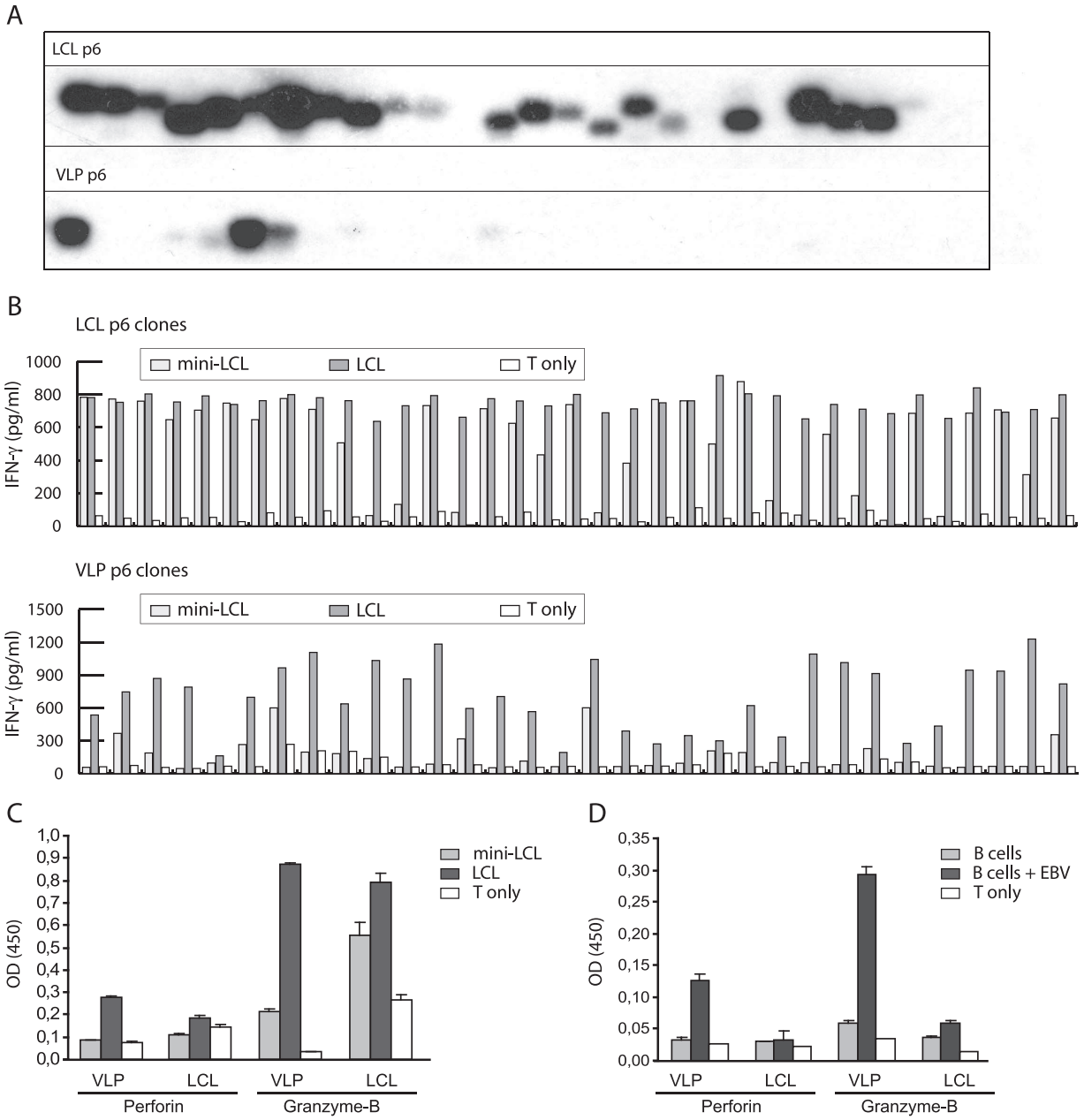


FIG. 7. Clonality and specificity of VLP- and LCL-stimulated $CD4^+$ T-cell lines. (A) Peripheral blood $CD4^+$ T cells from donor GB were stimulated six times with either autologous LCL-HS or VLP-pulsed autologous PBMC and analyzed for T-cell receptor Vβ expression by PCR, and then the PCR products were subjected to Southern blot hybridization. T-cell lines stimulated with VLP-pulsed PBMC were oligoclonal at this passage, while LCL-stimulated T-cell lines were still polyclonal. (B) $CD4^+$ T-cell clones were generated by limiting dilution from T-cell lines of donor GB at passage six after stimulation with either VLP-pulsed PBMC or LCL-HS. The specificity of the clones was determined by assessing their reactivity against autologous LCL and mini-LCL. After stimulation with VLP-pulsed PBMC, the majority of clones were specific for EBV lytic cycle proteins, as indicated by their exclusive reactivity against LCL. In contrast, most of the T-cell clones obtained from the LCL-stimulated T-cell line recognized both types of target cells and, thus, were not specific for EBV lytic cycle antigens. $CD4^+$ T-cell lines from donor JM were stimulated eight times with either LCL-HS (LCL) or VLP-pulsed PBMC (VLP) and subsequently tested for recognition of autologous LCL and mini-LCL (C) or primary B cells incubated with T EBV (D). Both T-cell populations displayed a similar cytolytic phenotype and secreted perforin and granzyme B upon antigen recognition. Importantly, B cells newly infected with EBV were recognized to a much larger extent by VLP than by LCL-stimulated T cells, suggesting that T-cell lines generated by stimulation with VLP-pulsed PBMC contain a higher proportion of virion antigen-specific T cells. These experiments were performed twice in two different donors with similar results. IFN-γ, gamma interferon; OD(450), optical density at 450 nm.

stimulated with LCL. To define the specificities of these T cells, the lines were cloned by limiting dilutions and outgrowing cultures tested for the recognition of autologous LCL versus autologous mini-LCL. Most of the clones that were derived from the T-cell lines stimulated with VLP-pulsed PBMC recognized EBV lytic cycle antigens as indicated by the selective recognition of LCL (Fig. 7B). In contrast, the majority of clones derived from LCL-stimulated T-cell lines reacted

against LCL and mini-LCL and therefore recognized non-lytic cycle antigens. Since the majority of endogenous latent EBV antigens are not efficiently presented on MHC-II (1, 22), these $CD4^+$ T cells most likely recognized cellular antigens. Similar results were obtained with T-cell lines from the two additional $EBV^+$ donors (data not shown). Thus, EBV-specific $CD4^+$ T cells were much more efficiently expanded when VLP-pulsed PBMC rather than LCL were used as stimulator cells. To test whether both T-cells populations were similarly able to contribute to the control of EBV infection, LCL- and VLP-stimulated T-cell lines were tested for their capacity to eliminate virus-infected target cells. Both T-cell populations were able to lyse LCL (data not shown), most likely by the granule exocytosis pathway because both lines secreted granzyme B and perforin upon antigen recognition. Whereas both lines efficiently recognized LCL, only LCL-stimulated lines secreted significant amounts of perforin and granzyme B in response to mini-LCL, which was probably a reflection of the autoreactive T-cell component in LCL-stimulated T-cell preparations (Fig. 7C). By contrast, when tested against freshly isolated primary B cells, only VLP-stimulated T cells recognized B cells that had been incubated for 24 h with WT EBV (Fig. 7D), suggesting that these lines are probably much more efficient in eliminating newly EBV-infected B cells and thus in controlling EBV infection.

## DISCUSSION

The recent identification of low levels of endogenous $CD4^+$ T cells as an important risk factor for the development of EBV-associated diseases in immunosuppressed patients (35) and of better clinical responses in patients with PTLD receiving EBV-specific T-cell lines that contained higher proportions of $CD4^+$ T cells (14) imply an important role for $CD4^+$ T cells in the control of EBV infection in vivo. EBV-specific $CD4^+$ T cells in LCL-stimulated T-cell preparations are almost exclusively directed against structural antigens of the virus (1), which are efficiently presented on MHC-II following the receptor-mediated uptake of released viral particles (2). These T cells are cytolytic and able to inhibit the outgrowth of LCL from newly EBV-infected B cells in vitro (15). Because the CD4/CD8 ratio in LCL-stimulated T-cell preparations is highly variable (36), this study aimed to define factors that affect the in vitro expansion of EBV structure antigen-specific $CD4^+$ T cells and to deduce strategies for the efficient ex vivo expansion of EBV-specific $CD4^+$ T cells for future use in the treatment of EBV-associated diseases.

As measured by lytic cycle protein expression, between 0 and 5% of cells in LCL cultures spontaneously become permissive for lytic viral replication (18). Within the group of LCL analyzed, the number of DNA-containing viral particles in cell culture supernatants varied about 40-fold, and these differences were reflected by variable levels of $CD4^+$ T-cell recognition, ranging from strong to undetectable. Thus, intrinsically low levels of virus production by LCL may compromise the expansion of lytic cycle antigen-specific $CD4^+$ T cells and contribute to the variable $CD4^+$ ratios in LCL-stimulated T-cell preparations.

The use of FCS as a medium supplement for culturing LCL for immunological applications has always been a major concern, since FCS-reactive $CD4^+$ T cells have been isolated from peripheral blood from many donors, but it remained unknown whether FCS-reactive T cells would constitute a significant proportion of the LCL-expanded $CD4^+$ T-cell population (24, 25). All $CD4^+$ T-cell lines that were repeatedly stimulated with LCL grown in FCS-supplemented media eventually showed FCS reactivity, even lines established from patients with acute infectious mononucleosis who are expected to have a strong antiviral T-cell response. Most likely, lytic cycle antigen-specific T cells are rapidly outnumbered by $CD4^+$ T cells that respond against FCS, probably because antigen is much more abundant. Although it is currently not known whether interindividual differences in FCS-specific $CD4^+$ T-cell precursor frequencies exist and contribute to the variable CD4/CD8 ratios in LCL-stimulated T-cell preparations, such T cells probably compromise the clinical efficacy of EBV-specific T-cell preparations by diminishing the number of virus-specific effectors. Xenogeneic immune responses are precluded when stimulator LCL are cultured in serum-free medium or medium supplemented with human serum, but despite new and improved medium formulations, the efficient propagation of LCL still requires the addition of serum (11). Owing to the high rate of EBV infestation in the adult population, human serum usually is derived from EBV-positive donors. Since EBV infection elicits strong humoral immune responses against many viral proteins, including glycoproteins essential for viral host cell adsorption and penetration (31), human serum may impair the uptake and subsequent presentation of virion antigens on MHC-II. In fact, serum from $EBV^+$ but not $EBV^-$ donors diminished T-cell recognition when B cells were pulsed with low titers of virus, suggesting that human serum impairs the presentation of virion antigens when viral particles become limiting, e.g., when virus production by stimulator LCL is intrinsically low or when LCL are treated with acyclovir to reduce virus production, as is currently performed in most clinical protocols (4, 32).

These findings indicated that the addition of excess amounts of EBV particles antagonizes the inhibitory effect of human serum and compensates for differences in virus production by different LCL, thereby facilitating the establishment of uniform and standardized stimulation conditions. Because the incubation of stimulator cells with WT EBV would pose an incalculable health risk to patients, the possibility of using genome-deficient EBV VLP was explored. EBV VLP produced by human cells in serum-free media are readily available in large quantities and transfer structural antigens as efficiently as WT EBV. Instead of LCL, PBMC pulsed with VLP were used as stimulators, because PBMC do not produce virus and are immediately available. Most importantly, the stimulation of $CD4^+$ T cells with LCL causes the expansion of virus-specific as well as autoreactive $CD4^+$ T cells (1, 12). Consequently, LCL-stimulated $CD4^+$ T-cell lines usually require 10 to 20 rounds of stimulation to become EBV specific (1). Because PBMC pulsed with VLP efficiently expanded virus-specific but not autoreactive $CD4^+$ T cells, EBV-specific $CD4^+$ T-cell lines already were obtained after four to six restimulations. Moreover, when tested against primary B cells that had been incubated with WT EBV, only VLP-stimulated T-cell preparations secreted substantial amounts of perforin and granzyme B, indicating that such

T-cell lines efficiently contribute to the control of EBV infection by eliminating cells newly infected with virus.

VLP-based vaccines have been used successfully to elicit immune responses against different viruses in vivo, most notably human papillomavirus (5, 38). Our results demonstrate that VLP also are useful for expanding the number of antigen-specific T cells in vitro, especially in those cases in which the number of known viral $CD4^+$ T-cell epitopes is too small to allow for peptide-based approaches.

Protective immunity against EBV probably requires $CD4^+$ and $CD8^+$ T-cell components. VLP-pulsed PBMC are unlikely to stimulate virus-specific $CD8^+$ T cells, because B cells are incapable of cross-presenting exogenous antigens on MHC-I (3) and because EBV-specific $CD8^+$ T cells barely target virion antigens (30). In order to obtain EBV-specific T-cell lines containing $CD4^+$ and $CD8^+$ components, the VLP stimulation approach may be combined either with conventional LCL stimulation protocols, such as by using acyclovir-treated LCL pulsed with VLP as stimulators, or with peptide stimulation approaches. The EBV-specific $CD8^+$ T-cell response is well characterized, and immunodominant epitopes have been defined for different HLA alleles (16, 21, 31). Using PBMC pulsed with these peptides as stimulators would obviate the lengthy procedure of generating LCL and significantly shorten the T-cell preparation process, which is critical due to the often rapid progression of PTLD. In addition, infusion of T-cells preparations enriched in disease-relevant specificities might improve the clinical efficacy of this adoptive T-cell therapy and hence the outcome of patients with PTLD.

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