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Virus-Specific Cytotoxic CD4+ T Cells for the Treatment of EBV-Related Tumors

Anna Merlo,* Riccardo Turrini,* Sara Bobisse,* Rita Zamarchi,† Rita Alaggio,‡ Riccardo Dolcetti,§ Josef Mauritner,¶ Paola Zanovello,*† Alberto Amadori,*† and Antonio Rosato*,†

Although adoptive immunotherapy with CD8+ CTL is providing clinically relevant results against EBV-driven malignancies, the effector role of CD4+ T cells has been poorly investigated. We addressed this issue in a lymphoblastoid cell line-induced mouse model of posttransplant lymphoproliferative disease (PTLD) by comparing the therapeutic efficacy of EBV-specific CD4+ and CD8+ T cell lines upon adoptive transfer. CD4+ T cells disclosed a long-lasting and stronger proliferative potential than CD8+ T cells, had a similar activation and differentiation marker profile, efficiently killed their targets in a MHC class II-restricted manner, and displayed a lytic machinery comparable to that of cognate CD8+ T cells. A detailed analysis of Ag specificity revealed that CD4+ T cells potentially target EBV early lytic cycle proteins. Nonetheless, when assessed for the relative therapeutic impact after in vivo transfer, CD4+ T cells showed a reduced activity compared with the CD8+ CTL counterpart. This feature was apparently due to a strong and selective downmodulation of MHC class II expression on the tumor cells surface, a phenomenon that could be reverted by the demethylating agent 5-aza-2’-deoxycytidine, thus leading to restoration of lymphoblastoid cell line recognition and killing by CD4+ T cells, as well as to a more pronounced therapeutic activity. Conversely, immunohistochemical analysis disclosed that HLA-II expression is fully retained in human PTLD samples. Our data indicate that EBV-specific cytotoxic CD4+ T cells are therapeutic in mice bearing PTLD-like tumors, even in the absence of CD8+ T cells. These findings pave the way to use cultures of pure CD4+ T cells in immunotherapeutic approaches for EBV-related malignancies. The Journal of Immunology, 2010, 184: 5895–5902.

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pstein-Barr virus is a human gammaherpesvirus characterzied by a marked B cell tropism. After primary infection that elicits a strong immune response, EBV establishes a strict latency in memory B cells where the virus resides lifelong (1). Alterations in the delicate balance between the transforming properties of EBV and the host immune control can directly lead or contribute to the development of B cell lymphomas, such as posttransplant lymphoproliferative disease (PTLD), Burkitt’s lymphoma and Hodgkin’s lymphoma (HL) (2), and other tumors (T cell and NK cell lymphomas, nasopharyngeal carcinoma, and gastric carcinoma) (3). Each EBV-associated malignancy is characterized by a distinct pattern of viral protein expression (3).

*Department of Oncology and Surgical Sciences and †Department of Pathology, University of Padova; ‡Istituto Oncologico Veneto, Istituto di Ricovero e Cura a CarattereScientifico, Padova; VR, Centro di Riferimento Oncologico, Istituto di Ricovero e Cura a CarattereScientifico, Aviano, Pordenone, Italy; and §Department of Pediatrics, Technische Universität München and Helmholtz Zentrum München, German Research Center for Environmental Health, Munich, Germany

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Address correspondence and reprint requests to Dr. Antonio Rosato, Department of Oncology and Surgical Sciences, University of Padova, Via Gattamelata, 64, I-35128 Padova, Italy. E-mail address: antonio.rosato@unipd.it

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Abbreviations used in this paper: BFA, brefeldin A; CMA, concanamycin A; decitabine, 5-aza-2’-deoxycytidine; EBNA, EBV-encoded nuclear Ag; FasL, Fas ligand; HL, Hodgkin’s lymphoma; LCL, lymphoblastoid cell line; MHC-II, MHC class II; PTLD, posttransplant lymphoproliferative disease; recombinant human; VLP, virus-like particle.

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The presence of viral Ags in malignant cells makes them a suitable target for immunotherapeutic approaches. Indeed, adoptive transfer of EBV-specific CTLs has proved to be safe and effective in both prophylaxis and treatment of PTLD (4, 5). The culture protocols currently used allow the generation of bulk populations, predominantly composed of CD8+ T cells, with a well-defined hierarchy of immunodominance in terms of the recognized viral epitopes (6).

On the basis of the encouraging results obtained in PTLD management, EBV-specific CTLs have been also used in nasopharyngeal carcinoma and HL patients with some good clinical outcomes, despite the restricted pattern of viral protein expression (7–9). This approach, however, cannot be applied to latency type I EBV-related malignancies, where only EBV-encoded nuclear Ag (EBNA1) is expressed. This protein, as a result of a glycline-alanine repeat that strongly impairs its processing and presentation in the context of MHC class I molecules, is poorly recognized by CD8+ CTls (10), except after cross-priming by professional APcs (11). By contrast, EBNA1 is naturally processed and presented in the context of MHC class II (MHC-II) molecules and can be recognized by CD4+ T cells as the immunodominant latent target (12, 13).

Thus far, CD4+ T cells have been traditionally viewed as indirect effectors, involved in priming antitumor responses, in maintaining function and growth of CD8+ T cells, and in orchestrating innate immunity, mainly through cytokine release (14). A more direct role for CD4+ T cells has been documented in mouse models, where CD4+ T cells mediate Fas-Fas ligand (FasL) or TRAIL/TRAIL receptor interactions and IFN-γ release as effector mechanisms (15). In contrast, these functions do not rely only on a direct recognition of target cells, because they are exerted also against cells lacking MHC-II expression (16). Nonetheless, CD4+ T cells can also exert a direct cytotoxic activity, even though most of available data deal with in vitro responses of clonal cultures (17, 18).
Although this feature was long considered an in vitro artifact, such an assumption has been overcome by the detection of cytotoxic CD4^+ T cells directly ex vivo, especially in the context of viral diseases (19). However, the physiological role and the potential contribution of these “new” effector cells to antitumor immunity and to adoptive immunotherapy are still obscure.

We aimed at elucidating whether EBV-specific CD4^+ T cells are per se able to induce effective therapeutic responses in a preclinical model and therefore should be included in adoptive therapy protocols. This is a relevant issue if we consider that, besides the above-mentioned data that EBV-specific CD4^+ T cells could be endowed of cytotoxic potential (17) and that a B cell lymphoma expresses HLA-II molecules, a higher percentage of CD4^+ T cells in the infusates was shown to correlate with a better outcome in PTLD patients (5).

We demonstrated that bulk cultures of virus-specific CD4^+ T cells exert a strong and specific MHC-II–restricted in vitro lytic activity mediated by cytotoxic granule exocytosis. Surprisingly, however, they showed a reduced in vivo therapeutic activity against lymphoblastoid cell lines (LCLs)-induced PTLD in SCID mice, as compared with the EBV-specific CD8^+ CTL counterpart obtained from the same donors. This feature is apparently due to a selective downmodulation of HLA-DR expression on the LCL after in vivo transfer, a phenomenon that could be reverted by epigenetic pharmacological treatment, leading to the restoration of LCL recognition and killing by CD4^+ T cells. As immunohistochemical analysis disclosed that HLA-II expression is fully retained in human PTLD, adoptive immunotherapy with CD4^+ CTLs could be explored as a potential new approach against EBV-associated malignancies.

**Materials and Methods**

**LCLs and EBV-specific CD4^+ and CD8^+ T cell lines**

EBV-transformed LCL and mini-LCL were generated by infection of PBMCs from HLA-typed EBV-seropositive healthy donors with culture supernatant from the EBV-producing marmoset cell line B95.8 (American Type Culture Collection, Manassas, VA) or with the mini-EBV strain (20), respectively, and maintained in complete RPMI 1640 medium (Euroclone, Pero, Milano, Italy) supplemented with 10% type AB Human Serum (Lonza BioWhittaker, Basel, Switzerland). Where indicated, LCLs were treated for at least 2 wk with 200 μM acyclovir (Sigma-Aldrich, St. Louis, MO). T cells lines were established using LCLs as stimulator cells, as described previously (4). After two to three stimulations, CD4^+ T cells were sorted using the CD4^+ T cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany), and both CD8^+ and CD4^+ T cells were cultured in parallel.

**Generation of alloreactive cultures**

Alloreactive cultures were established using LCL or mini-LCL as stimulator cells. Briefly, we seeded 2 x 10^6 PBMCs with irradiated allogenic LCL or mini-LCL at a responder to stimulator ratio of 4:1 in a 24-well plate in human serum complete medium supplemented with 10% U/ml recombinant human (rh)IL-2 (Proleukin; Chiron, Emeryville, CA). IL-2 was replenished twice weekly. Alloreactive cultures were restimulated with LCL or mini-LCL once a week. Cytotoxicity test was performed after three rounds of restimulation.

**Cytotoxicity assay**

Cytotoxicity assay of CD4^+ and CD8^+ T cells was determined in a standard 4-h 51Cr release assay, as reported previously (21). Autologous LCL, mini-LCL, PHA blasts, and K562 cell line were used as target cells. Where indicated, T cells were pretreated for 2 hr at 37°C with either 20 μM brefeldin A (BFA) or 100 nM concanavilin A (CMA; both from Sigma-Aldrich) and assayed in the presence of the drugs. To assess calcium dependence of cytolytic activity, 4 mM EGTA (Sigma-Aldrich) was added to the assay. For Ab blocking experiments, target cells were preincubated for 30 min at room temperature with 40 μg/ml HLA-I–specific mAb (W6/32) or HLA-II–specific mAb (Ti39; BD Pharamingen, San Diego, CA), whereas T cells were preincubated with 10 μg/ml anti-FasL mAb (NOK-1; BioLegend, San Diego, CA).

**Flow cytometry**

FITC- or PE-conjugated mAb against the following human Ags were used: CD4, CD8, CD25, CD38, CD69, TCR-αβ (WT31), TCR-γδ, and HLA-DR (L243) (BD Biosciences, San Jose, CA); CD3, CD16, and CD56 (BD Pharmingen); CD45RA and CD45RO (Caltag Laboratories, Burlingame, CA); CD19 (Coulter Clone, Beckman Coulter, Galway, Ireland); and HLA-ABC (W6/32; BioLegend). Intracellular cytokine staining was performed using the Cytofix/CytoPerm Plus Fixation/Permeabilization kit (with BD GolgiStop; BD Biosciences), anti-IFN-γ, anti–TNF-α mAb (IOTest; Immunotech/Beckman Coulter, Marseille Cedex, France), and anti-granulysin mAb (RF10; MBL). Cells were analyzed using an EPICS XL (Beckman Coulter) flow cytometer, and data were evaluated with FlowJo software (TreeStar, Ashland, OR).

**ELISA test**

To investigate the Th1/Th2 cytokine production profile of CD4^+ and CD8^+ T cells, we used SearchLight Human Th1/Th2 Cytokine Array (Thermo Scientific, Rockford, IL), according to the manufacturer’s instructions. Briefly, we seeded 2 x 10^6 effector cells and 2 x 10^6 autologous LCL in 96-well round-bottom plates. Cytokine secretion was measured after 5-h incubation. Alternatively, cytokine ELISA test was performed using the human TNF-α screening set (Thermo Scientific), according to the manufacturer’s instructions. Briefly, 1 x 10^6 PBMCs were seeded in 96-well round-bottom plates and pulsed with virus-like particles (VLPs) (22) or EBV-purified proteins (23). Because the viral supernatant contained FBS, negative control was FBS-pulsed PBMCs. After incubation, 1 x 10^5 responding CD4^+ T cells were added. Cytokine secretion was measured after 20 h.

**Analysis of perforin and granyme b mRNA expression**

Total RNA was isolated from 10^5 CD4^+ and CD8^+ T cells using the TRizol Reagent (Invitrogen Life Technologies, Grand Island, NY), cDNA was synthesized with Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies). Amplifications of the cDNA for granyme B was performed with the following primer pair: 5'-TGCAGAGGAAGAAGATTTTGC-GG-C3' (forward) and 5'-GAGGATGCAATGTTTCGTC-3' (reverse) and the following conditions: denaturation 15 s at 94°C, annealing 30 s at 69°C, and extension 1 min at 72°C for 30 cycles (24). Amplification of the cDNA for perforin was carried out with the primer pair 5'-ACCACGATGTTGACTGTCAGTTGTC-3' (forward) and 5'-GAAGGGACGCGCTACATTGTC-3' (reverse), and the following conditions: denaturation 1 min at 95°C, annealing 1 min 30 s at 63°C, and extension 2 min at 72°C for 34 cycles (24). The expected lengths of the amplified cDNA were 459 bp for perforin and 180 bp for granyme B. β-Actin PCR amplification was performed as a control of RT-PCR. Primers were purchased from MWG Biotec (Ebersberg, Germany); all other reagents were purchased from Applied Biosystems (Foster City, CA).

**In vivo experiments**

In vivo studies involved 6- to 8-wk-old female SCID mice (Charles River Laboratories, Calco, Como, Italy) housed in our specific pathogen-free animal facility. Procedures involving animals and their care were in conformity with institutional guidelines (D.L. 116/92 and subsequent implementing circulars). Effector cells (10^7) were administered i.p. 4 d after inoculation of 10^7 autologous LCL, and mice were evaluated for survival (25). To assess the role of IFN-γ in our experimental setting, mice were injected i.p. with a neutralizing anti-human IFN-γ mAb (7R2A4; total dose, 1 mg/mouse at days −1, −1, 0, 6, and 10 from injection with CD8^+ T cells; European Collection of Cell Cultures, Porton Down, U.K.) or with rhIFN-γ (30,000 IU/mice for 14 d, starting 4 d after LCL engrafment; PeproTech, Rocky Hill, NJ). To assess MHC expression, mice were inoculated i.p. with 10^6 LCLs that were subsequently recovered by peritoneal wash. Alternatively, after LCL injection, mice were treated with 5-aza-2-deoxycytidine (decitabine; 0.25 mg/kg once on day 0 and twice a day on days 1, 2, and 3; Pharmachemie B.V., Haarlem, Holland); next, they were injected with CD4^+ T cells on day 4 or sacrificed to recover LCL. The chosen dose of decitabine was well tolerated and did not inhibit the growth of LCL in mice (LCL versus LCL + decitabine; p = 0.1978; LCL group: n = 100, median survival = 29 d; LCL + decitabine group: n = 24, median survival = 30.5 d).

**In vivo imaging**

To track the fate of CD4^+ T cells in vivo, T cells were transduced with a lentiviral vector coding for the Firefly Luciferase reporter gene (26). Bioluminescence images were acquired at several time points after cell injection using a cooled charge-coupled device camera mounted on a high-magnification box (IVIS Lumina II Imaging System; Caliper Life Sciences, Alameda, CA). Ten minutes before imaging, animals were anesthetized and administered i.p. with 150 mg/kg d-luciferin (Caliper Life Sciences) in PBS. A region of interest was drawn over the abdomen of animals, and the signal...
intensity was measured as radian (total photon/cm²/steradian) using the LivingImage software (Caliper Life Sciences).

Human samples of PTLD

H&E-stained slides and cell blocks from 12 PTLD, including 3 early lesions, 1 polymorphic PTLD, and 8 monomorphic PTLD/lymphoma, according to World Health Organization classification 2001 (27), were retrieved from the institutional files of the Surgical Pathology Section of University of Padova (Padova, Italy). All samples were found positive for EBV. Consecutive 4-μm sections were collected from representative blocks and immunolabeled with the primary Abs HLA-DR (LN-3, mouse; dilution 1/300, no pretreatment; Novocastra, Newcastle, U.K.) and HLA-I (EP1395Y, rabbit, dilution 1/100, pretreatment with Bond Epitope Retrieval Solution 1 at 100°C, 30 min; Novus Biologicals, Littleton, CO). Ag was detected by incubation with labeled polymer and diaminobenzidine. All the immunolabeled sections were counterstained by Harris hematoxylin, dehydrated, cleared, and mounted.

Statistical analysis

Kaplan-Meier product limit method was performed to estimate the survival curves, and comparison of survival between groups was performed using the log-rank test. All p values were based on a two-sided testing, and statistical analyses were carried out with the MedCalc statistical package (version 8.1).

Results

EBV-specific CD4+ and CD8+ T cell lines

To generate EBV-specific CD4+ and CD8+ T cell lines, PBMCs from five healthy EBV-positive donors were cultured with autologous irradiated LCL and IL-2 and subsequently separated by immunomagnetic sorting. The resulting CD3+/CD4+ and CD3+/CD8+ double-positive cultures contained <1% of NK cells. For all five donors, CD4+ T cells grew more vigorously and for a longer time (>14 wk) in comparison with CD8+ T cells (Fig. 1A), which progressively lost their proliferative capacity after three to seven restimulations.

Immunophenotypically, all T cell lines tested were essentially similar (Supplemental Table I) to those previously described (28) and displayed an early/intermediate effector phenotype (29) at the time of adoptive transfer (data not shown). Moreover, the expression of FoxP3 in the CD4+ T cell lines was negligible, thus excluding the presence of regulatory T cells (data not shown).

Functional characterization showed that the lytic activity exerted by both EBV-specific CD4+ and CD8+ T cells against autologous LCL was specific, very high (Fig. 1B), and HLA-II and HLA-I restricted, respectively, as demonstrated by experiments with blocking Abs (Fig. 1C).

Conflicting results exist about the lytic mechanisms used by CD4+ T cells (17, 30). To assess these aspects in our cultures, the perforin cytolytic pathway was selectively blocked with CMA (31) and EGTA (17), whereas BFA and a blocking mAb to FasL were used to inhibit the Fas–FasL killing pathway (31). Cytotoxicity of both CD4+ and CD8+ CTLs was completely abolished by CMA and EGTA, whereas it was unaffected by BFA and anti-FasL mAb (Fig. 1D), thus indicating a preferential use of the perforin-based cytolytic pathway. Consistently, perforin and granzyme B transcripts were detected by RT-PCR in both CD4+ and CD8+ T cell lines (Supplemental Fig. 1A), as recently reported for allogstimulated CD4+ and CD8+ T cells (32). Notably, flow cytometry analysis showed a high proportion of CD4+ T cells expressing granulysin (range, 47.0–95.8%; median, 83.8%), a feature already described for EBV-specific CD4+ T cells (17, 33). In contrast, CD8+ T cells had a lower content of this molecule (range, 15.0–48.0%; median, 29.3%; Supplemental Fig. 1B). Overall, the CD4+ T cells we generated are endowed with an in vitro cytotoxic activity comparable in extent and mechanisms to that of CD8+ T cells.

We also characterized the profile of cytokine secreted by CD8+ and CD4+ T cells. Although CD8+ CTLs were strongly skewed toward a classic Th1 pattern, CD4+ T cells were characterized by an intermediate profile, comprising both Th1 and Th2 cytokine production (20). The lytic activity reduction against mini-LCL target cells transformed by an EBV defective for lytic cycle Ag production (20). The lytic activity reduction against mini-LCL, as compared with LCL (Fig. 2A), suggested a preferential recognition of viral Ags associated with the lytic cycle. Nonetheless, the remaining minimal recognition of mini-LCL does not allow to completely exclude the presence of a reactivity against latent Ags or putative B cell-associated Ags (34). Interestingly, the reduced cytotoxicity in mini-LCL compared with LCL was not due to an intrinsic lower susceptibility to lysis of mini-LCL, as both LCL and mini-LCL were equally lysed by alloreactive T cell cultures (Supplemental Fig. 3). Among all lytic cycle Ags, CD4+ T cells appeared to preferentially recognize those associated with the early phase, because LCL treated with acyclovir, a drug that impairs the progression to the late phase of the lytic cycle, were killed as efficiently as untreated LCL (Fig. 2B). This notion is further supported by the lack of response to VLPs, which contain almost exclusively virion structural proteins expressed late during the lytic cycle (Fig. 2C), and to a set of

FIGURE 1. Functional characterization of EBV-specific CD8+ and CD4+ T cell lines. A, Total cell counts of CD8+ (magnified in the inset) and CD4+ T cell lines before each restimulation with LCLs. B, Lytic activity of CD8+ (left panel) and CD4+ (right panel) T cell lines against autologous LCLs, PHA blasts, and K562, as assessed by a standard 4-h 51Cr release assay. C, MHC restriction was evaluated using anti–HLA-I and anti–HLA-II mAb. Cytotoxicity is reported as LUs; *p < 0.05; **p < 0.001, refer to statistically significant differences, respectively. D, Cytotoxicity was evaluated in the presence of reagents blocking perforin-based pathway (CMA and EGTA) and Fas/FasL-based pathway (BFA and anti-FasL mAb) (one representative experiment out of three).
To compare the in vivo therapeutic impact of CD4+ and CD8+ T cell therapeutic activity in an in vivo model of PTLD displayed by LCL (36).

NA1-specific responses, as epitopes of this protein are poorly of restimulation with LCL did not favor the reactivation of EB-negligible or absent (data not shown), likely because the protocol residual recognition of mini-LCL, whereas reactivity to EBNA1 was could be observed against EBNA2, which is in line with the re-cycle. With regard to recognition of latent Ags, a slight reactivity reactivity against Ags associated with the early phases of the lytic cycle. With regard to recognition of latent Ags, a slight reactivity could be observed against EBNA2, which is in line with the residual recognition of mini-LCL, whereas reactivity to EBNA1 was negligible or absent (data not shown), likely because the protocol of restimulation with LCL did not favor the reactivation of EB-NA1-specific responses, as epitopes of this protein are poorly displayed by LCL (36).

Therapeutic activity in an in vivo model of PTLD
To compare the in vivo therapeutic impact of CD4+ and CD8+ T cell lines, SCID mice were inoculated with 10^6 LCL i.p. and, 4 d later, with 10^6 effector cells by the same route (25). CD8+ T cells exerted a relevant therapeutic activity and improved survival of treated animals (p < 0.0001) compared with untreated mice (Fig. 2A). As a whole, our data indicate a preferential reactivity against Ags associated with the early phases of the lytic cycle. With regard to recognition of latent Ags, a slight reactivity could be observed against EBNA2, which is in line with the residual recognition of mini-LCL, whereas reactivity to EBNA1 was negligible or absent (data not shown), likely because the protocol of restimulation with LCL did not favor the reactivation of EBNA1-specific responses, as epitopes of this protein are poorly displayed by LCL (36).

1) Optical imaging of bioluminescent EBV-specific CD4+ T cells disclosed a rapid disappearance of the population upon in vivo transfer (Supplemental Fig. 4). Because the reduced survival of effector cells could explain the poor therapeutic activity, exogenous IL-2 was supplied to sustain CD4+ T cell survival in vivo (25), without achieving any significant increase in efficacy (data not shown).

2) As CD8+ T cells in vitro produce more IFN-γ than CD4+ T cells upon stimulation with LCL (as evaluated by ELISA tests and intracellular staining; Supplemental Figs. 2, 5A, respectively), we assessed a possible direct role of this cytokine on LCL viability and growth rate. Treatment with IFN-γ had no effect on LCL biology, either in vitro (data not shown) or in vivo (Supplemental Fig. 5B). The potential, indirect role of this cytokine in tumor eradication was investigated by administering IFN-γ-blocking Abs to mice receiving CD8+ T cells. Unlike previously reported data (37), we found that the therapeutic activity of transferred CD8+ T cells was not influenced by mAb to IFN-γ (Supplemental Fig. 5C).

3) We finally hypothesized that the timing of T cell administration could influence the therapeutic outcome. Indeed, adoptive transfer of CD4+ T cells in mice 6 h after LCL inoculation preserved a full antitumor activity (Supplemental Fig. 6), as compared with CD4+ T cells transferred at day 4.

We therefore argued that the reduced therapeutic efficacy of CD4+ T cells could be ascribed to LCL changes that occurred in vivo. In this regard, mouse proteins could be pinocytosed and processed by LCL, leading to the production of epitopes that may be loaded onto MHC-II molecules and thus mask EBV-derived epitopes similarly to what has been observed for LCL grown in FBS or FCS (Ref. 38 and our unpublished data). This phenomenon would progressively make LCL not recognizable by CD4+ T cells while retaining the capacity to interact with CD8+ T cells.
However, in vitro culturing of LCL with mouse serum for 4 d led only to a slight but not significant reduction in CD4+ T cells recognition (Supplemental Fig. 7). In contrast, if the therapeutic effects depend on the killing of the LCL following a direct interaction of the TCR with MHC-II molecules, then HLA expression would strongly influence the process. Quite unexpectedly, HLA molecule expression revealed a differential behavior following in vivo LCL inoculation. In fact, although HLA-I expression was essentially retained over time, HLA-DR molecules underwent a progressive and strong downmodulation (Fig. 3), a feature that could likely account for the lower in vivo activity of CD4+ CTLs when transferred 4 d after LCL inoculation.

In vivo modulation of HLA-II expression on LCL with epigenetic drugs

To restore MHC-II expression in LCL injected in SCID mice, rhIFN-γ was administered without, however, achieving any modification of surface staining (data not shown). In contrast, MHC-II gene expression is regulated by different epigenetic mechanisms, including DNA methylation and histone acetylation, involving either the MHC-II gene promoter or its transactivator (CIITA) gene promoter (39). As it has been reported that IFN-γ activity could ultimately depend on the methylation status of such promoters, IFN-γ could not be effective in upregulating MHC-II expression if they are methylated (40, 41). Animals were therefore treated with the demethylating agent decitabine, which was administered after LCL inoculation and up to day 3. At day 4, LCL cells were recovered by peritoneal washing and stained for MHC molecule expression. Decitabine treatment upregulated HLA-DR expression, albeit at a variable degree and only in a fraction of the population (HLA-DR: range, 35.5–66.9; median, 45.9%) (Fig. 4A), thus improving LCL recognition by CD4+ CTLs (Fig. 4B, 4C). Indeed, although LCLs recovered from untreated mice were poorly recognized, LCLs from decitabine-treated animals were significantly more susceptible to lysis and stimulated a higher percentage of IFN-γ– and TNF-α–producing CD4+ CTLs. We also assessed the effects of decitabine on HLA-I expression and CD8+ CTL-mediated recognition of LCLs. Decitabine induced only a limited upregulation of expression intensity of HLA-I molecules but did not modify the overall percentage of positive cells (data not shown). With regard to the recognition capacity of CD8+ T cells, decitabine treatment improved cytokine production but left the cytotoxic activity unaffected (Supplemental Fig. 8A, 8B). Next, we sought to assess whether the pharmacological treatment could positively impact on the therapeutic activity of adoptively transferred CD4+ CTLs, and we found that it significantly improved survival of mice in comparison with untreated animals (p < 0.0001) (Fig. 4D). Moreover, there was an increase in mean survival compared with that of animals injected with CD4+ T cells alone (48 versus 36 d, respectively), indicating that the increased in vitro recognition was paralleled by an improved in vivo therapeutic efficacy.

HLA-I and HLA-II expression in human PTLD

If a differential expression of MHC class I and MHC-II molecules would also characterize human PTLD, the possibility of exploiting the effector functions of EBV-specific CD4+ CTLs to contrast tumor growth would rapidly fade, because of the impossibility of an efficient recognition of target cells. In contrast, LCL biology could be
affected by mouse microenvironment through some unknown mechanisms, and therefore, the HLA-II downregulation could represent a bias limited to this experimental model. We then investigated by immunohistochemistry the expression of HLA-DR on a series of biopsies of human EBV-positive PTLD at different stages of development (Supplemental Table II). HLA-DR overall expression progressively increased from early lesions, including mononucleosis-like PTLD and plasmacytic hyperplasia, to overt lymphoma. Notably, HLA-DR–positive immunostaining was found on the large blasts representing the neoplastic cell component, which were present in the interfollicular areas in the mononucleosis-like PTLD and gradually subverted and substituted the lymphoid tissue architecture in the more advanced and aggressive forms of disease. HLA-DR was negative only in scattered cell elements undergoing a plasmacytoid differentiation. HLA-I was diffusely positive in both small lymphocytes and blasts, with no differences among the various types of PTLD (Fig. 5). Therefore, these data indicate that EBV-immortalized B cells do not downmodulate HLA-II molecules in human PTLD and hence can be regarded as a suitable target for CD4+ CTL adoptive immunotherapy.

Discussion

Although tumor-specific CD8+ T cells are still considered the mainstream effector component of adoptive immunotherapy (42, 43), several in vitro findings have clearly pointed out that CD4+ T cells are also endowed with Ag-specific lytic activity (18, 44) and therefore could per se be capable of effector functions. In EBV-related malignancies, virus-specific clonal or polyclonal lytic CD4+ T cell lines can block LCL proliferation and growth in vitro (17, 45). Prospectively, this could be highly relevant for the potentially “universal” EBV-associated EBNA1 tumor Ag, because EBNA1-specific cytotoxic CD4+ T cells may theoretically be used among the various types of PTLD (Fig. 5). Therefore, these data indicate that EBV-immortalized B cells do not downmodulate HLA-II molecules in human PTLD and hence can be regarded as a suitable target for CD4+ CTL adoptive immunotherapy.

![Figure 4](http://www.jimmunol.org/DownloadedFrom)

**FIGURE 4.** Decitabine treatment restores HLA-DR expression and LCL recognition by CD4+ T cells. A, Flow cytometry analysis of HLA-DR expression on LCLs from decitabine-treated mice (gray plot, isotype controls; black lines, HLA-DR staining). The inset reports data from a drug-untreated mouse. Data from one representative cell line of three tested are shown. B, Cytotoxicity exerted by CD4+ T cells against LCLs from decitabine-treated or untreated mice. Figure shows mean ± SD of five independent experiments. C, IFN-γ and TNF-α production by CD4+ T cells after challenge with LCLs from decitabine-treated or untreated mice. LCLs obtained as in B were used to stimulate CD4+ T cells in vitro. Percentage of positive cells was calculated on total gated CD4+ T cells. Mean ± SD of four independent experiments is shown. *p < 0.05; **p < 0.001, refer to statistical significant differences, respectively. D, Kaplan-Meier survival curves of mice inoculated with LCLs only (historical control group, n = 100) or with LCLs and CD4+ T cells (n = 22; see Fig. 3A) or with LCLs and CD4+ T cells plus decitabine (n = 26; median survival = 48 d; LCL versus CD4+ plus decitabine; p < 0.0001; CD4+ versus CD4+ plus decitabine; p = 0.049).

![Figure 5](http://www.jimmunol.org/DownloadedFrom)

**FIGURE 5.** HLA expression on human EBV-associated PTLD. Formalin-fixed, paraffin-embedded specimens of early lesions (left column), polymorphic PTLD (central column), and monomorphic PTLD/lymphoma (right column) were stained with H&E (top row) and evaluated by immunohistochemistry with Abs to HLA-I (central row) and HLA-DR (bottom row). The inset at the bottom right in some pictures reports a more detailed view of diagnostic representative fields. Magnification is reported in every picture. All PTLD variants were intensely positive for HLA-I in both small lymphocytes and large blasts. HLA-DR was selectively strongly expressed in residual germinal center cells and large blasts in all subtypes of PTLD.
to treat every EBV-driven malignancy. Importantly, before evaluating the feasibility of approaches based on induction and expansion of EBNA1-specific cytotoxic CD4+ T cells, a still open question is whether CD4+ T cells alone could replace CD8+ T cells with the same efficacy.

To address this critical issue, we decided to exploit a clinically relevant protocol to generate parallel cultures of EBV-specific CD8+ and CD4+ T cells to be transferred in a mouse model of PTLD. We report the successful generation, from different donors, of EBV-specific CD4+ T cell cultures characterized in vitro by a cytotoxic activity fully overlapping that of cognate CD8+ T cells. Surprisingly, however, the in vivo antitumor impact of the two T cell populations was considerably different, because CD4+ T cells alone showed only reduced efficacy. The activity of pure CD4+ T cell populations in tumor therapy has been mainly ascribed to cytokine release and recruitment of other effector cells (46). In our experimental setting, this mechanism can be in part ruled out because neither the exogenous administration nor the blocking of CD8+ T cell-secreted IFN-γ had any impact on LCL biology. In contrast, HLA-DR underwent a progressive downmodulation on the LCL upon in vivo inoculation, whereas HLA-I expression remained nearly unchanged, a phenomenon that could account for the reduced in vivo efficacy of CD4+ versus CD8+ T cells. Noteworthy, partial reversal of HLA-II downmodulation by decitabine improved LCL recognition by CD4+ T cells and prolonged survival in drug-treated mice. Overall, the concern about HLA-II downmodulation is likely due to a strong bias apparently limited only to the mouse model, as in human PTLD specimens HLA-DR is retained in all stages of disease and consistently expressed by tumor cells. Therefore, in terms of clinical applicability, CD4+ T cells could be, in principle, efficaciously administered to PTLD patients without any additional pharmacological treatment. This possibility is strongly supported by results of a recent case report, showing that in vivo transfer of a NY-ESO-1–specific CD4+ T cell clone induced strongly supported by results of a recent case report, showing that in vivo transfer of a NY-ESO-1–specific CD4+ T cell clone induced any additional pharmacological treatment. This possibility is in principle, efficaciously administered to PTLD patients without preclinical results consistently indicate that tumor Ag-specific express MHC-II molecules (48). On the whole, both clinical and pended on cross-priming or on a direct recognition of target cells. In in our experience, this mechanism can be in part ruled out because neither the exogenous administration nor the blocking of CD8+ T cell-secreted IFN-γ had any impact on LCL biology. In contrast, HLA-DR underwent a progressive downmodulation on the LCL upon in vivo inoculation, whereas HLA-I expression remained nearly unchanged, a phenomenon that could account for the reduced in vivo efficacy of CD4+ versus CD8+ T cells. Noteworthy, partial reversal of HLA-II downmodulation by decitabine improved LCL recognition by CD4+ T cells and prolonged survival in drug-treated mice. Overall, the concern about HLA-II downmodulation is likely due to a strong bias apparently limited only to the mouse model, as in human PTLD specimens HLA-DR is retained in all stages of disease and consistently expressed by tumor cells. Therefore, in terms of clinical applicability, CD4+ T cells could be, in principle, efficaciously administered to PTLD patients without any additional pharmacological treatment. This possibility is strongly supported by results of a recent case report, showing that in vivo transfer of a NY-ESO-1–specific CD4+ T cell clone induced any additional pharmacological treatment. This possibility is in principle, efficaciously administered to PTLD patients without preclinical results consistently indicate that tumor Ag-specific express MHC-II molecules (48). On the whole, both clinical and pended on cross-priming or on a direct recognition of target cells. In
Supplementary Figure 1. Expression of lytic molecules. A, Perforin and granzyme B expression. Figure shows perforin (left panels) and granzyme B (right panels) expression as assessed by RT-PCR in PBMC (positive control) and CD4+ and CD8+ T cell lines of two representative donors. The HT-29 human colon adenocarcinoma cell line was used as a negative control. β-actin amplification was carried out as control of the RT-PCR reaction. B, Granulysin expression. CD8+ (left panel) and CD4+ (right panel) T cell lines were fixed, permeabilized and stained with anti-granulysin mAb. The grey plot indicates the background obtained with an irrelevant, isotype-matched control antibody. Percentage of positivity and relative mean fluorescence intensity is reported. Figure shows a representative donor out of 5 tested.
**Supplementary Figure 2.** Cytokine production profile. Figure shows cytokine production by EBV-specific CD4\(^+\) and CD8\(^+\) T cells in response to LCL, as assessed by ELISA test. Data reported refer to one donor, representative of all donors tested.
Supplementary Figure 3. Susceptibility to lysis of LCL and mini-LCL. Alloreactive cultures, generated using allogenic LCL (allo LCL) or the corresponding mini-LCL (allo mini-LCL) as stimulators, were tested for lytic activity against LCL and mini-LCL. Both cell lines were lysed at the same extent. Results are representative of 3 different cultures.
Supplementary Figure 4. Survival of CD4⁺ T cells after adoptive transfer in SCID mouse. Figure shows bioluminescence images of *Luciferase*-transduced CD4⁺ T cells at different time points after i.p. injection in SCID mouse.
Supplementary Figure 5. IFNγ does not play a therapeutic role in anti-EBV CD4+ T cell adoptive immunotherapy. A, In vitro IFNγ production by CD4+ and CD8+ T cells after stimulation with autologous LCL and PMA-ionomycin. Effector cells were stimulated for 5 h and then fixed, permeabilized and stained for intracellular cytokine. Percentage of positive cells was calculated on total gated CD4+ or CD8+ T cells. Figure shows mean ± SD of 5 independent experiments while * and ** refer to statistical significant differences with P < 0.05 and P < 0.001, respectively. B, Kaplan-Meier survival curves of SCID mice inoculated with autologous LCL with or without recombinant human IFNγ. Four days after i.p. injection of 10⁷ LCL cells, animals were treated with the cytokine, according to the schedule reported in Methods section. Mice were left untreated (LCL, n = 7), or received IFNγ (n = 7). The administration of IFNγ did not affect the in vivo growth rate of LCL (median survival of control group = 31 days; median survival of treated group = 32 days, P = 0.165). C, Kaplan-Meier survival curves of mice inoculated with LCL only (control group, n = 11), with LCL and CD8+ T cells (CD8+ group, n = 5) or with LCL and CD8+ T cells plus anti-human IFNγ neutralising antibody (CD8+ + anti-IFNγ group, n = 5). Antibody administration did not reduce the therapeutic activity of adoptively transferred CD8+ T cells (median survival of control group = 24 days; median survival of CD8+ group = 53 days; median survival of CD8+ + anti-IFNγ group = 53 days; control group vs. CD8+ group, P = 0.0008; control group vs. CD8+ + anti-IFNγ group, P = 0.80).
Supplementary Figure 6. CD4⁺ T cells given shortly after tumor inoculation display a high therapeutic activity. Kaplan-Meier survival curves of mice inoculated with LCL only (control group, n = 7), or with LCL and CD4⁺ T cells 6 hours after LCL engraftment (CD4⁺ day 0 group, n = 9). The adoptive transfer of CD4⁺ T cells few hours after LCL significantly improved survival of treated mice (median survival of control group = 31 days; median survival of CD4⁺ day 0 group = 63 days, \( P < 0.0001 \)), as compared to the same effector population administered at day 4.
Supplementary Figure 7. Exogenous antigens do not interfere with CD4⁺ T cell-mediated EBV recognition in LCL. Lytic activity of CD4⁺ T cells against autologous LCL grown in medium supplemented with human or mouse serum, as assessed by a standard 4-h $^{51}$Cr release assay. Proteins in mouse serum, which could be pinocytosed and presented by LCL in the context of MHC-II molecules, did not displace EBV-derived epitopes, as LCL were recognised and killed at an almost similar extent. Figure shows mean ± SD of 4 independent experiments.
Supplementary Figure 8. Effect of decitabine treatment in CD8+ T cell-mediated recognition of LCL. A, Cytotoxicity exerted by CD8+ T cells against LCL from decitabine-treated or untreated mice. Figure shows mean ± SD of 5 independent experiments. B, IFNγ and TNFα production by CD8+ T cells after challenge with LCL from decitabine-treated or untreated mice. LCL cells obtained as in A were used to stimulate CD8+ T cells in vitro. Percentage of positive cells was calculated on total gated CD8+ T cells. Figure shows mean ± SD of 4 independent experiments, while * and ** refer to statistical significant differences with P < 0.05 and P < 0.001, respectively.
## Supplementary Table I: Phenotypic analysis of EBV-specific CD4\(^+\) and CD8\(^+\) T cell lines.

<table>
<thead>
<tr>
<th>Marker</th>
<th>CD4(^+) Range</th>
<th>Median</th>
<th>CD8(^+) Range</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD25</td>
<td>77.0%-99.6%</td>
<td>99.4%</td>
<td>2.4%-64.6%</td>
<td>6.7%</td>
</tr>
<tr>
<td>CD38</td>
<td>54.5%-97.3%</td>
<td>35.2%</td>
<td>67.4%-95.4%</td>
<td>80.7%</td>
</tr>
<tr>
<td>CD45RA</td>
<td>1.9%-12.1%</td>
<td>4.4%</td>
<td>2.6%-9.7%</td>
<td>7.0%</td>
</tr>
<tr>
<td>CD45RO</td>
<td>89.9%-96.5%</td>
<td>95.8%</td>
<td>88.8%-95.8%</td>
<td>94.0%</td>
</tr>
<tr>
<td>CD69</td>
<td>47.0%-81.1%</td>
<td>79.6%</td>
<td>2.9%-27.3%</td>
<td>15.5%</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>11.1%-88.4%</td>
<td>32.7%</td>
<td>89.5%-98.4%</td>
<td>97.3%</td>
</tr>
<tr>
<td>TCR(\alpha\beta)</td>
<td>81.5%-99.1%</td>
<td>98.4%</td>
<td>76.1%-95.7%</td>
<td>92.3%</td>
</tr>
<tr>
<td>TCR(\gamma\delta)</td>
<td>0.0%-2.0%</td>
<td>0.2%</td>
<td>0.0%-0.4%</td>
<td>0.25%</td>
</tr>
</tbody>
</table>

Range and median value of phenotypic marker expression of EBV-specific CD4\(^+\) and CD8\(^+\) T cell lines from 5 different donors.
Supplementary Table II: Clinicopathological characteristics of patients with EBV-related PTLD at different stages of development.

<table>
<thead>
<tr>
<th>Pt. number</th>
<th>Sex /Age (year of diagnosis)</th>
<th>Diagnosis</th>
<th>Site</th>
<th>HLA-II</th>
<th>HLA-I</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (2006, Fig. 5)</td>
<td>M, 16 yr</td>
<td>PTLD early lesions</td>
<td>palatine tonsil</td>
<td>10-15% of blasts in the interfollicular areas; all blasts found positive*</td>
<td>100%</td>
</tr>
<tr>
<td>2 (2004)</td>
<td>M, 7 yr</td>
<td>PTLD early lesions</td>
<td>tonsil</td>
<td>10-15% of blasts in the interfollicular areas; all blasts found positive*</td>
<td>100%</td>
</tr>
<tr>
<td>3 (2006)</td>
<td>M, 2 yr</td>
<td>PTLD early lesions</td>
<td>pharyngeal and palatine tonsil</td>
<td>10-15% of blasts in the interfollicular areas; all blasts found positive*</td>
<td>100%</td>
</tr>
<tr>
<td>4 (2006)</td>
<td>F, 12 yr</td>
<td>polymorphic PTLD (non lymphoma)</td>
<td>lymph node</td>
<td>60% of blast cells in the whole population; all blasts found positive.</td>
<td>100%</td>
</tr>
<tr>
<td>5 (2006, Fig. 5)</td>
<td>M, 53 yr</td>
<td>monomorphic PTLD</td>
<td>lymph node</td>
<td>95% of blast cells in the whole population; all blasts found positive.</td>
<td>100%</td>
</tr>
<tr>
<td>6 (1998, Fig. 5)</td>
<td>M, 13 yr</td>
<td>PTLD lymphoma</td>
<td>lymph node</td>
<td>60% of blast cells in the whole population; all blasts found positive; scattered plasmacells and plasmacytoid elements found negative.</td>
<td>95%</td>
</tr>
<tr>
<td>7 (1998)</td>
<td>M, 10 yr</td>
<td>PTLD lymphoma</td>
<td>lymph node</td>
<td>80% of blast cells in the whole population; all blasts found positive; scattered plasmacells and plasmacytoid elements found negative.</td>
<td>98-100%</td>
</tr>
<tr>
<td>8 (1998)</td>
<td>M, 12 yr</td>
<td>PTLD lymphoma</td>
<td>adenoid fragment</td>
<td>70% of blast cells in the whole population; all blasts found positive.</td>
<td>98-100%</td>
</tr>
<tr>
<td>9 (1998)</td>
<td>F, 16 yr</td>
<td>PTLD lymphoma</td>
<td>lymph node</td>
<td>50-60% of blast cells in the whole population; all blasts found positive.</td>
<td>100%</td>
</tr>
<tr>
<td>10 (1999)</td>
<td>M, 16 yr</td>
<td>PTLD lymphoma</td>
<td>lymph node</td>
<td>90% of blast cells in the whole population; all blasts found positive.</td>
<td>100%</td>
</tr>
<tr>
<td>11 (2003)</td>
<td>M, 10 yr</td>
<td>PTLD lymphoma</td>
<td>lymph node</td>
<td>75% of blast cells in the whole population; all blasts found positive.</td>
<td>100%</td>
</tr>
<tr>
<td>12 (2004)</td>
<td>M, 1 yr</td>
<td>PTLD lymphoma</td>
<td>small bowel</td>
<td>90% of blast cells in the whole population; all blasts found positive.</td>
<td>100%</td>
</tr>
</tbody>
</table>

* residual germinal centre cells were diffusely positive.