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Successful *In Vitro* Priming of EBV-Specific CD8+ T Cells Endowed with Strong Cytotoxic Function from T Cells of EBV-Seronegative Children

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Epstein-Barr virus (EBV)-seronegative transplant recipients are at high risk of developing EBV-associated post-transplant lymphoproliferative disorder (PTLD), and would maximally benefit from an EBV-directed T-cell therapy for prevention or treatment of PTLD. So far, efforts to activate CD8+ EBV-specific cytotoxic T lymphocytes (CTL) endowed with high specific cytotoxicity from EBV-seronegative children have failed. We compared the CD8+ CTL priming efficiency of three different modified activation protocols, based on lymphoblastoid cell lines (LCL) stimulation potentially enhanced by either LCL presentation through dendritic cells, or selection of IFN-γ+ cultured cells, or culture in the presence of rhIL-12 and rhIL-7, according to the standard protocol for reactivation of EBV-specific CTL. We found that only specific LCL stimulation in the presence of rhIL-12 and rhIL-7 was able to reproducibly expand EBV-specific CD8+ CTL endowed with strong cytotoxic activity from truly EBV-seronegative children. The lines thus activated, which included specificities toward EBV latent and lytic proteins, showed high percentage CD8+ T cells, with <10% naïve CD8+/CCR7+/CD45RA+ cells. Overall, the total number of CD8+ central memory cells, and of CCR7 T-cell effectors was comparable to that observed in healthy EBV-seropositive controls. In conclusion, it is feasible to activate EBV-specific CD8+ CTL with suitable characteristics for *in vivo* employment from EBV-seronegative children.

Key words: CD8+ T-cell priming, cytotoxic T lymphocytes, Epstein-Barr virus, pediatric transplantation, post-transplant lymphoproliferative disorder

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Introduction

Post-transplant lymphoproliferative disorders (PTLD) are life-threatening complications of immunosuppression in hematopoietic stem cell transplant (HSCT) and solid organ transplant (SOT) recipients (1–3), mostly associated with proliferation of Epstein-Barr virus (EBV)-infected B cells, whose uncontrolled expansion in immunocompetent individuals is prevented by cytotoxic T lymphocytes (CTL) (4–5).

Restoration of EBV-specific immunocompetence by infusion of EBV-specific CTL, reactivated *ex vivo* from EBV-seropositive individuals through stimulation with autologous EBV-transformed lymphoblastoid cell lines (LCL) (6), has proved safe and effective in preventing or treating PTLD after HSCT (7–10). More recent data indicate that this strategy may also be of value in the treatment of PTLD developing after SOT (10–13). EBV-naïve SOT recipients and, albeit less frequently, patients receiving HLA-haploidentical HSCT from a seronegative donor or cord blood SCT, are at higher risk of developing PTLD, due to failure to mount a protective immune response in the context of immune deficiency (14,15). However, the potential application of T-cell therapy in this high-risk population is limited by the difficulty in obtaining EBV-specific CTL from seronegative individuals lacking specific memory T cells (4,16,17).

Recent studies have demonstrated that it is feasible to activate EBV-specific CTLs in adult EBV-naïve individuals, by prolonged LCL stimulation (18) or by priming with dendritic cells (DC) loaded with apoptotic/necrotic LCL (19). However, priming of EBV-specific CTL in children resulted in the expansion of CD4+ T cells (18). Although it has been demonstrated that prevention of early-phase EBV-induced B-cell proliferation *in vitro* requires CD4+ effector T cells (20), there are little data regarding their effects *in vivo*. Conversely, several studies have shown the central role

of CD8+ CTL in cell-mediated containment of EBV infection (4,5,21) and data in the mouse model documented that transfer of CD8+ T cells raised against human LCL can prevent the formation of tumors after infusion of LCL from the same individual (22). Moreover, recovery from PTLD after antiviral therapy and reduction of immunosuppression in renal transplant recipients were shown to be associated with a sustained recovery of circulating EBV-specific CD8+ T cells (23), and, in our clinical experience, SOT patients with poor response to EBV-specific CTL infusions had a majority of CD4+ CTL in the infused line [(12) and Comoli P, personal observation].

Since EBV-specific CTL of the CD8+ phenotype have been recognized to be crucial for recovery from established PTLD, we endeavored to develop a protocol of EBV-CTL priming that would allow optimal expansion and high specific cytotoxic activity of CD8+ CTL. In particular, we compared the CD8+ CTL priming efficiency of three different modified activation protocols, based on LCL stimulation potentially enhanced by either LCL presentation through DC, or selection of IFN-γ + cultured cells, or culture in the presence of recombinant human (rh)IL-12 and rhIL-7, to the standard protocol for reactivation of EBV-specific CTL from EBV-seropositive individuals. We, then, analyzed the quality of the resulting EBV-specific response with these three different approaches.

Materials and Methods

Blood donors

Peripheral blood samples were collected from six EBV-seronegative patients with end-stage renal failure receiving dialysis while awaiting kidney transplantation. Seven adult EBV-seropositive donors were used as controls in some experiments. Characteristics of patients and controls are reported in Table 1.

Determination of EBV status was evaluated by serology and viral DNA analysis. Anti-EBV antibody titers in serum against viral capsid antigen (VCA) and nuclear antigens (EBNA) were performed by ELISA (anti-VCA IgG and IgM ELISA Kit, DiaSorin, Saluggia, Italy; anti-EBV EBNA IgG ELISA Kit, Biotest, Dreieich, Germany). Titers >24 AU/mL were considered indicative of VCA IgM and IgG positivity, while an arbitrary index >1.2 was indicative of EBNA IgG positivity, according to manufacturers' instructions. EBV DNA was quantified using an original quantitative polymerase chain reaction assay (12).

Patients or guardians gave written informed consent at the time of enrolment; the study was conducted according to Institutional guidelines, and approved by the Institutional Review Board.

LCL and DC production

PBMC were isolated by Ficoll-Hypaque density gradient centrifugation (12), and used either fresh or cryopreserved. For generation of LCL, PBMC were incubated with EBV-containing supernatant from the B95-8 cell line (American Type Culture Collection, Rockville, MD, USA) in the presence of 800 ng/mL of cyclosporin-A (Sandoz Pharmaceuticals, Basel, Switzerland) in RPMI supplemented with 10% fetal calf serum. Cells were continuously cultured for 3–4 weeks, following a previously described protocol (12).

Table 1: Characteristics of the subjects enrolled into the study at the time of peripheral blood collection for EBV-specific CTL generation

									CD8+ CTL activation			
Donors	Sex	Age	HLA	VCA IgM	VCA IgG	EBNA IgG	EBV DNA	ELISPOT	Standard	DC-LCL	IFN-γ + selection	Standard + IL-7/IL-12
Seronegative patients												
1	M	10	A2,2 B8,18	–	–	–	neg	75 ± 7	Yes	Yes	No	Yes
2	F	10	A3,29 B13,44	–	–	–	neg	57 ± 2	No	No	No	Yes
3	F	10	A1,3 B35,55	–	–	–	neg	30 ± 6	No	ND	ND	Yes
4	F	7	A2,68 B13,44	–	–	–	neg	35 ± 2	No	ND	No	Yes
5	M	9	A2,32 B18,39	–	–	–	neg	43 ± 5	No	No	ND	Yes
6	F	27	A2,3 B60,60	–	–	–	neg	60 ± 3	Yes	Yes	ND	Yes
									2/6	2/4	0/3	6/6
Seropositive donors	2 F, 5 M	28 ± 17	Positive for A2 and/or B8 and/or B35	–	+	+	neg	429 ± 196	7/7	ND	ND	ND

DC were generated from peripheral blood monocytes as previously described (24). After 6–7 days incubation, cells were recovered, phenotyped to assess the degree of maturity and either cryopreserved for later use or pulsed with apoptotic/necrotic B-LCL. After pulsing with apoptotic/necrotic LCL, our DC, displayed an activated phenotype, with up-regulation of major histocompatibility complex (MHC) and costimulatory molecules (prepulsing: CD83: 26 ± 20%, CD80: 47 ± 20%, CD86: 34 ± 18%, CD40: 32 ± 31%, HLA-DR: 87 ± 12%, MFI 2793 ± 892; post-pulsing: CD83: 52 ± 23%, CD80: 62 ± 14%, CD86: 59 ± 24%, CD40: 63 ± 27%, HLA-DR: 98 ± 0.5%-MFI 4129 ± 1089).

EBV-specific CTL activation

EBV-specific CTLs were activated and expanded *in vitro* from frozen PBMC of EBV-seronegative subjects according to the following stimulation protocols: (I) standard protocol for reactivation of EBV-specific CTLs from EBV-seropositive individuals, according to a method previously reported (7,12). In detail, PBMC were plated in 2 mL X-VIVO 20 medium (BioWhittaker, Walkersville, MD, USA) with 2% autologous plasma, at 2×10^6 cells per well and stimulated with irradiated autologous LCL at a responder-to-stimulator (R:S) ratio of 40:1; (II) 2×10^6 responder PBMC per well were co-cultured with DC pulsed with apoptotic autologous LCL at a R:S ratio of 20:1. Apoptosis was obtained by γ irradiation (15 000 rads), and immature DC were loaded with apoptotic LCL according to a method previously described (19); (III) PBMC were plated and stimulated as in protocol I. At day +12, selection of IFN-γ + T cells by immunomagnetic microspheres coated with anti-IFN-γ monoclonal antibody (mAb) (Miltenyi, Bergisch Gladbach, Germany), and restimulation with autologous LCL were performed according to protocol I; (IV) PBMC were plated at the same conditions as protocol I, in the presence of 10 ng/mL rhIL-7 and 10 pg/mL rhIL-12 (both, R&D Systems).

After 10 days for protocols I, II and IV, and 12 days for protocol III, cultures were restimulated with irradiated autologous LCL at an R:S ratio of 4:1. Starting on day 14, 20 U/mL rhIL-2 (Hoffman-La Roche, Basel, Switzerland) were added to the wells twice weekly, and the cultures were subsequently restimulated weekly with irradiated autologous LCL in the presence of rhIL-2. Starting from day +28, effector cells were examined for phenotype, for IFN-γ production by ELISPOT assay, and for EBV specificity in cytotoxicity assays.

Flow cytometry

mAbs used to characterize cultured cells were: anti-CD3 FITC, PE, PerCP-Cy5.5, anti-HLA-DR PE, anti-CD8 FITC, PE, PerCP-Cy5.5, anti-CD56 PE, anti-TRCγ δ FITC, anti-CD4 PE, PerCP-Cy5.5, anti-CD19 FITC, anti-CD20 PE, anti-CD45 FITC, anti-CD27 PE, anti-CD62L PE, anti-CCR7 FITC, anti-CD45RA PE, anti-CD1 PE, anti-CD14 FITC, anti-CD83 PE, anti-CD80 PE, anti-CD86 FITC (Becton Dickinson, Mountain View, CA, USA); anti-CD69 PE (Serotec, Oxford, UK). Appropriate isotype-matched controls were included. Cytofluorimetric analysis was performed by means of direct immunofluorescence on a FACScan flow cytometer (Becton Dickinson).

ELISPOT assay

ELISPOT assays to determine the frequency of IFN-γ-secreting PBMC were performed following a method previously described (25). PBMC, thawed and cultured overnight in RPMI-fetal calf serum (FCS) medium before use in the assay, or cultured CTL, were seeded at 10^5 cells/well in the absence or in the presence of EBV-LCL or 2 μg/mL of EBV antigen peptides. In particular, we employed the following latent phase protein EBNA3A peptides: A2.1-restricted LLDFVRMGV and SVRDRLARL; B8.1-restricted FLRGRAYGL; B35.1-restricted YPLHEQHGM (Roche Diagnostics, Milano, Italy), a peptide mix containing 15-mer peptides spanning the EBV LMP-2 protein (Jerini, Berlin, Germany), and A2.1-restricted lytic phase protein BMLF1 peptide GLCTLVAML (Proimmune, Oxford, UK). IFN-γ-producing spots were counted using an Elispot reader (Bioline, Torino, Italy). The number of spots per well was calculated after subtraction of assay background, quantitated as the average of 24 wells containing only sterile complete medium, and of specific background, quantitated as the sum of cytokine spots associated with responders alone or responders plated with dimethyl sulfoxide solvent control, as appropriate.

Cytotoxicity assay

Whenever required, CD3+/CD4+ and CD3+/CD8+ cells were selected from effector cell suspension by positive selection with magnetic microspheres coated with anti-CD4 and anti-CD8 mAbs (Miltenyi). Cytotoxic activity was measured in a standard ^{51}Cr-release assay, as previously described (12), against autologous and allogeneic LCL, autologous PHA blasts and Daudi cell lines. For HLA class I and class II blocking experiments, target cells for cytotoxicity assays were incubated with 30 μL of the murine anti-human HLA class I mAb W6/32 or HLA class II mAb CR3/43 (Dako, Glostrup, Denmark) for 30 min at room temperature.

Results

EBV-specific CD8+ CTL can be generated only in a fraction of EBV-seronegative donors, using the standard method or stimulation with DC loaded with apoptotic LCL.

The ability of autologous LCL to induce a primary response in our EBV-seronegative donors was first tested through activation by the standard protocol. The total expansion of LCL-stimulated cell lines during the first 4 weeks of culture ranged from 2.7- to 12-fold (mean 7.9 ± 3.3).

Cell lines, analyzed after four LCL stimulation rounds, showed a higher expansion of the CD3+ CD4+ subset compared with the prevalence of CD8+ CTL observed in EBV-seropositive donors (Table 2). CD4+ cells had a naïve/memory/effector population distribution comparable to that of healthy seropositive individuals (Table 3). CD3+ CD8+ lymphocytes accounted for 35 ± 10% of total cell recovery (Table 2). CD8+ T cells in the five EBV-seronegative children were either low in number or in a large proportion TCRγ δ+ and/or CD56+, while in the EBV-seronegative adult the majority revealed a CD8bright TCR-γ δ- CD56- phenotype (data not shown). Within the CD8+ T-cell population, 9 ± 12% of cells were of the naïve phenotype. As shown in Figure 1, these T-cell lines displayed scarce lysis toward autologous LCL. EBV-specific, HLA-restricted cytotoxicity, present albeit at low levels in 2/5 EBV-seronegative children, was mainly mediated by CD4+ cells, while lysis displayed by the CD8+ subset was MHC-unrestricted (Figure 1 and 2B). However, the T-cell lines from the EBV-seronegative adult and from 1 EBV-seronegative child, in line with the observed phenotype, were able to exert some EBV-specific, HLA class I-restricted killing mediated by the CD8+ subset (Table 1, Figure 2B and 2C), though not comparable to that observed in healthy EBV-seropositive individuals.

In an attempt to effectively activate or increase CD8+ CTL number and specific function, we compared three different stimulation protocols, all based on priming by

Table 2: Phenotype of CTL lines generated from EBV-seronegative patients using different activation protocols

	CD8+	CD4+	TCR γ δ+	TCR γ δ+CD8+	CD56+ CD3+	CD56+ CD3−
Seronegative patients						
Standard	35 ± 10*	50 ± 18	25 ± 19	12 ± 11	16 ± 4	8 ± 8
DC-LCL	43 ± 19	42 ± 18	29 ± 20	14 ± 7	9 ± 8	6 ± 7
IFN-γ+	11 ± 5	77 ± 10	8 ± 7	0.5 ± 0.1	5 ± 4	3 ± 3
+IL7/IL12	62 ± 18	20 ± 8	29 ± 6	14 ± 3	9 ± 2	5 ± 4
Seropositive donors						
Standard	80 ± 12	17 ± 10	9 ± 7	7 ± 4	4 ± 3	3 ± 3

*Data are reported as % positive cells.

autologous LCL as in the standard method, although potentiated through either presentation of apoptotic/necrotic LCL by DC, or selection of an activated IFN-γ+ T-cell subset, or addition of rhIL-12 and rhIL-7 at the time of priming.

Co-culture with autologous DC loaded with apoptotic/necrotic LCL did not significantly increase either the number or cytotoxicity of CD8+ T cells. In particular, CD4+ and CD8+ T-cell populations were present in equal numbers, with 29 ± 20% CD3+ TCRγ δ+ cells and low numbers of CD3-CD56+ cells (Table 2). While DC-LCL were able to induce a primary CD8+ response in the EBV-seronegative adult, and the EBV-seronegative child who showed partial activation of CD8+ CTL with the standard protocol (CD8+ CD69+ cells: 60% and 37%, respectively; CD8 cell-mediated cytotoxicity at a 10:1 E:T ratio: 36% and 40%, respectively, vs. 76 ± 15% in healthy EBV-seropositive controls), they failed to prime EBV-specific CTL in two EBV-seronegative children tested who had not shown activation after standard stimulation (Figure 2b and 2c vs. 2a). In the patient from the latter group reported in Figure 2, 75% of CD8+ cells were naïve CCR7+/45RA+ T lymphocytes, while CD8+ effector subsets, amounting to 20% of the T cells, did not lyse autologous LCL (specific lysis at 10:1 E:T ratio: 9%). Moreover, mean expansion of DC-LCL-stimulated cell lines during the first 4 weeks of culture was lower than that observed with standard protocol (mean 3.5-fold, range 2.5–4.6).

Stimulation with LCL followed by IFN-γ+ cell selection fails to expand EBV-specific CTL in EBV-seronegative donors

Since we were able to activate EBV-specific CD8+ CTL with the standard method in 1 EBV-seronegative child, whose PBMC showed the highest frequency of IFN-γ-secreting cells upon stimulation with autologous LCL in a ELISPOT assay (Table 1), we determined whether EBV-specific cytotoxic activity mediated by CD8+ T cells could be revealed by selectively expanding T cells that became IFN-γ+ in response to stimulation with autologous LCL, a method that has proved effective in expanding antigen-specific populations from PBMC of seropositive individuals (26).

After 12-day stimulation of 50 × 10^6 PBMC with autologous LCL, we observed a mean % IFN-γ+ cells of 0.04 ± 0.06 in the EBV-seronegative children tested. Following selection of IFN-γ+ lymphocytes, we obtained a mean recovery of 1.6 × 10^6 ± 0.6 × 10^6 cells (mean IFN-γ+ cells on CD4+ T cells: 8 ± 5%; mean IFN-γ+ cells on CD8+ T cells: 3 ± 3%) that were further cultured through three rounds of LCL stimulation. No expansion of the selected IFN-γ+ cells was observed after repeated stimulation with LCL (mean growth rate: 0.1 ± 0). Moreover, this protocol did not succeed in eliciting either CD8+ or CD4+ CTL in all three EBV-seronegative children tested. The cells recovered from 4-week cultures included a majority of CD3+ CD4+ lymphocytes, while more than 50% of the CD8+ cells showed a naïve CCR7+/45RA+ phenotype (Table 2). These T cells did not show any measurable lysis of autologous or allogeneic LCL (Figure 1).

EBV-specific CD8+ CTL can be generated reproducibly from EBV-seronegative children by stimulation with LCL in the presence of rhIL-7 and rhIL-12

Based on previous observations from our group in the setting of leukemia-directed CTL activation (27) and BK virus-specific CD8+ CTL expansion (24), we proceeded to determine whether we could boost EBV-specific CD8 CTL induction through the synergistic action of rhIL-12, which has been demonstrated to enhance T-cell cytotoxicity (28), coupled with rhIL-7, a cytokine that promotes CD8+ T-cell proliferation and survival (29). Using this procedure, EBV-specific CD8+ CTL were successfully generated from all six EBV-seronegative donors.

Cell lines activated from the six EBV-seronegative donors showed a median expansion at 4 weeks of 12-fold (range 3.9–24). Phenotypic analysis indicated that the majority of cells were CD3+ CD8+ (Table 2). Among the CD3+CD8+ T-cell population, only a small minority, comparable to that observed in healthy seropositive controls, showed a CCR7+ CD45RA+ naïve phenotype (in line with the 7 ± 2% T cells that were found to be CD8+/CD45RA+/CD45RO−), while the numbers of CCR7− CD45RA− effector memory and CCR7− CD45RA+ terminal effector CD8+ T cells were 53 ± 14% and 35 ± 18%, respectively (Table 3). While only 3.5 ± 3% of CD8+ CTL in the seropositive controls were CD45RA+/CD45RO+, we found 28.5 ± 7% CD45RA/RO double positive CD8+ cells in the CTL activated from EBV-seronegative patients. Overall, the total

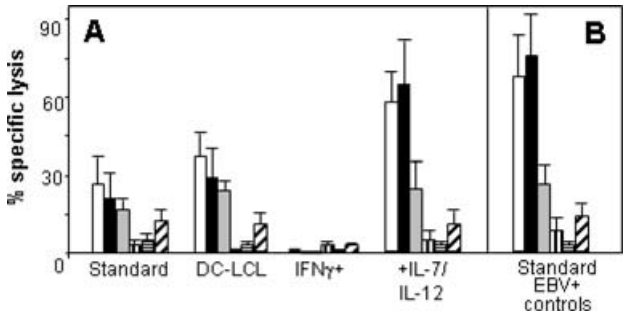

Figure 1: Comparison of EBV-specific cytotoxicity mediated by CTL lines activated *in vitro* with different stimulation protocols. CTL lines reactivated from the six EBV-seronegative patients with the four stimulation protocols (panel A) or from seven EBV-seropositive healthy controls with standard method (panel B) were tested in a chromium release assay against autologous LCL (white bars), HLA-mismatched LCL (vertically striped bars), autologous PHA blasts (horizontally striped bars), or Daudi cells (diagonally striped bars). Cytotoxicity of CD8+ (black bars) and CD4+ (grey bars) T cells positively selected from bulk cultures is also reported. Results of each experiment represent mean cytotoxicity at a 10:1 E:T ratio. Each bar represents the mean cytotoxicity of the 6 EBV-seronegative or 7 EBV-seropositive subjects ± SD.

Table 3: Memory subsets of CTL lines generated from EBV-seronegative patients using different activation protocols

	On CD8+						On CD4+				
	CD69	CD62L	CCR7+ CD45RA+	CCR7+ CD45RA−	CCR7− CD45RA−	CCR7− CD45RA+	CD69	CCR7+ CD45RA+	CCR7+ CD45RA−	CCR7− CD45RA−	CCR7− CD45RA+
Seronegative patients											
Standard	32 ± 10*	6 ± 3	9 ± 12	8 ± 8	60 ± 15	23 ± 14	29 ± 13	8 ± 7	11 ± 9	71 ± 16	10 ± 12
DC-LCL	38 ± 15	ND	24 ± 33	7 ± 4	38 ± 21	30 ± 11	25 ± 18	12 ± 8	11 ± 7	53 ± 4	22 ± 15
IFN-γ +	10 ± 6	ND	52 ± 8	15 ± 12	26 ± 12	7 ± 7	63 ± 29	19 ± 10	18 ± 12	49 ± 33	14 ± 8
+IL7/IL12	53 ± 19	7 ± 5	7 ± 4	7 ± 6	53 ± 14	35 ± 18	11 ± 7	11 ± 4	13 ± 7	68 ± 10	10 ± 8
Seropositive donors											
Standard	43 ± 20	18 ± 8	4 ± 4	7 ± 7	79 ± 18	10 ± 7	10 ± 7	6 ± 8	12 ± 9	69 ± 14	13 ± 6

*Data are reported as % positive cells.

proportion of CD8+ T-cell effectors (88%) was comparable to that observed in healthy EBV-seropositive controls (89%). However, while the majority of EBV-specific CD8+ effectors in the EBV-seropositive individuals are CD45RA− (79 ± 18%), in our EBV-seronegative donors >40% virus-specific CD8+ CTL were CD45RA+ (Table 3). The number of CD8+ T cells that were also perforin+ in the seronegative cohort were 51 ± 15%, as compared to 65 ± 17% in the healthy seropositive controls.

In all cases, the CTL lines showed strong lysis of the autologous LCL [57 ± 16% mean lysis at effector:target (E:T) ratio of 10:1], while little or no reactivity was observed against HLA-mismatched LCL (5 ± 2% mean lysis), HLA-mismatched PHA blasts (mean lysis <1%) or autologous PHA blasts (2.5 ± 1% mean lysis). Lysis of Daudi cell line at the same E:T ratio was found to be 10 ± 7%, comparable to what observed in healthy controls (Figure 1). Cytotoxicity was mainly CD8+ T-cell-mediated, and was reduced in the presence of anti-HLA class I mAb, although some CD4+ T-cell-mediated lysis of autologous LCL, blocked by anti-HLA class II mAb, was also observed (Figures 1 and 2). The median increase of cytotoxicity mediated by CD8+ T cells observed in the five EBV-seronegative children was 5-fold (from a mean lysis of 14 ± 10% with the standard protocol to 66 ± 30% with the addition of rhIL-7 and rhIL-12 at a E:T ratio of 10:1).

As suggested by the cytotoxicity assay, cells recovered from cell cultures and stimulated for 24 h with autologous LCL showed high levels of IFN-γ production in a ELISPOT assay (mean value of 387 ± 103 spots/10^5 cells) (Figure 3).

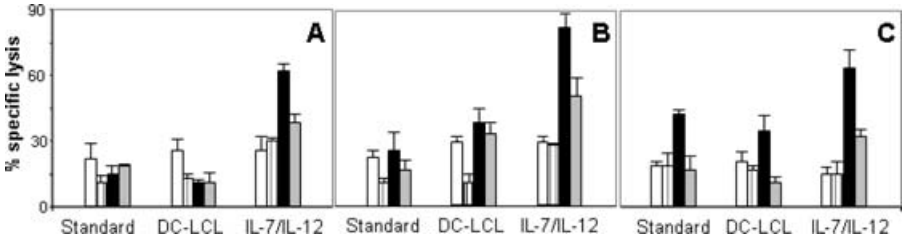

Figure 2: Stimulation with LCL in the presence of rhIL-12 and rhIL-7 activates EBV-specific CTL endowed with high CD8+, HLA-class I-restricted cytotoxic activity from both EBV-seronegative adult and children donors. CD4+ (white bars) and CD8+ (black bars) T cells isolated from bulk cultures of EBV-specific CTL activated from two EBV-seronegative children (panel A: patient 2; panel B: patient 1) and from the EBV-seronegative adult (panel C) were tested against autologous LCL. Cytotoxicity of CD4+ T cells in the presence of anti-HLA class II monoclonal antibody (striped bars) and of CD8+ T cells in the presence of anti-HLA class I monoclonal antibody (gray bars) is also shown. Results represent mean cytotoxicity at a 10:1 E:T ratio ± SD.

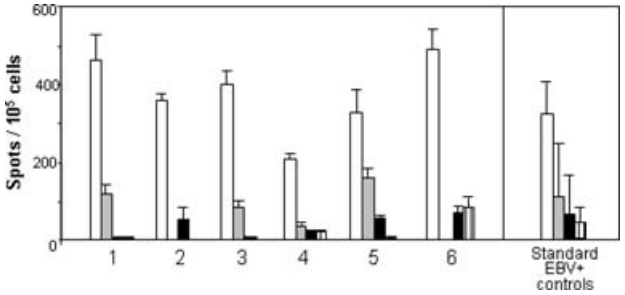

Figure 3: Antigen specificity of the EBV-specific CTL activated from the five EBV-seronegative individuals by LCL stimulation in the presence of rhIL-12 and rhIL-7. The frequency of IFN-γ-secreting lymphocytes was measured in bulk EBV-specific CTL, in response to EBV LCL (white bars), EBV EBNA3A peptides (grey bars), and EBV LMP-2 protein peptide mix (black bars) and BMLF peptide GLC (striped bars). On the horizontal axis, results from the six consecutive patients and from healthy controls are reported. IFN-γ-secreting cells are represented as number of spots/10^5 T cells (mean spots of triplicate experiments).

In order to determine the EBV protein specificity of the primed EBV-specific cell lines, we analyzed IFN-γ release in response to EBNA3 and LMP2 peptides in a ELISPOT assay. We observed specific responses to the EBNA3 peptides in the cell lines from 4/5 HLA-A02, and/or B08, and/or B35 EBV-seronegative donors, while IFN-γ production in response to challenge with a 15-mer peptide mix spanning the LMP2 protein sequence was observed in the cell lines from 4/6 EBV-seronegative donors.

Discussion

EBV-seronegative transplant recipients are at high risk for the development of fatal EBV-associated PTLD, and would maximally benefit from an EBV-directed T-cell therapy. In the case of SOT recipients, EBV-specific CTL could be re-activated from patients at the time of diagnosis (11–12). However, in some cases, high viral load in peripheral blood B-cells hampers LCL generation (12) or, more importantly, the rapid progression of PTLD precludes the possibility of waiting the time necessary for the expansion procedure. Therefore, CTL preparation prior to EBV infection could represent a useful tool in the EBV-seronegative transplant cohort. The standard method, successfully employed to reactivate EBV-specific CTL from virus-seropositive individuals, fails to efficiently expand EBV-specific, HLA class I-restricted CD8+ CTL with high cytotoxic capacity from virus-seronegative individuals (4,16,18). Recently, two studies have demonstrated successful generation of EBV-specific CTL lines displaying CD8+ T-cell-mediated cytotoxicity from adult EBV-seronegative subjects, either by the standard protocol or by stimulation with DC loaded with apoptotic/necrotic LCL (18–19). These findings were either attributed to the presence of environmental antigen-specific CTL precursors cross-reacting with EBV antigens or to failure to mount a serological response upon previous EBV exposure (18). In line with these data, we could also activate and expand EBV-specific CTL exerting HLA class I-restricted CD8 T-cell-mediated lysis from the virus-seronegative adult included in the study, using both the standard protocol or stimulation with LCL-loaded DC. The cytotoxicity of these CTL, however, was lower than that observed in healthy seropositive individuals. Thus, their capacity of preventing development and/or progression of PTLD may not be optimal.

No protocol has so far succeeded in priming and expanding EBV-specific CD8+ CTL lines from seronegative children, who include the highest percentage of EBV-naïve individuals. Savoldo et al. succeeded in activating CTL from seronegative children by stimulating with autologous LCL and selecting activated CD25+ T cells at day 9–11 after priming (18). The majority of these CTL were CD4+ and HLA class II-restricted, a finding likely indicating primary activation in true naïve pediatric subjects (16). These cells, which likely play a central role in inhibiting proliferation of newly EBV-infected B cells (20), and might contribute help to the CD8+ CTL response, have not been proven to control established PTLD *in vivo*. Therefore, we elected to

develop a method that could allow activation and expansion of CD8+ CTL. As already shown by Savoldo et al. (18), both standard method and stimulation with LCL-loaded DC alone failed to reproducibly expand CD8+ CTL from EBV-seronegative children. The latter observation may have been due to a suboptimal maturation of DC, though similar results have also been shown by others (19). Similarly, selection of the small fraction of IFN-γ + cells, activated upon stimulation with LCL, did not result in an expansion of the CD8+ CTL subset nor in the enhancement of specific cytotoxicity. Rather, in comparison to the other activation protocols, these CTL lines included the highest percentages of naïve CD4+ and CD8+ T cells.

Metes et al. and Popescu et al. reported increased numbers of CD8+ T cells in EBV-specific CTL activated from adult EBV-seronegative individuals by stimulation with LCL or LCL-loaded DC in the presence of rhIL-12 (19,30). However, the specific cytotoxicity exerted by those CTL, reactivated from EBV-seronegative adults, was of small magnitude. Since, in a previous work, we demonstrated that only the association of rhIL-12 with rhIL-7 was able to consistently induce the priming of leukemia-reactive CD8+ CTL in naïve donors (27), we elected to combine rhIL-7 with rhIL-12 in the LCL priming phase, to evaluate whether we obtained activation of CD8+ EBV-specific CTL endowed with cytotoxic potential comparable to that observed in EBV-seropositive individuals. With this modified approach, we could obtain EBV-specific CD8+ CTL that exerted optimal levels of CD8+ T-cell-mediated cytotoxicity from all EBV-seronegative children tested. These results are noteworthy, since the pediatric seronegative subjects we employed were not healthy immunocompetent children, but patients with end-stage renal failure undergoing dialysis, a condition that has been associated with immune deficiency (31). In addition, priming with LCL in the presence of rhIL-12 and rhIL-7 generated EBV-CTL with a higher specific cytotoxicity also from the EBV-seronegative adult, likely due to the higher number of perforin+ CD8+ T lymphocytes (data not shown) included in the activated CTL. The presence of the two cytokines similarly favors EBV CTL activation by LCL-loaded DC (data not shown). However, since cross-presentation of EBV antigens by DC does not seem mandatory in our *in vitro* system, different from that observed *in vivo* (32), the use of LCL represents a simpler strategy for expansion of EBV-specific CTL, since DC preparation requires larger amounts of blood, which may be difficult to obtain from children.

Among the effectors contained in the CTL lines activated with our approach, we observed a higher number of CCR7− CD45RA+ CD8+ T cells than that commonly observed in EBV-specific CTL reactivated from seropositive individuals. While cells belonging to this subset have often been considered terminally differentiated effectors (33), it has been recently proposed that EBV-specific CD8+ CD45RO+ T cells may reexpress CD45RA antigen after resolution of primary infection, and that these CD45RA re-expressing cells are apoptosis-resistant memory CD8+ T lymphocytes (34). Therefore, the CCR7−/CD45RA+/CD8+ lymphocytes we observed could be the *in vitro* counterpart of CD45RA reexpression after primary EBV infection *in vivo*.

In conclusion, we demonstrate that it is possible to reproducibly generate and expand EBV-specific CD8+ CTL endowed with strong cytotoxic activity from truly EBV-seronegative children, by means of autologous LCL stimulation in the presence of rhIL-12 and rhIL-7. The CTL thus obtained, whose specific activity is comparable to that observed in healthy seropositive controls, seem suitable for use in T-cell therapy protocols for prevention or treatment of PTLD after HSCT and SOT. In particular, the presence of memory T-cell subsets should guarantee long-term restoration of specific immunity in HSCT recipients, while the excellent expansion potential of these CTL offers the possibility to infuse high numbers of CD8+ effectors mediating optimal cytotoxic activity. This point might be crucial for clinical response in the case of SOT recipients, since the immunosuppressive drugs administered to these patients exert a stronger inhibition of CTL activation and expansion, rather than cytotoxic activity (35–36).

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