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Monocyte-Derived Dendritic Cells Generated After a Short-Term Culture with IFN-α and Granulocyte-Macrophage Colony-Stimulating Factor Stimulate a Potent Epstein-Barr Virus-Specific CD8$^+$ T Cell Response[1]

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Cellular immune responses are crucial for the control of EBV-associated lymphoproliferative diseases. To induce an anti-EBV cell-mediated immunity, we have used dendritic cells (DCs) generated by a 3-day culture of human CD14$^+$ monocytes in the presence of GM-CSF and type I IFN (IFN-DCs) and pulsed with peptides corresponding to CTL EBV epitopes. The functional activity of IFN-DCs was compared with that of APCs differentiated by culturing monocytes for 3 days with GM-CSF and IL-4 and indicated as IL-4-DCs. Stimulation of PBLs from EBV-seropositive donors with EBV peptide-pulsed autologous IFN-DCs resulted in a stronger expansion of specific T lymphocytes producing IFN-γ with respect to stimulation with peptide-loaded IL-4-DCs, as assessed by ELISPOT assays. When purified CD8$^+$ T cells were cocultured with EBV peptide-pulsed IFN-DCs or IL-4-DCs, significantly higher levels of specific cytotoxic activity were observed in CD8$^+$ T cell cultures stimulated with IFN-DCs. Injection of peptide-pulsed IFN-DCs into SCID mice transplanted with autologous PBLs led to the recovery of a significantly greater number of EBV-specific human CD8$^+$ T cells from the spleen and the peritoneal cavity with respect to that recovered from mice injected with peptide-pulsed IL-4-DCs. Moreover, a significant delay in lymphoma development was observed when peptide-pulsed IFN-DCs were injected into SCID mice reconstituted with PBMCs endowed with a high capability of lymphoma induction, whereas injection of unpulsed IFN-DCs was ineffective. Our results indicate that IFN-DCs efficiently promote in vitro and in vivo the expansion of CD8$^+$ T lymphocytes acting as cytotoxic effectors against EBV-transformed cells. ***The Journal of Immunology,*** **2003, 170: 5195–5202.**

Epstein-Barr virus is an ubiquitous human γ-herpesvirus that persists for life by establishing a latent infection in resting memory B cells (1). EBV is the etiologic agent of infectious mononucleosis and has been associated with a number of human malignancies, including Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease, and lymphoproliferative disorders in immunosuppressed individuals (1). The cell-transforming ability of EBV is believed to be determined by the coordinated expression of latent genes (1, 2). In particular, EBV-associated posttransplant lymphoproliferative disorders (PTLD)[4] of B cells arising in transplant recipients are characterized by the expression of the full set of latent Ags, i.e., the EBV-encoded nuclear Ags (EBNAs), leader protein nuclear Ag, and the latent membrane protein (LMP)-1 and LMP-2 latent membrane Ags (2). The crucial importance of EBV-specific CTLs, and in particular of CD8$^+$ T cell effectors recognizing EBNA Ags, for the control of this type of latent infection is strongly suggested by much evidence. In fact, EBV-associated PTLD usually arise as a consequence of the impairment of T cell-mediated immunity caused in transplant patients by the immunosuppressive therapy (2), and complete regression of PTLD can be achieved by reducing or discontinuing the immunosuppressive regimen (3). Furthermore, adoptive transfer of EBV-specific CTLs in bone marrow transplant patients has been reported to be effective for the prophylaxis and treatment of immunoblastic lymphoma (4–6).

The use of Ag-loaded dendritic cells (DCs) is now considered a particularly attractive strategy for ex vivo as well as in vivo elicitation of CTL responses. Recent studies have indicated that DCs loaded with EBV Ags can represent more physiological and efficient APCs for the in vitro expansion of EBV-specific T cells with respect to the EBV-transformed lymphoblastoid cell lines (LCL) (4) currently used (7–11). Fully mature DCs proved to be powerful stimulators of EBV-specific T cell responses (7–11), whereas immature DCs were not as efficient (9, 10).

Recently, a single-step 3-day culture of human monocytes in the presence of GM-CSF and type I IFN was reported to lead to the differentiation of APCs (IFN-DCs) exhibiting phenotypic and functional properties typical of activated partially mature DCs (12, 13). In particular, IFN-DCs have been shown to strongly promote a Th cell type 1 response and to induce a potent primary immune response in chimeric SCID mice reconstituted with human PBLs (12, 13). However, the capability of IFN-DCs to stimulate Ag-specific CD8$^+$ T cell responses has not been addressed yet. In the

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[4] Abbreviations used in this paper: PTLD, posttransplant lymphoproliferative disorder; BMLF-1, product of *Bam*HI-M leftward open reading frame-1; DC, dendritic cell; EBNA, EBV-encoded nuclear Ag; HS, human serum AB; hu, human; LCL, lymphoblastoid cell line; LMP, latent membrane protein.

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present study, we asked whether IFN-DCs pulsed with HLA class I-restricted EBV-derived peptides could efficiently expand in vitro EBV-specific $CD8^+$ T cell precursors and CTL effectors. We also evaluated the ability of EBV peptide-pulsed IFN-DCs to elicit in vivo a cell-mediated immunity capable of protecting SCID mice from the development of EBV-induced immunoblastic B cell lymphomas, occurring in these animals after transplantation of PBMCs from EBV-positive donors and closely resembling EBV-associated lymphoproliferative disorders arising in immunocompromised individuals (14). In this study, we show that IFN-DCs efficiently stimulate both in vitro and in vivo EBV-specific $CD8^+$ T lymphocytes acting as cytotoxic effectors.

Materials and Methods

Donors

LL, FZ, FB, and AB were healthy EBV-seropositive donors, as determined by the standard ELISA (DiaSorin, Saluggia, Italy). All donors were typed according to standard HLA serotyping procedures.

Cell lines

LCL were derived from PBMCs of donors, using the supernatant of the marmoset line B95-8 added in the presence of 1 μg/ml cyclosporin A in RPMI 1640 (EuroClone, Paignton, U.K.) supplemented with 10% FCS, 2 mmol/L L-glutamine, 100 IU/ml penicillin/streptomycin, 1 mM sodium pyruvate, 1 mM nonessential amino acids, and 10 mM HEPES (all from EuroClone). The T2 TAP-deficient HLA-A2-positive cell line was cultured in IMDM (EuroClone) supplemented with 10% FCS, 10^{-5} M 2-ME, L-glutamine, penicillin/streptomycin, sodium pyruvate, nonessential amino acids, and HEPES (EuroClone). All cell lines were tested for the absence of mycoplasma by *Mycoplasma* PCR ELISA (Roche Diagnostics Gmbh, Mannheim, Germany).

DCs and T cells

Separation of monocytes and PBLs was obtained by standard Ficoll/Percoll density gradient centrifugation. DC preparations were obtained, as previously described (12). Briefly, DCs were generated from immunomagnetically purified $CD14^+$ monocytes (MACS Monocyte Isolation Kit; Miltenyi Biotec, Bergisch Gladbach, Germany) by 3-day culture in GM-CSF (500 U/ml) (PeproTech, London, U.K.) and either IL-4 (250 U/ml) (R&D Systems, Minneapolis, MN) or consensus IFN-α (1000 U/ml), a synthetic type I IFN kindly provided by Amgen (Thousand Oaks, CA).

For subset purification, $CD8^+$ T cells were positively isolated from Percoll-PBL by immunomagnetic CD8 microbeads using the MACS system (Miltenyi Biotec).

Peptides and peptide pulsing

The synthetic peptides used in this study correspond to HLA class I-restricted CTL epitopes of different EBV proteins (Table I). The influenza virus matrix protein 58-66 peptide was used as positive control of HLA-A2.1-restricted $CD8^+$ T cell responses. Peptides were synthesized by Tecnogen (Naples, Italy). Lyophilized peptides were dissolved in PBS-10% DMSO (stock concentration 500 μg/ml) and stored at −20°C. For peptide pulsing, stimulator or target cells were incubated with peptides (10 μg/ml) in serum-free RPMI 1640 medium for 3 h at 37°C, washed, and added to responder cells.

T cell stimulation

For PBL stimulation, peptide-pulsed DCs were seeded in replicate wells of 96-well plates at 10^4 cells/well in 100 μl of RPMI 1640 medium supplemented with 5% human serum AB (HS) (EuroClone), 2 mmol/L L-glutamine, 100 IU/ml penicillin/streptomycin, 1 mM sodium pyruvate, 1 mM nonessential amino acids, and 10 mM HEPES (complete RPMI 1640 medium). Responder PBLs (10^5 cells/well) were added in 100 μl of complete RPMI 1640 medium. For $CD8^+$ T cell stimulation, unpulsed or peptide-pulsed DCs (10^5 cells/well) were cocultured in 24-well tissue culture plates with $CD8^+$ purified cells (10^6 cells/well) in 2 ml of complete RPMI 1640 medium. Both PBL-DC and $CD8^+$-DC cultures were restimulated on days 7 and 14, as described for the first stimulation. Human rIL-2 (Collaborative Biomedical Products, Bedford, MA) was added on day 5 after the first stimulation (10 U/ml), and on day 3–5 after each restimulation (100 U/ml).

ELISPOT assay

ELISPOT assays were performed on day 0, before stimulation of donors' PBLs with peptide-pulsed DCs, and on days 7 and 14, i.e., 7 days after the first and the second stimulation, respectively. MultiScreen-HA plates (Millipore, Bedford, MA) were coated with 5 μg/ml of mouse capture mAbs anti-human IFN-γ or anti-human IL-4 (BD PharMingen, San Diego, CA) for 2 h at room temperature. Plates were then washed four times with PBS-0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO) and blocked with PBS-10% HS for 60 min at room temperature. Fresh PBLs (10^5 cells/well) were incubated with unpulsed or peptide-pulsed autologous monocytes (2×10^4 cells/well). DC-stimulated PBLs (2×10^4 or 5×10^4 cells/well) were incubated with unpulsed or peptide-pulsed autologous monocytes or T2 cells as APCs, at responder to stimulator ratios ranging from 1:1 to 5:1. As positive controls, the same number of cells was plated with 1 μg/ml PHA. After incubation at 37°C for 20 h (IFN-γ assay) and 48 h (IL-4 assay), plates were extensively washed with PBS-0.25% Tween 20, and incubated for 2 h at room temperature with 100 μl of 2 μg/ml biotinylated secondary mAbs anti-human IFN-γ or anti-human IL-4 (BD PharMingen). After extensive washing, 50 μl streptavidin-HRP conjugate (BD PharMingen) was added to the wells, and the plates were incubated for 90 min at room temperature. Colorimetric reaction was obtained using 3-aminoethylcarbazole solution (Sigma-Aldrich) as a substrate. The number of spots was automatically determined with the use of a computer-assisted video image analyzer (AID ELISPOT reader; AID GmbH, Strassberg, Germany). The number of peptide-specific T cells was calculated by subtracting the mean number of spots induced by unpulsed APCs from mean number of spots induced by peptide-pulsed APCs.

Cytotoxicity assays

$CD8^+$ responders were tested for their cytolytic activity on day 21 in standard 4-h ^{51}Cr release assays. Unpulsed or peptide-pulsed LCL were labeled with 100 μCi sodium-^{51}chromate (PerkinElmer Life Sciences, Boston, MA), extensively washed, and used as target cells (4000 targets/well) at various E:T ratios, as indicated. The percentage of specific ^{51}Cr release was calculated as follows: ((mean experimental cpm − mean spontaneous cpm)/(mean maximum cpm − mean spontaneous cpm)) × 100%. Spontaneous release was <20% of maximum release.

Table I. *Details of donor HLA typing and CTL epitope peptides from EBV proteins used in this study*

Donors and HLA Typing	CTL Epitope Sequence	EBV Protein (residues)	Abbreviation	HLA Restriction	Ref.
FZ: A3 A32 B35 B53	RLRAEAQVK	EBNA-3A (603–611)	RLR	A3	15
	YPLHEQHGM	EBNA-3A (458–466)	YPL	B35	16
	AVLLHEESM	EBNA-3B (488–496)	AVL	B35	17
FB: A11 A26 B7 B18	RPPIFIRRL	EBNA-3A (379–387)	RPP	B7	18
	IVTDFSVIK	EBNA-3B (416–424)	IVT	A11	19
	AVFDRKSDAK	EBNA-3B (399–408)	AVF	A11	19
LL: A2	VLQWASLAV	gp350 (842–850)	VLQ	A2	20
	GLCTLVAML	BMLF-1 (280–288)	GLC	A2	21
	CLGGLLTMV	LMP-2 (426–434)	CLG	A2	22
	SVRDRLARL	EBNA-3A (596–604)	SVR	A2	16
	LLDFVRFMGV	EBNA-3C (284–293)	LLD	A2	23

Immunophenotypic analysis

Fresh, unstimulated $CD8^+$ T cells and DC-stimulated $CD8^+$ T cells were washed, resuspended in PBS containing 1% HS, and incubated with fluorochrome-conjugated anti-human CD27 and anti-human CD45RO or anti-human CD45RA mAbs (BD PharMingen) for 30 min at 4°C. Cells were analyzed by flow cytometry. Data were collected and analyzed by using a FACSort (BD Biosciences, Bedford, MA) flow cytometer, and data analysis was performed by CellQuest software (BD Biosciences).

In vivo expansion of EBV-specific $CD8^+$ T cells and their quantification by IFN-γ ELISPOT assays

CB17 *scid/scid* female mice (Charles River, Italia Calco, Italy) were used at 4 wk of age. On day 0, monocytes and PBLs were separated from donor PBMCs by standard Ficoll/Percoll density gradient centrifugation. Freshly isolated PBLs were injected i.p. into recipient mice at 40×10^6 cells/mouse. On the same day, $CD14^+$ monocytes were immunomagnetically purified, and a portion of cells was frozen, whereas the rest was cultivated for 3 days in the presence of GM-CSF and IFN-α or IL-4 for DC differentiation. On day 3, two groups of mice were injected i.p. with peptide-pulsed IFN-DCs or IL-4-DCs (2×10^6 cells/mouse). One group of mice was left untreated as a control. Seven days later, DC-injected mice were given a boost dose (2×10^6 cells/mouse) of peptide-pulsed IFN-DCs or IL-4-DCs, differentiated from frozen $CD14^+$ monocytes. After 7 days, mice were sacrificed and total cells recovered from spleens or peritoneal lavages of each group of mice were pooled and stimulated in vitro with donor-derived peptide-pulsed LCL at a stimulator to responder ratio of 1:10. Three days later, the cocultures were restimulated by an overnight incubation with unpulsed or peptide-pulsed immature DCs, obtained after cultivation of donor monocytes with GM-CSF and IL-4 for 6 days, at a stimulator to responder ratio of 1:10. After incubation, human $CD8^+$ T cells were positively selected from the cocultures with MACS Micro Beads (Miltenyi Biotec GmbH) and tested in ELISPOT assays for the production of IFN-γ (EuroClone). Briefly, 100 μl of cell suspension containing 1×10^4 cells, in RPMI 1640 supplemented with 2 mM L-glutamine and 10% heat-inactivated FCS, was dispensed in each well of 96-well plates coated with anti-IFN-γ Ab. After an overnight incubation and cell lysis, trapped cytokine molecules were revealed by incubation with a secondary biotinylated detection Ab and developed by incubation with streptavidin-alkaline phosphatase and then with 5-bromo-4-chloro-3-indolyl phosphate substrate in a gel overlay. Colored spots were enumerated on an inverted microscope at a magnification of ×40. Data were expressed as IFN-γ spots/10^4 $CD8^+$ cells. Student's *t* test was used for calculating the significance of the differences in the number of EBV-specific $CD8^+$ T cells detected in the cell populations deriving from the various groups of mice.

Human (Hu)-PBL-SCID mouse model

CB17 *scid/scid* female mice (Charles River) were used at 4 wk of age. All work with animals conformed to European Community guidelines. PBMCs were obtained from peripheral blood of donors AB and FB, two monozygotic twins. On day −3, $CD14^+$ monocytes from donor AB were immunomagnetically purified and cultivated, as described above, in the presence of GM-CSF and IFN for 3 days. On day 0, PBMCs from donor FB were injected i.p. into recipient mice at 40×10^6 cells/mouse. Three hours later, mice were injected i.p. with unpulsed or peptide-pulsed IFN-DCs (2×10^6 cells/mouse). One group of mice was left untreated as a control of lymphomagenesis. Seven days later, IFN-DC-injected mice were given a boost dose of unpulsed or peptide-pulsed IFN-DCs. ANOVA test paired with Tukey-Kramer comparison was used for calculating the significance of the differences in the survival times of mice.

Results

Peptide-pulsed IFN-DCs efficiently stimulate the in vitro expansion of EBV-specific T cells

Table I reports the HLA typing of the three healthy EBV-seropositive donors and the sequence of the EBV peptides, known to be CTL epitopes (15–23), used in this study. In all the experiments described in this work, the DC preparations were generated from immunomagnetically purified $CD14^+$ monocytes by 3-day culture in GM-CSF and IFN (IFN-DCs) or IL-4 (IL-4-DCs). The DC preparations exhibited morphological and phenotypic features similar to those previously reported (12, 13).

In a first set of experiments, we evaluated the capability of IFN-DCs or IL-4-DCs to expand in vitro EBV-specific $CD8^+$ T cells from PBLs of the three donors. To this end, IFN-DCs or IL-4-DCs were pulsed with HLA class I-restricted EBV peptides (Table I) and used to stimulate autologous PBLs. The EBV peptide-specific T cell frequencies were analyzed in IFN-γ or IL-4 ELISPOT assays, performed on freshly isolated PBLs and after each of two subsequent stimulations with peptide-pulsed DCs. No IL-4-producing peptide-specific T cells were ever detected. In contrast, a significant expansion of peptide-specific T cells secreting IFN-γ was induced after the second PBL stimulation (Fig. 1). In the cases of donors FZ and FB, a stronger amplification of T cells reactive against all the selected EBNA-3A or EBNA-3B peptides was induced after stimulation of autologous PBLs with IFN-DCs as compared with IL-4-DCs. In the case of donor LL, a similar expansion of T cells specifically recognizing the product of *Bam*HI-M leftward open reading frame-1 (BMLF)-1 (GLC) and EBNA-3C (LLD) peptides was induced in the cultures stimulated by IFN-DCs or IL-4-DCs, with respect to the precursor frequencies detected in unstimulated cultures. In contrast, a significant increase in the frequencies of T cells reactive against the LMP-2 (CLG), EBNA-3A (SVR), and gp350 (VLQ) peptides was observed only in PBL cultures stimulated with IFN-DCs, with respect to the basal frequencies of these precursors. Only a weak expansion of T cell precursors specific for the LMP-2 (CLG), and no expansion of T cells reactive against the EBNA-3A (SVR) and gp350 (VLQ) peptides, was detected in PBL cultures stimulated with IL-4-DCs. Collectively, the results of the ELISPOT assays indicated that EBV peptide-pulsed IFN-DCs were highly efficient in stimulating the expansion in vitro of T cells reactive against EBV CTL epitopes.

Peptide-pulsed IFN-DCs induce EBV-specific CTLs and promote the expansion of memory $CD8^+$ T cells

We then evaluated the ability of IFN-DCs or IL-4-DCs to amplify in vitro EBV-specific CTL effectors. To this end, $CD8^+$ T cells purified from PBLs of donors LL and FB were stimulated with autologous IFN-DCs or IL-4-DCs pulsed with the relevant peptides. After three stimulations, the $CD8^+$ T cells were assayed for their cytotoxic activity against autologous LCL, or HLA-mismatched LCL in the case of donor LL, either pre-exposed or unexposed to the relevant peptide (Fig. 2). In the case of donor LL, we chose to stimulate the $CD8^+$ T cells with DCs pulsed with the BMLF-1 (GLC) peptide based on the results of the ELISPOT assays, revealing that this peptide was able to promote the best expansion of specific T cell precursors (Fig. 1). After stimulation of $CD8^+$ cells from donor LL with BMLF-1 peptide-pulsed DCs, similar levels of specific cytotoxic activity were exerted by cell lines stimulated with either IFN-DCs or IL-4-DCs (Fig. 2, *left panel*). Stimulation of $CD8^+$ cells from donor LL with BMLF-1 peptide-loaded DCs generated an effector population with a strong epitope-specific component, as indicated by both the levels of incremental lysis of autologous LCL targets preloaded with the BMLF-1 peptide over the lysis of unloaded autologous LCL and by the absence of specific lysis of peptide-pulsed allogeneic HLA-$A2^-$ LCL (Fig. 2, *left panel*). When $CD8^+$ cells from donor FB were repeatedly stimulated with autologous IFN-DCs or IL-4-DCs, both loaded with the same EBNA-3A- or EBNA-3B-derived peptides used for stimulation of PBLs, a strong expansion of an epitope-specific polyclonal effector population was observed exclusively in the cultures stimulated with peptide-pulsed IFN-DCs, as indicated by the levels of incremental lysis of autologous peptide-loaded LCL vs autologous unloaded targets (Fig. 2, *right panel*). The cultures of $CD8^+$ cells from donor FB were analyzed for the expression of CD45RO, CD45RA, and CD27 markers, before and after stimulation with IL-4- or IFN-DCs (Fig. 3). A similar

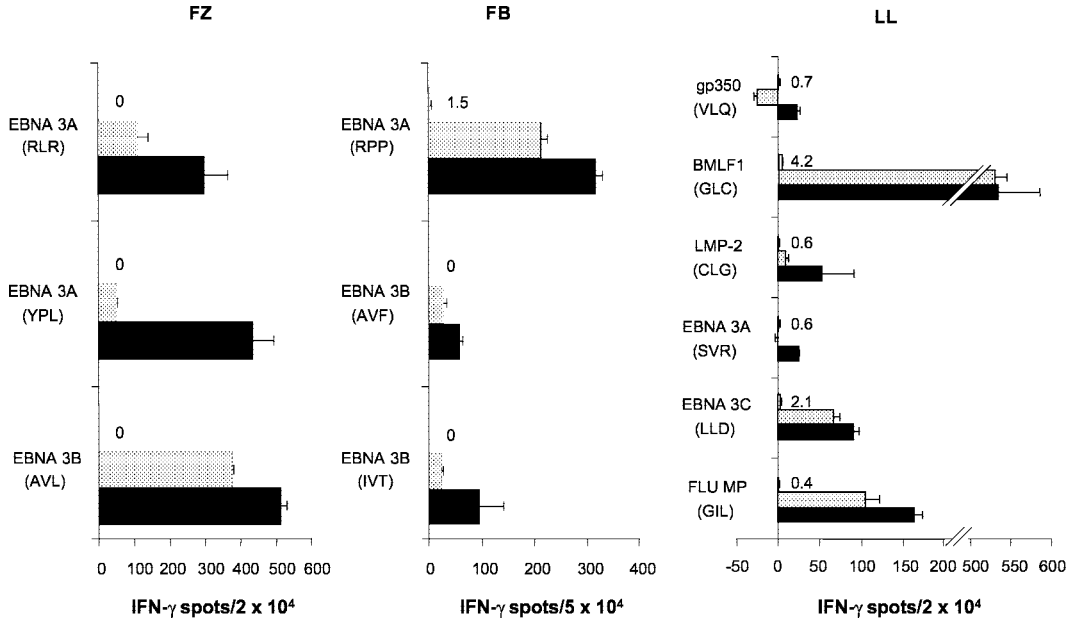

FIGURE 1. In vitro expansion of EBV-specific T cells by stimulation with peptide-pulsed IFN-DCs or IL-4-DCs. IFN-DCs or IL-4-DCs derived from EBV-seropositive donors were pulsed with the indicated HLA class I-restricted EBV peptides and cocultured with autologous PBLs. On day 7, T cells were restimulated with peptide-pulsed DCs, generated from cryopreserved monocytes. The figure shows the frequencies of peptide-reactive T cells evaluated in IFN-γ ELISPOT assays performed on day 0 (open bars and numbers), using freshly isolated PBLs as responder cells and autologous monocytes as peptide-presenting cells, and 7 days after the first restimulation of PBLs with autologous peptide-pulsed IFN-DCs (filled bars) or IL-4-DCs (dotted bars) (day 14), using autologous monocytes (donors FZ and FB) or T2 cells (donor LL) as peptide-presenting cells. Each bar represents the mean spot number of triplicates ± SD per 2×10^4 or 5×10^4 responder cells. The number of peptide-reactive cells was calculated by subtraction of mean spot numbers induced by autologous unpulsed monocytes or T2 cells from mean spot numbers induced by peptide-pulsed presenting cells. One representative experiment of three is shown.

increase in the percentage of $CD8^+$ cells with a memory phenotype ($CD45RA^-CD27^+$) was observed after two rounds of stimulation with peptide-pulsed IL-4-DCs (*E*) or IFN-DCs (*F*), with respect to unstimulated $CD8^+$ cells (*B*) and $CD8^+$ cells stimulated with unpulsed IL-4-DCs (*C*) or IFN-DCs (*D*). In the cultures stimulated with unpulsed DCs, the number of $CD8^+$ cells rapidly declined after the second stimulation, preventing their further stimulation and phenotypic analysis. Interestingly, higher levels of $CD45RA^-$ $CD27^+$ cells were detected in $CD8^+$ cultures subjected to three stimulations with peptide-pulsed IFN-DCs (*H*) as compared with cultures stimulated with peptide-pulsed IL-4-DCs (*G*), in which a sharp decrease in the percentage of $CD8^+$ cells with memory phenotype was observed after the third stimulation. Collectively, these observations suggest that IFN-DCs can efficiently promote in vitro the expansion and survival of memory phenotype $CD8^+$ cells acting as cytotoxic effectors.

Injection of peptide-pulsed IFN-DCs into hu-PBL-SCID mice promotes the in vivo expansion of EBV-specific CD8+ T cells and limits the development of EBV-associated lymphomas

Finally, we asked whether IFN-DCs loaded with EBV peptides could efficiently expand in vivo EBV-specific $CD8^+$ T lymphocytes capable of inhibiting lymphomagenesis in the hu-PBL-SCID

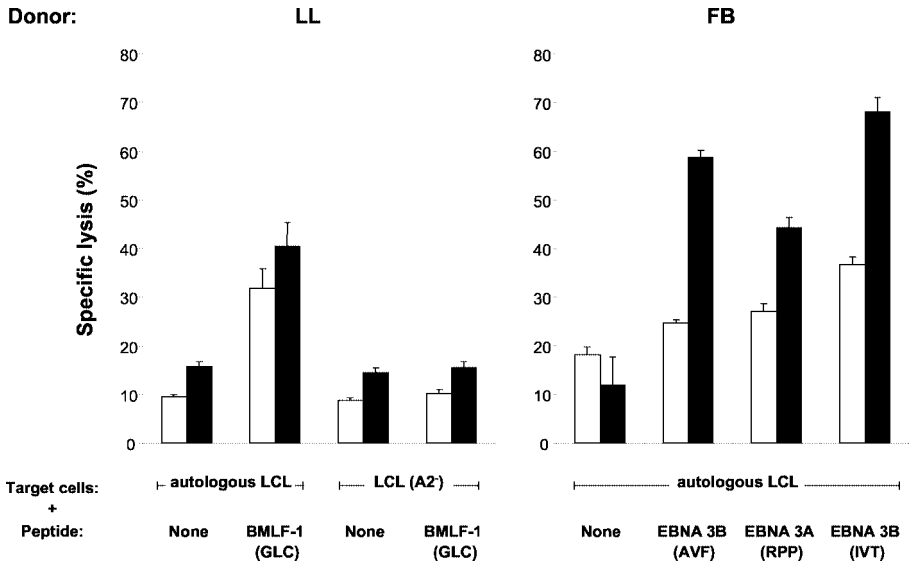

FIGURE 2. Cytotoxic activity of $CD8^+$ T cells stimulated with peptide-pulsed IFN-DCs or IL-4-DCs. $CD8^+$ T lymphocytes were immunomagnetically purified from PBLs and stimulated on days 0, 7, and 14 with peptide-pulsed IFN-DCs (filled bars) or IL-4-DCs (open bars) at a stimulator to responder ratio of 1:10. Seven days after the third stimulation, $CD8^+$ cells were assayed for cytotoxic activity against autologous or allogeneic unpulsed or peptide-pulsed LCL in standard 4-h ^{51}Cr release assays. Results are expressed as percentage of specific lysis at the 10:1 (donor LL) or 20:1 (donor FB) E:T ratio. One representative experiment of three is shown.

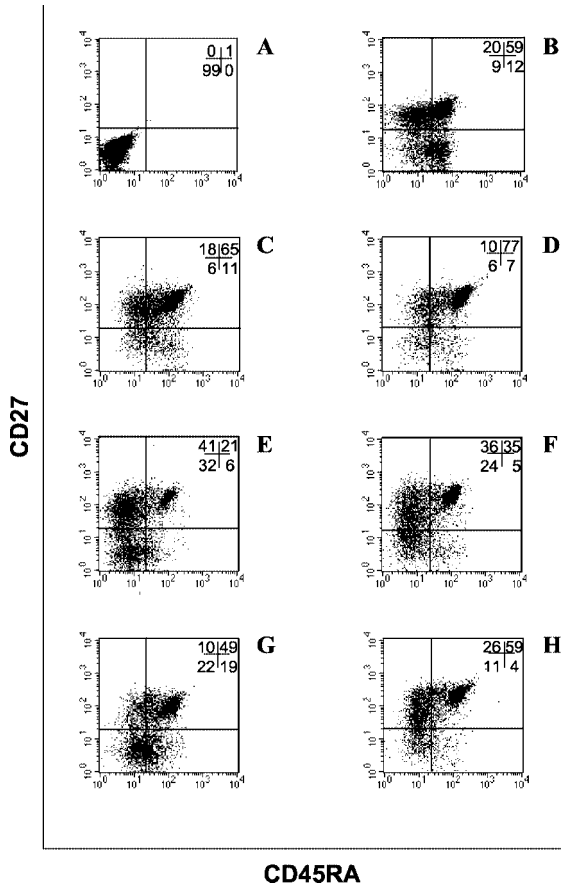

FIGURE 3. Sustained expansion of $CD8^+$ T cells with a memory phenotype after stimulation with peptide-pulsed IFN-DCs. $CD8^+$ T lymphocytes purified from donor FB PBLs were stimulated with unpulsed or peptide-pulsed IFN-DCs or IL-4-DCs and double stained with anti-CD27 and anti-CD45RA or anti-CD45RO Abs. In all cases, the expression of CD45RO and CD45RA was found to be mutually exclusive. *A*, Representative dot-plot FACS analysis of $CD8^+$ T lymphocytes stained with isotype-matched control Abs. *B–H*, Dot-plot FACS analysis of $CD8^+$ T lymphocytes stained with anti-CD45RA and anti-CD27 Abs at the following time points: before stimulation (*B*): 7 days after the second stimulation with unpulsed IL-4-DCs (*C*), unpulsed IFN-DCs (*D*), peptide-pulsed IL-4-DCs (*E*), peptide-pulsed IFN-DCs (*F*); 7 days after the third stimulation with peptide-pulsed IL-4-DCs (*G*) or IFN-DCs (*H*).

Table II. *Effect of injection of EBV peptide-pulsed IFN-DCs on lymphoma development in SCID mice transplanted with PBMCs from donor FB*[a]

Vaccine	Mean Day of Death (± SD)	
None	58.2 (± 9.7)	NS
Unpulsed IFN-DCs	65.0 (± 16.9)	NS
Peptide-pulsed IFN-DCs	90.6 (± 2.3)	$p < 0.01$

[a] CB17 *scid/scid* female mice were reconstituted with 4×10^7 PBMCs from donor FB. Three hours after reconstitution, the mice were divided into three groups. The first group of mice received no further treatment, whereas mice in the second and third groups were injected i.p. with, respectively, 2×10^6 unpulsed IFN-DCs or IFN-DCs pulsed with a pool of EBNA-3A- and EBNA-3B-derived peptides. These peptides were the same used for in vitro stimulations of donor FB PBL (see Fig. 1). Seven days later, a boost dose (2×10^6 cells) of unpulsed or peptide-pulsed IFN-DCs was injected i.p. in the second and third groups of mice, respectively. The DCs utilized in this experiment were derived from $CD14^+$ monocytes obtained from donor AB, the identical twin of donor FB. There were six mice per group. All mice eventually died of lymphomas, as assessed at necropsy.

chimeric model (14). To answer this question, we chose to reconstitute SCID mice with peripheral blood cells isolated from donor FB, as preliminary experiments had indicated that i.p. injection of SCID mice with 40×10^6 PBMCs from this donor led to the development of lymphomas in the totality of the injected animals in ~7–8 wk, placing FB in the high-incidence donor category (24). Two i.p. injections of peptide-pulsed IL-4-DCs or IFN-DCs into SCID mice transplanted with autologous PBLs led to the recovery of EBV-specific human $CD8^+$ T cells from the spleen and the peritoneal cavity of the treated mice (Fig. 4). However, the number of EBV-specific $CD8^+$ cells recovered from mice injected with peptide-pulsed IFN-DCs was significantly greater than that rescued from mice given peptide-pulsed IL-4-DCs, in both the spleen and the peritoneal cavity (Fig. 4). To assess whether injection of peptide-pulsed IFN-DCs could affect EBV-associated lymphomagenesis, SCID mice were first reconstituted with total PBMCs from donor FB and then given two subsequent i.p. injections of unpulsed or peptide-pulsed IFN-DCs, differentiated from $CD14^+$ monocytes of the monozygotic twin, AB, of donor FB. Table II shows that two i.p. injections of EBV peptide-pulsed IFN-DCs into SCID mice previously reconstituted with syngeneic PBMCs caused a highly significant prolongation of survival time as compared with what observed for unvaccinated SCID mice and

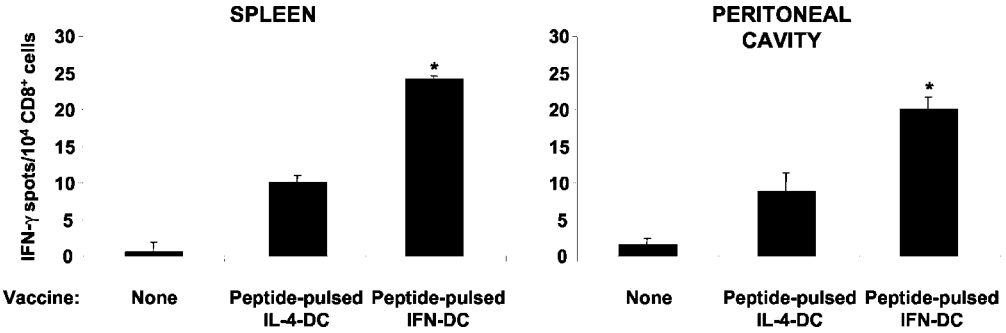

FIGURE 4. In vivo expansion of EBV-specific $CD8^+$ T cells after injection of peptide-pulsed IFN-DCs or IL-4-DCs into SCID mice transplanted with human PBLs. Fresh PBLs isolated from donor FB were injected i.p. into SCID mice at 40×10^6 cells/mouse. Three days later, two groups of mice were injected i.p. with IFN-DCs or IL-4-DCs (2×10^6 cells/mouse) autologous to the PBLs, both pulsed with the same pool of EBNA-3A- and EBNA-3B-derived peptides used for in vitro stimulation of donor FB PBLs (see Fig. 1). The third group of mice was left untreated as a control. Seven days later, DC-injected mice were given a boost dose (2×10^6 cells/mouse) of peptide-pulsed IFN-DCs or IL-4-DCs. After seven days, mice were sacrificed, and total cells recovered from spleens or peritoneal lavages of each group of mice were pooled and stimulated in vitro, as described in *Materials and Methods*. The figure shows the number of peptide-specific human $CD8^+$ T cells detected in IFN-γ ELISPOT assays after in vitro restimulation with unpulsed or peptide-pulsed immature DCs, derived from donor FB. Each bar represents the mean spot number ± SD per 10^4 human $CD8^+$ T cells. The number of peptide-specific cells was calculated by subtraction of mean spot numbers induced by autologous unpulsed DCs from mean spot numbers induced by peptide-pulsed DCs. *, $p < 0.01$ vs the number of EBV-specific $CD8^+$ T cells derived from mice injected with peptide-pulsed IL-4-DCs.

for mice vaccinated with unpulsed IFN-DCs. These results indicated that IFN-DCs were efficient in stimulating the in vivo expansion of EBV-specific $CD8^+$ T lymphocytes capable of limiting the development of EBV-associated lymphomas.

Discussion

Adoptive transfer of EBV-specific CTL lines hold particular promise as treatment option for therapy or prophylaxis of PTLD in bone marrow or solid organ transplant recipients (4–6, 25, 26). Usually, the EBV-specific CTL lines are obtained by repeated in vitro stimulation of PBMCs with autologous LCL, a process requiring 4–6 wk and leading to a preferential expansion of T cells recognizing dominant EBV Ags, in particular EBNA-3A, EBNA-3B, and EBNA-3C proteins (27). In both the therapeutic and prophylactic treatment of transplant recipients with EBV-specific CTLs, the adoptive transfer of CTL lines containing a wide spectrum of anti-EBV T cell reactivities would be preferable to achieve a more effective control of EBV-transformed B cells in vivo and to reduce the possibility of immune escape by growing tumors. The in vitro expansion of the individual's EBV-specific T cell repertoire may be optimally induced by physiologic APCs loaded with EBV Ags ex vivo. Importantly, such APCs could be used to actively immunize patients at risk of developing PTLD. The active immunization approach would be particularly useful to induce a protective immunity to EBV in seronegative patients before the transplant as well as in solid-organ transplant recipients, in which the EBV lymphoproliferative lesions are generally of recipient origin and, therefore, transfer of donor-derived EBV-specific T cells is not a treatment option (1, 2). Moreover, active immunization might better promote the development of a long-lived T cell memory with respect to adoptive transfer of T cell lines repeatedly stimulated in vitro and mainly consisting of effector type cells committed to die rapidly after Ag encounter in vivo. Although persistence of EBV-specific T cells for relatively long period of times has been observed after adoptive transfer of CTLs (5), protective levels of memory T cells may last longer in actively immunized patients, who would be able to repeatedly generate CTLs after in vivo exposure to EBV Ags.

DCs are at present considered the best APC candidates for the development of immunotherapy strategies against chronic infectious diseases as well as malignancies. In fact, DCs are the most potent type of APCs, due to their optimal ability to initiate and modulate the immune response (28). The use of DCs as APCs for the in vitro expansion of EBV-specific T cells has been supported by recent studies, in which EBV Ags were loaded onto DCs using different modalities, such as peptide pulsing (7), infection with recombinant viral vectors (8, 9), pulsing with LCL cell lysates (10), or exposure to necrotic or apoptotic LCL (11). In all of these studies, mature DCs efficiently promoted the expansion of EBV-specific T cells with cytotoxic activity and, when compared with immature DCs (9, 10), exhibited a superior stimulatory activity.

In our study, we evaluated the capability of IFN-DCs pulsed with EBV-derived CTL epitope peptides to act as efficient stimulators of EBV-specific $CD8^+$ T cell precursors and CTL effectors. The potential of IFN-DCs as highly active APCs for the priming of a Th1 immune response has been recently indicated (12, 13), but no evidence of their capability to induce Ag-specific $CD8^+$ T cell responses was yet available. Our results indicate that IFN-DCs efficiently promote the expansion of Ag-specific IFN-γ-producing memory $CD8^+$ T cells. In fact, for the majority of the EBV CTL epitopes tested, strong increases in the frequencies of specific IFN-γ-producing T cell precursors were obtained after two stimulations of PBLs with peptide-pulsed IFN-DCs, with respect to the basal levels detected in unstimulated PBLs (Fig. 1). Particularly high frequencies of T cells reactive to the EBNA-3A, EBNA-3B, and EBNA-3C epitopes were obtained after stimulation of donors' PBLs with autologous peptide-pulsed IFN-DCs, as expected on the basis of the marked skewing of memory $CD8^+$ responses toward immunodominant epitopes of EBNA-3 latent Ags in healthy EBV carriers (27, 29). Interestingly, a notable expansion of T cells reactive to the HLA-A2-restricted EBNA-3A (SVR) and LMP-2 (CLG) epitopes was obtained after stimulation of PBLs from donor LL with peptide-pulsed IFN-DCs. This observation appears of relevance with respect to the efficiency of IFN-DCs as APCs. In fact, primary as well as memory T cell reactivities to the subdominant EBNA-3A (SVR) and LMP-2 (CLG) epitopes used in this study are not generally detected after in vitro stimulation with LCL (29). More recently, it has been shown that T cell effectors specific for subdominant LMP-2 epitopes could be expanded by using mature peptide-pulsed DCs as stimulators (7, 9). The IFN-DC-induced increase in the frequency of T cells reactive to the LMP-2 (CLG) epitope favorably compares with the increase observed in one of these studies after stimulation with fully mature DCs (9), further supporting the notion that the functional behavior of IFN-DCs closely resembles that of mature DCs. The results on the expansion of T cells specific for lytic cycle Ag epitopes obtained after stimulation of PBLs from donor LL also point out the efficiency of IFN-DCs as APCs. In fact, whereas similar high frequencies of T cells reactive to the BMLF-1 (GLC) epitope were obtained using IFN-DCs or IL-4-DCs as stimulators, the expansion of T cells specific for the gp350 epitope was promoted exclusively by IFN-DCs. Several studies have demonstrated that responses to lytic cycle Ags, including BMLF-1, are detectable in CTL memory (21, 30, 31) and can dominate those to latent cycle Ags (32), as in the case of donor LL, whose basal frequency of T cells reactive against the BMLF-1 (GLC) epitope largely exceeded that of each latent cycle Ag reactivity tested. Moreover, BMLF-1 (GLC) epitope-specific T cells can be easily detected in sensitive ELISPOT assays by adding the corresponding peptide to total PBMCs as responders (30, 32), indicating that relatively inefficient APCs, such as the IL-4-DCs used in this study, can stimulate T cells reactive to this immunodominant epitope. In contrast, gp350-specific memory CTLs are maintained in healthy EBV carriers at very low frequencies, as in the case of donor LL, and successful expansion of gp350 (VLQ) epitope-specific memory CTLs could be achieved only after repeated stimulation of PBMCs with T2 cells presensitized with the corresponding peptide (20). The ability of IFN-DCs to promote the expansion of memory T cells reactive toward LMP-2 or lytic cycle Ags is relevant for their possible use as cellular adjuvants in immune interventions against EBV-associated malignancies. In fact, reactivation of LMP-2-specific T cells is potentially important for an effective cell-mediated immune control of latently infected cells, as LMP-2 is the only Ag expressed by EBV-infected blood memory B cells from healthy donors (1) and is expressed in most types of EBV-associated malignancies (2). In contrast, expansion of T cell precursors recognizing BMLF-1, an early protein functioning as a *trans* activator of other lytic cycle genes (17), or gp350 would contribute to the immune control of latently infected B cells undergoing spontaneous reactivation of lytic cycle.

The ability of peptide-pulsed IFN-DCs to expand specific IFN-γ-secreting T cell precursors was paralleled by their capacity to reactivate epitope-specific CTLs when used as stimulators of purified $CD8^+$ T cells (Fig. 2). In accordance with the IFN-DC- vs IL-4-DC-induced expansion of the corresponding T cell precursors detected in the ELISPOT assays (Fig. 1), similar levels of BMLF-1 (GLC)-specific cytotoxic activity were induced after stimulation of $CD8^+$ T cells from donor LL with IFN-DCs or IL-4-DCs, whereas strong CTL responses to the EBNA-3A and EBNA-3B epitopes

could be reactivated from donor FB $CD8^+$ T cells exclusively by IFN-DCs. Interestingly, the stronger reactivation of latent epitope-specific CTLs from donor FB $CD8^+$ T cells induced by IFN-DCs as compared with IL-4-DCs was paralleled by the persistence in the IFN-DC-stimulated cell lines of a $CD45RO^+CD45RA^-CD27^+$ cell subset, which in contrast was not sustained in the IL-4-DC-stimulated cell lines (Fig. 3). This observation strongly suggests that IFN-DCs promoted the expansion of latent epitope-specific effectors exhibiting the $CD45RO^+CD45RA^-CD27^+$ phenotype. Although this phenotype has been considered to identify memory cells with low or no cytolytic activity within circulating $CD8^+$ T cells (33), cytotoxic effector cells can be generated from sorted $CD45RA^-CD27^+$ cells after in vitro restimulation with Ag (33). Moreover, Hislop et al. (34) have recently demonstrated that in healthy EBV carriers latent epitope-specific $CD8^+$ T cells are strongly polarized toward a $CD45RO^+CD45RA^-$ memory phenotype and can exert a significant cytolytic activity against latent epitope-loaded target cells, despite the uniform CD27 expression.

The ability of IFN-DCs to act as potent inducers of both expansion of specific precursor T cells and cytotoxicity is most likely the consequence of the high expression of CD80 and CD86 molecules (12), enabling IFN-DCs to provide strong costimulatory signals. The sustained expansion of memory EBV-specific CTLs induced by IFN-DCs when cocultured with purified autologous $CD8^+$ T cells might also be mediated by IL-15, which was previously shown to be secreted in significant amounts by IFN-DCs, but not by IL-4-DCs (12). In fact, IL-15 concentrations similar to those found in IFN-DC supernatants have been demonstrated to increase the DC-induced expansion of Ag-specific memory CTLs (35), and DC-derived IL-15 has been indicated as a factor capable of bypassing the requirement for $CD4^+$ cell help in the in vitro expansion of memory CTLs (35, 36).

The ability of IFN-DCs to promote the expansion of EBV-specific $CD8^+$ effector cells in vivo is indicated by the results of IFN-DC-based vaccination of SCID mice reconstituted with peripheral blood cells from donor FB (Fig. 4 and Table II). In our experimental setting, the highly significant delay in lymphoma development observed in mice receiving two injections of peptide-pulsed IFN-DCs with respect to control mice and mice receiving unpulsed IFN-DCs (Table II) can only result from the in vivo expansion and activation of EBV-specific CTLs. This conclusion is also supported by the recovery of notable numbers of EBV-specific $CD8^+$ T cells from SCID mice transplanted with PBLs from donor FB and injected with autologous peptide-pulsed IFN-DCs (Fig. 4). The efficacy of EBV-specific cytotoxic $CD8^+$ T cells in protecting SCID mice from the growth of EBV-associated lymphomas has been documented in previous studies (37, 38), in which high numbers of EBV-specific CTLs expanded in vitro were injected into SCID mice transplanted with autologous LCL. To the best of our knowledge, our study represents the first evidence that a DC-based vaccination can be effective in limiting the development of EBV-induced immunoblastic B cell lymphomas in SCID mice inoculated with PBMC from EBV-seropositive donors.

Collectively, our results indicate that IFN-DCs represent a useful tool for a rapid in vitro expansion of EBV-specific CTLs to be used in adoptive immunotherapy of EBV-associated malignancies and, more importantly, support the possible use of IFN-DCs as cellular adjuvants for active immunization strategies aiming at the immune control of EBV-associated lymphoproliferative disorders in immunosuppressed individuals. More general implications for the use of IFN-DCs in immune interventions against nonvirus-associated human malignancies may derive from ongoing studies devoted to assess the efficiency of IFN-DCs as stimulators of cell-mediated immunity against poorly immunogenic human tumor-associated Ags.

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