Research Article

Broadening Specificity and Enhancing Cytotoxicity of Adoptive T Cells for Nasopharyngeal Carcinoma Immunotherapy

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Abstract

Although promising, clinical responses to adoptive immunotherapy for nasopharyngeal carcinoma (NPC) are still limited by the restricted number of Epstein–Barr virus (EBV) antigens that can be targeted and their poor immunogenicity. Our previous work indicated that the immunogenic features of the NPC-associated viral antigen BARF1 may be exploited for immunotherapeutic purposes. Nevertheless, T-cell lines obtained with current protocols include only negligible numbers of BARF1-specific cytotoxic T lymphocytes, pointing to the need to enrich these effectors in BARF1 specificities. Considering that in B lymphocytes BARF1 is mainly a lytic EBV antigen, we tested different EBV lytic-cycle inducers (TPA/butyric acid, doxorubicin, and cisplatin) used at suboptimal concentrations for their ability to upregulate BARF1 expression in lymphoblastoid B-cell lines (LCL), the commonly used antigen-presenting cells, without compromising their survival. The LCLs treated with doxorubicin (DX-LCL) can reproducibly and efficiently generate EBV-specific effectors enriched in BARF1 specificities from both healthy donors and NPC patients. These DX-LCLs also had more pronounced immunogenic properties, including HLA class I upregulation and expression of immunogenic cell death markers, such as enhanced calreticulin exposure and HMGB1 release. In particular, doxorubicin triggers an HMGB1 autocrine/paracrine loop with its receptor, TLR4, which is also upregulated in DX-LCLs and is responsible for NF-κB activation and a delayed apoptosis that allows a prolonged stimulation of EBV-specific T-cell precursors. This protocol may thus constitute a valid alternative to the use of engineered LCLs to generate EBV-specific T-cell lines for adoptive immunotherapy, being relatively simple, easily upgradable to Good Manufacturing Practice standards, and therefore more broadly applicable. Cancer Immunol Res; 4(5); 431–40. ©2016 AACR.

Introduction

Nasopharyngeal carcinoma (NPC) is a malignant tumor arising from the mucosal epithelium of the nasopharynx, which is characterized by a multifactorial etiology and a peculiar geographical distribution. In fact, NPC is relatively rare in most parts of the world, particularly in Europe and North America, while it has a high incidence in southern China and southeast Asia, where it constitutes a significant health problem (1, 2). NPC is characterized by several histopathologic entities, with the undifferentiated form being the most frequent (3, 4). The standard treatment for NPC is radiotherapy with or without chemotherapy, depending on disease stage. Although excellent control can be achieved with a combination of radiotherapy and chemotherapy, local recurrence still occurs in 5% to 10% of cases (3, 4), and prognosis of patients with metastatic disease remains poor (3, 4). Hence, new low-toxicity therapeutic strategies are strongly needed to improve the clinical control of NPC.

In addition to dietary, environmental, and genetic risk factors, this malignancy shows a strong association with Epstein–Barr virus (EBV), as supported by the observation that the EBV genome is monoclonal and detected in tumor cells of virtually all cases of NPC (5, 6). EBV infections in NPC cells are characterized by the so-called type II latency, with the expression of a limited set of latent proteins, including EBNA1, LMP-1, LMP-2, and BARF1, which may cooperatively contribute to EBV-driven epithelial cell transformation. These viral antigens are almost constantly, albeit heterogeneously, expressed by NPC cells and have become attractive targets for immunotherapy. Adoptive infusion of autologous EBV-specific T cells has recently emerged as an effective tool for the treatment of EBV-driven malignancies, and its efficacy in lymphoid tumors such as posttransplantation lymphoproliferative disease has been convincingly demonstrated (7). Phase I/II clinical studies have also provided evidence supporting the potential application of cytotoxic T lymphocyte (CTL)-based therapy to NPC patients, either as a single treatment for recurrent or metastatic disease or in combination with chemotherapy (7–10). Nevertheless, the rate of clinical responses obtainable in NPC patients by adoptive immunotherapy is unsatisfactory. These disappointing findings may be due to the limited set of viral antigens expressed by NPC cells, as well as to their poor immunogenicity, as shown by the subdominance of specific T-cell...
responses usually elicited by LMP-1 and LMP-2 (11). These limitations are stimulating the exploration of novel strategies to improve the efficacy of adoptive immunotherapy for NPC. Genetic manipulation of dendritic cells and lymphoblastoid cell lines (LCL), the conventional antigen-presenting cells (APC) used in current protocols, was recently shown to induce EBV-specific CTL cultures enriched in specificities for LMP antigens able to mediate enhanced clinical responses in patients with lymphoma (12), however, the applicability of such procedure is limited to very few specialized centers.

One of the possible strategies to overcome these limitations is the exploitation of additional viral proteins expressed by NPC cells, which may serve as tumor-associated antigens to be targeted for improved CTL induction and expansion. The oncogenic EBV protein BARF1 is expressed in the majority of NPC cases (13–16) and may constitute an attractive therapeutic target. In particular, we have previously demonstrated that NPC patients show strong spontaneous CD4+ and CD8+ T-cell responses against BARF1 protein and derived epitopes, indicating that BARF1 is a naturally immunogenic protein that may be targeted by specific CTLs (17). Indeed, we have also shown that BARF1-specific CTLs can be easily generated from EBV+ donors, an important prerequisite to exploit BARF1 immunogenicity for immunotherapeutic purposes. Nevertheless, EBV-specific T-cell lines generated by current protocols based on the use of autologous EBV+ LCLs as APCs show only very limited specificities for BARF1, if any (17).

On these grounds, we performed a study aiming at developing a new and broadly applicable protocol for adoptive immunotherapy of NPC, based on the generation of T-cell lines enriched in BARF1-specific effectors. Considering that in B lymphocytes BARF1 is mainly a lytic EBV antigen (18), we devised a strategy based on the use of low doses of EBV lytic cycle inducers to upregulate BARF1 in LCLs without inducing massive cell apoptosis or a complete EBV lytic replication, to allow these cells to effectively present BARF1 peptides together with other target epitopes. To these ends, we investigated different EBV lytic cycle inducers, such as TPA with butyric acid, doxorubicin, and cisplatin. After treatment, LCLs were γ-irradiated 80 Gy before the first stimulation, and 40 Gy before each other restimulation. IL2 (3 ng/mL) was added to the culture starting from day 14, and fresh medium was replaced every 3 days. Effector clones were cocultured with APCs at a 40:1 T-cell:LCL ratio.

**Materials and Methods**

**Cell lines and culture conditions**

The following cell lines, all obtained in 2010 to 2012, were used: c666.1 NPC (obtained from T. Ooka, Lyon; Granta-519 mantle cell lymphoma (ACC-342, DMSZ); T2-A2 (kind gift from M.G. Masucci, Stockholm); K562 (ACC-10, DMSZ); donor-derived EBV-infected LCLs, generated by in vitro transformation of B cells with the B95-8 EBV strain. Short tandem repeat (STR) loci for cell lines authentication were evaluated for all cell lines in our laboratory in 2011 and at the end of the study in 2014, by using the GenePrint10-System (Promega). All cell lines were cultured in RPMI-1640 (Gibco), containing 10% fetal bovine serum (Gibco), 2 mmol/L of l-glutamine, 100 μg/mL of streptomycin and 100 IU/mL of penicillin (Sigma), with the exception of Granta-519, cultured in complete Dulbecco’s Modified Eagle Medium (Cambrex). Donor- and patient-derived BARF1-specific CTL lines were cultured in CellGro GMP DC (CellGenix), supplemented with 100 μg/mL of streptomycin and 100 IU/mL of penicillin (Sigma). LCLs were seeded in complete RPMI-1640 medium (5 × 10^5 cells/mL) and treated with either 20 ng/mL of 12-O-tetradecanoyl-phorbol-1-acetate (TPA) and 5 μmol/L of sodium butyrate (NaB; Sigma) for 48 hours or doxorubicin, 25 mmol/L for 6 hours and cisplatin, 5 μmol/L for 6 hours. After doxorubicin and cisplatin treatment, cells were washed once and cultured in fresh complete medium for further 24 hours. Doxorubicin and cisplatin were provided by the anticancer drug unit of our institution.

**Generation of EBV-specific CTL lines**

Donor- and patient-derived BARF1-specific CTLs were generated and weekly restimulated using as APCs autologous LCLs treated or not with suboptimal concentrations of TPA + NaB, doxorubicin, or cisplatin. After treatment, LCLs were γ-irradiated 80 Gy before the first stimulation, and 40 Gy before each other restimulation. IL2 (3 ng/mL) was added to the culture starting from day 14, and fresh medium was replaced every 3 days. Effector clones were cocultured with APCs at a 40:1 T-cell:LCL ratio.

**Multispectral imaging flow cytometry**

The following fluorescent-conjugated monoclonal antibodies were used: anti–CD8-PC7 (mouse IgG1; clone SFC121Thy2D3, Beckman Coulter), anti–CD19-PE (mouse IgG1; clone HB19, eBioscience), anti–granzyme-β–FITC (mouse IgG1; clone GB11) (BD Pharmingen). To determine the T:APC conjugate formation (CD8+ T cells and autologous B-LCL) and to enumerate granyme-β–granules, a cytotoxicity assay was performed. Briefly, after 2 hours of coculture, autologous LCL + T cells (1.5 × 10^6 cells/conjugate) were stained with anti–CD8-PC7 and anti–CD19-PE antibodies in an appropriate volume of 10% rabbit serum and PBS. Then, cells were incubated with fixation/permeabilization buffer for 30 minutes at 4°C, washed twice, and labeled with anti–granyme-β–antibody in the presence of 2% rabbit serum in PBS at 4°C for 45 minutes and after two washes cells were resuspended in PBS with 1% paraformaldehyde. Cells were run on ImageStreamX cytometer using the INSPIRE software and images were analyzed using the IDEAS software (Aminis). Cells were excited with a 488-nm laser (30 mW intensity). Brightfield, side scatter, and fluorescent cell images were acquired at ×40 magnification. Only events with brightfield area >30 μm^2 (excluding debris) and nonsaturating pixels were collected. In T:APC binding experiments, 3 × 10^4 events were collected for each sample. Cells were gated for focused populations and doublets containing at least one T cell were gated from among all cells. Intracellular granyme-β granules formation was determined by subcellular localization and spot count experiments. Events (3,000) were collected for each sample. Cytoplasmic localization of the granules was measured using the IDEAS software “internalization algorithm,” defined as...
the ratio between intensity inside the cell and the intensity of the entire cell. The inside of the cell is defined by the "erosion mask" that fits the cell membrane. Cells containing small concentrated fluorescent spots have positive scores, whereas cells showing little/diffuse fluorescence have negative scores. Only viable cells were selected on the basis of morphology. Single-stained controls were used to compensate fluorescence between channel images on a pixel-by-pixel basis.

For patients and donors, generation of HLA-A*0201-expressing c666.1 NPC cells, quantitative real-time PCR, immunoblotting, cytotoxicity assays, HMGB1-release quantification, and TLR-4 neutralization experiments refer to Supplementary Material.

Results

Doxorubicin upregulates BARF1 mRNA expression

Different EBV lytic cycle inducers were used at suboptimal concentrations to induce a mainly abortive lytic cycle and upregulate BARF1 expression in LCLs without inducing major apoptotic effects. As a first step, we used the TPA + NaB combination as the most commonly used treatment to trigger EBV lytic reactivation (2, 21). Healthy donor–derived LCLs treated for 48 hours with TPA + NaB showed significantly increased BARF1 mRNA expression (2.5 mean fold-increase, \( P < 0.05 \); Fig. 1A). Nevertheless, the treatment also induced a broad upregulation of both latent (LMP-1, EBNA-1, EBNA-2) and lytic (ZEBRA, EA) EBV genes, together with a highly productive lytic cycle (Fig. 1A; Supplementary Fig. S1A). Doxorubicin-treated LCLs (DX-LCLs) showed a marked increase in BARF1 mRNA expression levels and a lower, but still significant (\( P < 0.05 \)), upregulation of other EBV genes without inducing a massive EBV lytic replication (Fig. 1A; Supplementary Fig. S1A). Notably, doxorubicin had less pronounced proapoptotic effects as compared with the other drugs investigated, as shown by PARP cleavage analysis (Fig. 1B).

Figure 1.

A, doxorubicin (DX) enhances BARF1 expression. qRT-PCR for EBV lytic and latent gene expression performed on LCLs treated with TPA + NaB, doxorubicin, or cisplatin (CSP). mRNA fold expression was normalized relative to control LCLs (Ctrl-LCLs). Mean of three independent experiments. **, \( P < 0.05 \) statistical significance for fold increase of BARF1 mRNA expression versus other EBV genes. \( *, P < 0.05 \) denotes statistical significance of fold increase versus Ctrl-LCLs (Student \( t \) test). B, DX-LCLs show a limited late apoptosis. PARP cleavage was assessed at the end of treatment. Whole-cell lysates (50 \( \mu \)g) were analyzed by immunoblotting for the indicated proteins; GAPDH was a loading control. DX-LCLs did not affect T-cell differentiation. C, differentiation status of CD3\(^+\)CD4\(^+\) and CD3\(^+\)CD8\(^+\) was assessed by analysis of CCR7 and CD45RA expression in early-stage cultures (day 10), and after the last stimulation (day 35). D, percentages of terminally differentiated (Temra, CCR7\(^+\)CD45RA\(^-\)), effector memory (EM, CCR7\(^+\)CD45RA\(^-\)), central memory (CM, CCR7\(^+\)CD45RA\(^-\)), and naive (CCR7\(^+\)CD45RA\(^-\)) are shown. Left histogram shows CD8\(^+\)/CD4\(^+\) ratio within CD3\(^+\) lymphocytes at day 35. Center and right histograms show CD4\(^+\) and CD8\(^+\) T-cell phenotypes, respectively. Mean of three independent experiments.
EBV-specific CTL differentiation is unaltered by DX-LCLs

EBV-specific CTL lines were generated by priming healthy donor-derived peripheral blood mononuclear cells (PBMC) with autologous LCLs either untreated or treated with TPA + NaB, doxorubicin, or cisplatin. CD8⁺/CD4⁺ ratio and T-cell differentiation profile were monitored by multiparametric flow cytometry analysis at days 10 and 35 (Fig. 1C). The percentage of CD8⁺ T cells ranged from 49% to 82% between the first and the last stimulation. Comparable increases in CD8⁺ T cells were observed at day 35, (67%–77%; C6/C7%), whereas CD4⁺ cells ranged between 6% and 11% (±5%; Fig. 1D). Combined analysis of CCR7 and CD45RA demonstrated that 80% to 90% of CD8⁺ and 69% to 77% of CD4⁺ T cells displayed an effector memory phenotype (EM, CCR7⁺CD45RA⁻; Fig. 1D). Analysis of additional EM markers (CD28 and CD62L) demonstrated a high percentage of CD28⁺CD8⁺ T cells in all cultures, indicating that CD8⁺ T cells are not exhausted. About 50% to 60% of CD8⁺ T cells also express the CD62L L-selectin. Analysis of CTL cultures obtained from four different donors did not reveal any significant difference in the distribution of CD28 and CD62L in the CCR7⁻/CD45RA⁻CD8⁺ EM population (Supplementary Fig. S1B). Similar findings were observed for CD4⁺ T cells (not shown). Therefore, no significant differences were observed with regard to the differentiation profile of the four CTL cultures.

CTLs generated with doxorubicin-treated LCLs kill T2-A2 cells with exogenous or endogenous BARF1-peptides

EBV-specific CTL cultures were tested for their ability to recognize and kill T2-A2 target cells loaded with known EBV-derived peptides in a HLA-A*0201–restricted fashion (Fig. 2A and B). In particular, T2-A2 cells were loaded with two BARF1-derived peptides, p23 and p49 (17), or with the LMP-1–derived YLQ peptide (11). CTLs generated with doxorubicin-treated LCLs (DX-LCLs) were able to specifically kill both BARF1- and LMP-1–loaded T2-A2 inducing a higher percentage of specific lysis as compared with CTLs obtained with TPA + NaB- or cisplatin-treated LCLs (CSP-LCLs; Fig. 2B). CTL responses against EBNA-1 (FMV) and LMP-2 (CLG) EBV peptide epitopes were also investigated. While LMP-2 targets were efficiently recognized by both DX- and cisplatin-CTLs (CSP-CTL; P < 0.05), but not by CTRL- or TPA + NaB-CTLs,

Figure 2.

A, DX-CTLs specifically kill T2-A2 cells loaded with BARF1-derived peptides. Induction of BARF1 (p23 and p49) and LMP1 (YLQ) peptide-specific T-cell responses from three representative out of five HLA-A*0201⁺ donors (20:1 Effector:target (E:T) ratio). Specific lysis was calculated by subtracting the lysis of empty T2-A2 cells. B, box plot representing cytolysis assay performed on T2-A2 cells loaded with BARF1 or LMP-1 peptides. *, P ≤ 0.05 versus the other CTL lines in experiments carried out on 5 independent HLA-A*0201⁺ donors. C, DX-CTLs show high specific killing against BARF1-endogenously expressing tumor cells. Cytotoxic activity of CTLs derived from two representative out of five HLA-A*0201⁺ donors against c666.1-Wt (negative control) and c666.1-A2, Granta-519, and K562 (specificity control) cells (20:1 E:T ratio). HLA-A*0201 restriction was confirmed with the antibody to HLA-A*0201 cr11.351. D, box plot of cytolysis assay results from CTL cultures of five independent HLA-A*0201⁺ donors tested against BARF1-endogenously expressing tumor cell lines (*, P ≤ 0.05 versus other targets; **, P ≤ 0.05 versus other targets; ***, P ≤ 0.05 versus other targets).
FMV-loaded cells were lysed at comparable levels by all different CTL cultures investigated (Supplementary Fig. S1C). To assess the ability of DX-CTLs to specifically recognize and kill NPC cells spontaneously expressing EBV antigens, the HLA-A–negative NPC c666.1 cell line was engineered to stably express HLA-A/B2701 (c666.1-A2; Supplementary Fig. S2A). As shown in Fig. 2C and D, DX-CTLs were more efficient and specific in killing NPC cells expressing BARF1 endogenously, whereas no killing was observed against the parental, HLA-A–negative c666.1-Wt cells. Conversely, control, TPA+NaB– and CSP-CTLs induced only low levels of specific lysis against c666.1-A2 (Fig. 2D). The limited extent of K652 cell killing further ensured the specificity of lysis (Fig. 2E).

EBV-specific cytotoxic activity was also assessed by enumerating the formation of LCL–T cell doublets by multispectral imaging flow cytometry. After incubation of effector T cells with peptide-pulsed autologous LCLs, we observed that DX-CTLs and, particularly, BARF1 peptide–loaded LCLs generated the highest frequency of effector–target doublets (Fig. 3A, *P ≤ 0.05 DX-CTLs vs. all other CTL cultures). Stimulation with the LMP-1 YLQ peptide elicited comparable numbers of doublets in all cultures except for CTLs generated with untreated LCLs, which showed a lower number of doublets (Fig. 3A).

In the same experimental conditions, the intracellular content of granzyme-β granules was evaluated in all CTL cultures by spot-counting analysis. As shown in Fig. 3B, if compared with CTRL-, TPA+NaB– and CSP-CTLs, a significantly higher absolute number of granzyme-β granules was detected in DX-CTLs (P ≤ 0.05) stimulated with empty LCLs or with LCLs pulsed with viral peptides, indicating that DX-LCLs are generally more efficiently armed (see the scale of different graphs in Fig. 3B). Notably, DX-CTLs stimulated with BARF1 peptide–pulsed LCLs showed an even higher number of granzyme-β granules as compared with those stimulated with LCLs loaded with LMP-1 peptides (P ≤ 0.05; Fig. 3B).

Doxorubicin enhances LCL immunogenicity

To characterize the mechanisms underlying the enhanced functional properties of DX-LCLs as APCs, we investigated the possible ability of doxorubicin to upregulate HLA class I expression. Considering that γ-irradiation is currently used to inactivate LCLs
before CTL stimulation and that this procedure was shown to increase HLA expression (22, 23), we investigated the possible synergism between doxorubicin and \( g \)-irradiation. HLA-A\(^{0201} \) mRNA expression was therefore monitored by qRT-PCR in LCLs before and 24 hours after \( g \)-irradiation (Fig. 4A). DX-LCLs revealed a marked increase in HLA-A\(^{0201} \) expression (mean \( = 2.3 \) fold), an effect that persisted also after 24 hours (Fig. 4A). No significant changes were detected in TPA\(+\)NaB– and CSP-LCLs compared with control LCLs (mean \( = 1.3- \) and 1.0-fold expression, respectively) at both time points. HLA-A\(^{0201} \) upregulation in DX-LCLs was also confirmed by flow cytometry analysis at 24 hours after \( g \)-irradiation (Fig. 4B).

Recent evidence indicates that doxorubicin is able to induce immunogenic cell death (ICD) both in vitro (24) and in vivo (25, 26), an effect mainly mediated by damage-associated molecular patterns (DAMP), including cell surface–exposed calreticulin (CRT), secreted ATP, and released high mobility group box 1 protein (HMGB1; ref. 27). We therefore explored the possibility that doxorubicin treatment may induce the expression of molecules potentially able to enhance LCL immunogenicity, even at doses inducing only a limited cell death. Analysis of critical ICD markers demonstrated that doxorubicin induces an early (28) CRT exposure (3 hours), with about 50% of cells displaying a CRT membrane localization. Conversely, only slight increases in CRT exposure were observed in TPA + NaB– and CSP-LCLs as compared with control LCLs (Fig. 4B). Release of HMGB1 in LCL culture medium was measured by ELISA at the end of treatment as a late ICD marker (ref. 29; Fig. 4C). Notably, the extent of HMGB1 release by DX-LCLs was about 3-fold higher than that of control LCLs, whereas a moderate decrease was observed in both TPA + NaB– and CSP-LCLs (about 0.8-fold change).

To verify whether the observed ICD effects persisted or were enhanced upon \( g \)-irradiation, expression of the HSP70 and HSP90 proteins was assessed by immunoblotting immediately after \( g \)-irradiation (t0) and 24 hours after \( g \)-irradiation (t24; ref. 30; Fig. 4D). Notably, in DX-LCLs, HSP protein levels are unchanged after \( g \)-irradiation and show a marked increase at t24. On the contrary, expression levels of both HSP70 and HSP90 proteins decrease in TPA\(+\)NaB– and CSP-LCLs at t0 and t24, respectively (Fig. 4D). To verify whether the enhanced HMGB1 release showed by DX-LCLs could trigger receptor–dependent responses in an autocrine/paracrine fashion, we evaluated the expression of its functional receptor, TLR4, and of its downstream adaptor molecule Myeloid Differentiation primary response 88 (MyD88; ref. 26). Flow cytometry analysis showed that doxorubicin and cisplatin markedly increased the number of TLR4-expressing cells (63.3% and 54.4%, respectively) compared with control LCLs (38.1% of positive cells), whereas TPA + NaB

![Figure 4.](image-url)

A, doxorubicin (DX) upregulates HLA-A\(^{0201} \) expression in LCLs. Relative quantitation by qRT-PCR of HLA-A\(^{0201} \) mRNA expression in LCLs before (NI, not irradiated; \( t = 0 \)) and 24 hours after \( g \)-irradiation (t24). Data are normalized relative to Ctrl-LCLs. B, HLA-A\(^{02} \), CRT, and TLR4 cell-surface expression is increased in DX-LCLs. Expression of HLA-A\(^{02} \) (top) and TLR4 (bottom) was analyzed by flow cytometry at t24, whereas CRT expression (center) was investigated after 3 hours. Isotype control (dashed line), Ctrl-LCLs (gray area), and treated-LCLs (black line) are shown. C, doxorubicin increases HMGB1 release. Histograms represent the normalized fold increase of HMGB1 released by treated versus untreated LCLs. Experiments were performed in triplicate and done at least three times on different donor-derived LCLs. D, doxorubicin upregulates HSP-70, HSP-90, and MyD88. Analysis was performed with PARP cleavage immediately after \( g \)-irradiation (t0), and 24 hours after \( g \)-irradiation (t24). GAPDH shows equal protein loading. E and F, doxorubicin activates NF-\( \kappa \)B and delays apoptosis onset. Expression of p50, p65, phospho-p65, MyD88, and cleaved caspase-3 was evaluated at the end of doxorubicin treatment; cleaved PARP was assessed at the end of doxorubicin treatment and at 48 hours.
only slightly affected TLR4 expression (Fig. 4B). In addition, immunoblotting analysis revealed that doxorubicin treatment upregulates MyD88 expression in LCLs at t0, an effect much more evident at t24 (Fig. 4D), whereas a significant decrease in MyD88 protein levels was observed in TPA+NaB- and CSP-LCLs at t0, which became almost undetectable at t24 (Fig. 4D). Moreover, PARP cleavage analysis demonstrated that the late phases of the apoptotic cascade in DX-LCLs occurred only 24 hours after γ-irradiation, whereas a cleaved PARP is already detectable at t0 in TPA+NaB- and CSP-LCLs, which is almost completely fragmented at t24. To assess the possible role of the TLR4/MyD88 pathway in the delayed onset of apoptosis observed for DX-LCLs, we investigated caspase-3 and PARP cleavage, MyD88 expression, and NF-kB activation in DX-LCLs in the presence or absence of a TLR4-neutralizing antibody. Interestingly, the presence of the anti-TLR4 antibody was able to counteract not only the doxorubicin-dependent MyD88 upregulation, but also the activation of some downstream transcription factors, such as NF-kB, as shown by analysis of the expression of the NF-kB p50 and phospho-p65 subunits (Fig. 4E). Furthermore, a higher expression of cleaved caspase-3 together with slightly decreased levels of the full-length PARP form was observed in cells exposed to doxorubicin for 24 hours as compared with doxorubicin-only LCLs, consistently with a delayed onset of the apoptotic cascade in the presence of a functional TLR4-dependent signaling (Fig. 4E). It is noteworthy that a significant increase of PARP cleavage was observed only 48 hours after doxorubicin exposure, an effect that was markedly more evident in the presence of an anti-TLR4 antibody, supporting a role for TLR4-dependent signaling in slowly sustaining LCL survival after doxorubicin treatment (Fig. 4E and F).

Lytic activity of DX-CTLs generated from NPC patients

To validate our results in the NPC setting, we verified whether doxorubicin was also able to enhance the immunogenicity of LCLs generated from NPC patients. As for healthy donor-derived LCLs, doxorubicin treatment significantly upregulated BARF1 and LMP-1 mRNA levels (Supplementary Fig. S2B), and enhanced HLA-A′0201, TLR4 expression, and CRT exposure also in LCLs derived from four different NPC patients (Supplementary Fig. S2C). Furthermore, a strong upregulation of the ICD markers HSP70, HSP90, and MyD88 was observed in DX-LCLs from NPC patients at t0 (Supplementary Fig. S2D). In addition, HMGB1 release was also significantly increased by doxorubicin in patient-derived LCLs as compared with untreated LCLs (Supplementary Fig. S2E). To assess the ability of DX-LCLs to efficiently prime and induce EBV-specific effector cells, we generated CTLs from four different NPC patients using autologous untreated and DX-LCLs as stimulators. Cytotoxicity assays were performed using T2-A2 cells loaded with BARF1- or LMP-1-derived peptides. As shown in Fig. 5, DX-CTLs displayed higher specificity and cytotoxic activity to BARF1 peptides compared with CTLs induced by untreated LCLs (20%–40% and 10%–20% specific lysis, respectively). Notably, DX-CTLs from NPC patients also showed an enhanced killing ability against LMP-1-loaded targets (Fig. 5A), further supporting the enhanced immunogenic features of DX-LCLs. More importantly, DX-CTLs were able to recognize and specifically kill tumor cells expressing BARF1 endogenously, in particular the c666.1-A2 NPC cells and Granta 519 lymphoma cells (50%–60% and 20%–30% specific lysis, respectively; Fig. 5B).

Discussion

In the present study, we report the development of a new and broadly applicable protocol allowing an efficient generation and expansion of EBV-specific CTLs enriched in specificities for BARF1, to be used for adoptive immunotherapy of NPC. Exploitation of the immunogenicity of this viral protein constitutes a relevant improvement of current protocols, which were so far designed to target mainly LMP-1 and LMP-2 proteins, but not BARF1. Indeed, T-cell cultures generated with conventional protocols are virtually devoid of BARF1-specific effectors. Considering that BARF1 is expressed by the majority of NPCs from both endemic and nonendemic areas (13–16), the availability of a protocol able to

![Figure 5](https://example.com/figure5.png)

Figure 5.
DX-CTLs elicit strong EBV-specific responses to both BARF1-and LMP-1-derived peptides, and to BARF1 endogenously expressing NPC cells. A, peptide-specific responses of CTLs generated by DX-LCLs from 2 HLA-A′0201 NPC patients. Cytotoxicity assays were performed against T2-A2 cells loaded with the indicated peptides (20:1 effector:target ratio); specificity was obtained by subtracting empty T2-A2 lysis. B, cytotoxic activity of patient-derived CTLs against the c666.1-Wt, c666.1-A2, Granta-519, and K562 cells. HLA-A′0201 restriction was confirmed with the cr11.351 antibody (not shown). Granta-519 cells may be more variably lysed and generally to a lesser extent than c666.1-A2, consistently with the nonconstitutive and markedly reduced (about 3–4-fold, not shown) expression of BARF1 in these cells.
generate EBV-specific CTLs efficiently targeting BARF1 may also enhance the still unsatisfactory rates of clinical responses obtainable by adoptive immunotherapy in this setting (9, 10).

Our protocol exploits the properties of doxorubicin, a commonly used antineoplastic drug, to promote an abortive EBV reactivation and induce ICD in treated LCLs (31, 32). In fact, we demonstrate that LCL treatment with subcytotoxic concentrations of doxorubicin is able to upregulate BARF1 expression without inducing massive EBV lytic replication, while preserving the expression of other relevant EBV latency targets, particularly LMP-1. Consistently, DX-LCLs efficiently generate and expand T-cell effectors able to specifically recognize and kill both autologous targets presenting BARF1-derived epitopes and NPC cells endogenously expressing BARF1 in an HLA class I–restricted fashion. Notably, DX-CTLs not only retain the ability to recognize subdominant LMP-1 epitopes, but also show an increased lytic efficiency against LMP-1–expressing targets as compared with effectors generated by conventional protocols.

Interesting insights were obtained by counting granzyme-B granules in EBV-specific T cells, which demonstrated that DX-CTLs include a substantial fraction of effectors readily activated and “armed” upon stimulation with BARF1 epitope peptides. Intriguingly, also TPA + NaB– or CSP-CTLs displayed a relatively higher number of granzyme-B–producing T cells when these effectors were cultured in the presence of BARF1-loaded LCLs, consistent with the relative immunodominance of BARF1 with respect to LMP-1. Moreover, in the presence of BARF1-presenting targets, DX-CTLs formed the highest number of doublets with target LCLs. These findings together support the conclusion that DX-CTLs are highly activated effectors with a broader and more efficient killing potential as compared with T-cell cultures generated with untreated, TPA + NaB– or CSP-LCLs.

The increased ability of DX-LCLs to generate EBV-specific CTLs with higher efficiency could be at least in part related to the prolonged survival of LCLs as compared with those treated with other lytic cycle inducers. Notably, persistence of live DX-LCLs in cocultures with autologous T lymphocytes is comparable with that of untreated LCLs, suggesting that treatment with subcytotoxic doses of doxorubicin preserves the LCL integrity necessary for an optimal interaction with EBV-specific T-cell precursors. Nevertheless, our results also indicate that other mechanisms are probably involved in the increased immunogenicity of DX-LCLs. In this respect, our finding that only DX-LCLs show increased HLA-A*0201 mRNA and protein levels is of particular relevance considering the central role of these molecules in antigen presentation. Intriguingly, nanomolar concentrations of doxorubicin were shown to induce a complex protein ubiquitination response without inhibiting proteasome activities (33), suggesting that the resulting intracellular accumulation of damaged proteins may induce quantitative and/or qualitative changes in the immunogenetic epitope repertoire. Our results are therefore consistent with the possibility that DX-LCLs have enhanced immunogenic properties also because of their ability to induce a more efficient and prolonged presentation of tumor-associated antigen epitopes. Considering that doxorubicin is able to induce ICD (34–36), we explored the hypothesis that doxorubicin could induce functional changes presumably responsible for the enhanced immunogenic features of LCLs even at doses unable to induce early apoptotic effects. Consistent with the ability of doxorubicin to modulate the expression/release of DAMPs both in vitro and in vivo (26), we demonstrate that only doxorubicin was able to elicit an early CRT exposure at LCL surface, one of the functional hallmarks of ICD (35, 37). In the setting of ICD, CRT serves as an “eat me” signal and promotes the immunogenicity of dying tumor cells. In line with the notion that CRT exposure is an early event in ICD, doxorubicin-enhanced CRT exposure was observed at a time point when no evidence of overt apoptosis was present. Extracellular HMGB1 is another critical mediator of ICD, being released by cells in response to various stimuli, including proapoptotic drugs (25, 35, 38), and exerting functional hallmarks of ICD (35, 37). In the setting of ICD, CRT serves as an “eat me” signal and promotes the immunogenicity of dying tumor cells. In line with the notion that CRT exposure is an early event in ICD, doxorubicin-enhanced CRT exposure was observed at a time point when no evidence of overt apoptosis was present. Extracellular HMGB1 is another critical mediator of ICD, being released by cells in response to various stimuli, including proapoptotic drugs (25, 35, 38), and exerting

Figure 6. Proposed mechanisms underlying the enhanced immunogenicity of DX-induced LCLs. Doxorubicin (DX) acts at different levels to improve the function of LCLs as APCs for the generation of EBV-specific CTLs. Doxorubicin upregulates HLA-A*02 surface expression and induces ICD, as shown by enhanced CRT surface exposure and HMGB1 release. Doxorubicin also upregulates the expression of TLR4, thus activating an autocrine/paracrine HMGB1–TLR4 loop that enhances LCL survival through NF-κB activation. These survival signals counteract for a certain period of time the proapoptotic signals induced by the drug, resulting in a delayed apoptosis of DX-LCLs, which may thus have prolonged interactions with EBV-specific T-cell precursors. Globally, these effects result in enhanced stimulation and activation of T cells, which also show the ability to specifically recognize and kill BARF1–expressing tumor cells.
direct effects on CD4+ T cells promoting Th1 polarization, T-cell expansion, and survival. Nonetheless, HMGB1 may also act indirectly by inducing functional changes on APCs. In particular, it has been shown that HMGB1 released by damaged or apoptotic cells may bind to TLR4 on dendritic cells, thereby mediating downstream signaling through MyD88, suggesting the occurrence of an autocrine/paracrine loop in the in vitro LCL culture. This local stimulation leads to enhanced NF-κB activation, as shown by upregulation of the p50 subunit and enhanced levels of the p65 phosphorylated form. Notably, experiments carried out with a TLR4-neutralizing antibody demonstrated that both the NF-κB activation and the delayed onset of apoptosis observed in DX-LCLs, but not in TPA + NaB- or CSP-LCLs are dependent on TLR4 triggering. On these grounds, our results support the possibility that the enhanced HMGB1 release induced by doxorubicin may result in the activation of an autocrine/paracrine loop in LCLs able to promote a prolonged survival and an enhanced immunogenicity of these APCs (Fig. 6).

We have also investigated the effects of doxorubicin on the expression of HSP70 and HSP90, key chaperone molecules playing important roles in signaling, protein function, trafficking, and turnover (39), and being also functionally involved in protein and epitope folding. Our findings indicate that only doxorubicin, but not TPA + NaB- or cisplatin, induces a marked upregulation of these proteins, even in the absence of overt apoptosis. This may be an additional mechanism underlying the enhanced immunogenicity of DX-LCLs and their antigens.

One of the major advantages of our innovative protocol resides in its easy upgradability to GMP standards, considering that no molecular engineering of cells is required, and that doxorubicin is a drug already and broadly used in the clinics. It is noteworthy that our protocol does not alter the differentiation phenotype of EBV-specific CTls that are generated and showed a high efficiency and reproducibility also when PBMCs from NPC patients were used. These features are relevant to ensure the feasibility and the broad applicability of our protocol. We are currently investigating whether the broader antigen specificity of DX-CTLs is not restricted to viral proteins but also involves cellular antigens whose targeting may further enhance the therapeutic potential of these effectors.

In conclusion, our results provide the rationale to investigate the safety and efficacy of DX-LCL–induced EBV-specific CTls in a phase I/II clinical study of adoptive immunotherapy for NPC patients. Our final goal is to assess whether our protocol is able to enhance the rate of clinical responses to adoptive immunotherapy in NPC patients, particularly for those with relapsed or refractory disease.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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