Human Epstein-Barr Virus (EBV)-specific Cytotoxic T Lymphocytes Home Preferentially to and Induce Selective Regressions of Autologous EBV-induced B Cell Lymphoproliferations in Xenografted C.B-17 *Scid/Scid* Mice

By João F. Lacerda,*‡ Marc Ladanyi,§ Diane C. Louie,‖ José M. Fernandez,* Esperanza B. Papadopoulos,‡ and Richard J. O'Reilly*‡

*From the *Bone Marrow Transplant Laboratory; ‡Bone Marrow Transplant Service, Department of Pediatrics and Medicine; and Laboratories of §Diagnostic Molecular Pathology and ‖Surgical Pathology, Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York 10021*

Summary

C.B-17 *scid/scid* (severe combined immunodeficiency [SCID]) mice inoculated with peripheral blood lymphocytes from Epstein-Barr virus (EBV)-seropositive donors, or with EBV-transformed lymphoblastoid B cell lines (EBV-LCL), develop lethal human EBV^+ B cell lymphoproliferative disorders (EBV-LPD) with characteristics similar to those arising in immunodeficient patients. Using this model, we examined the capacity of human effector cells to control human EBV-LPD. SCID mice received rabbit anti-asialo GM1 antiserum to abrogate endogenous natural killer-cell function. Preliminary experiments showed that adoptive transfer of peripheral blood mononuclear cells (PBMC), purified T cells, interleukin (IL) 2–activated PBMC or anti-CD3–activated T cells derived from EBV-seropositive donors did not result in improved survival of treated mice (in vivo effector/target ratio 2:1 to 1:1). In contrast, EBV-specific cytotoxic T lymphocytes (CTL), derived from EBV-seropositive donors and expanded in vitro, exhibited strong EBV-specific and HLA-restricted activity both in vitro and in vivo. SCID mice inoculated intraperitoneally with autologous but not with HLA-mismatched EBV-LCL had significantly improved survival relative to untreated mice after inoculation of EBV-specific CTL either intraperitoneally ($P < 0.001$) or intravenously ($P < 0.001$) (in vivo effector/target ratio 1:1). SCID mice bearing large subcutaneous EBV^+ tumors and treated intravenously with 10^7 EBV-specific CTL achieved complete tumor regression. Both CTL- and CTL–plus–IL-2–treated mice survived significantly longer than untreated animals or animals treated with IL-2 alone ($P = 0.004$ and $P < 0.02$, respectively). SCID mice bearing two subcutaneous EBV^+ tumors, one autologous and the other HLA mismatched to the EBV-specific CTL donor, had regression of only the autologous tumor after intravenous infusion of 10^7 EBV-specific CTL. Moreover, we could demonstrate preferential homing of PKH26-labeled EBV-specific CTL to autologous but not to HLA-mismatched EBV^+ tumors as early as 24 h after intravenous adoptive transfer. Immunophenotypic analyses also demonstrated preferential infiltration of T cells into the autologous EBV^+ tumor in SCID mice bearing both the autologous and either fully HLA-mismatched or genotypically related haplotype-sharing EBV^+ tumors. The human T cells infiltrating EBV^+ tumors were $CD3^+$ and, predominantly, $CD8^+CD4^-$. Our results indicate that EBV-specific CTL preferentially localize to and infiltrate EBV^+ tumors bearing the appropriate HLA antigens and thereafter induce targeted regressions of disease.

EBV is a ubiquitous human herpesvirus that latently infects B cells of most of individuals by adulthood (1, 2). Persistent infection usually has no clinical consequences among immunocompetent individuals. However, patients with severe congenital or acquired deficiencies of cell-mediated immunity are at increased risk for developing

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EBV-induced B cell lymphoproliferative disorders (EBV-LPD)[1] (1, 2). In organ allograft recipients, these disorders tend to be polyclonal, of either donor or host origin, and may regress spontaneously when administration of immunosuppressive drugs is curtailed (2, 3). In contrast, bone marrow transplant (BMT) patients frequently present either with oligoclonal or monoclonal disorders, which are almost uniformly refractory to treatment with antineoplastic or antiviral drugs (2, 4–7).

The possibility of transferring resistance to virus-induced tumors by adoptive cell therapy has been demonstrated in murine models (8). In 1992, Riddell et al. (9) also showed that adoptive transfer of CMV-specific T cell clones could restore anti-CMV immunity in human BMT recipients. More recently, we have reported a series of five patients that developed EBV-LPD of donor B cell origin after a T cell–depleted BMT who achieved durable complete remissions after infusions of PBMC derived from their EBV-seropositive bone marrow donor (10). In formulating this approach, we reasoned that EBV-specific T cells, which circulate at high frequency in EBV-seropositive individuals (11), might be able to multiply in the EBV^+ lymphoma-bearing BMT recipient and induce regressions of disease. Indeed, we have subsequently shown that these donor PBMC infusions induced a marked increase in the number of EBV-specific cytotoxic T cell precursors (CTLp) in the circulation (12). In addition, Servida et al. (13) and Rooney et al. (14) have demonstrated regressions of EBV-LPD in BMT recipients after infusions of in vitro–generated EBV-specific T lymphocytes, again implicating these cells as the effectors of the regressions observed.

Mutant C.B-17 mice homozygous for the autosomal recessive mutation *scid* (SCID mice) are severely deficient in B and T lymphocytes (15, 16). However, SCID mice have normal myeloid, APC, and NK cell function (17–19). Because of their inability to generate functional lymphocytes, SCID mice are permissive to the engraftment and growth of human lymphocytes and several human tumors (19–21). Human lymphocytes have also been shown to remain functional in the SCID mouse after adoptive transfer (21). Inoculation of SCID mice with PBL from EBV-seropositive donors or with EBV-transformed lymphoblastoid cell lines (EBV-LCL) results in the development of EBV-induced immunoblastic B cell lymphomas with characteristics similar to EBV-LPD arising in immunocompromised hosts (20, 22, 23).

Based on these observations, we hypothesized that this animal model might provide an informative system for evaluating the antilymphoma activity of different human effector cell populations. Accordingly, we have established both intraperitoneal and subcutaneous models for the growth of human EBV-LPD in SCID mice to examine the effects of infusions of different types of human effector cells. These studies demonstrate that in vitro–expanded EBV-specific T cells, derived from normal EBV-seropositive individuals, can induce regressions of autologous but not of HLA-mismatched human EBV-LPD when administered intraperitoneally or intravenously into tumor-bearing mice. Moreover, using supravital labeling techniques and immunophenotyping studies, we could show preferential homing and infiltration of EBV-specific T lymphocytes into autologous subcutaneous tumors in animals bearing both autologous and either HLA-mismatched or genotypically related haplotype-sharing HLA-matched EBV^+ tumors.

[1] *Abbreviations used in this paper:* α-asialo GM1, anti-asialo GM1 antiserum; BMT, bone marrow transplant; CM, complete medium; CTLp, CTL precursor; EBV-LCL, EBV-transformed lymphoblastoid B cell line; EBV-LPD, EBV-induced B cell lymphoproliferative disorder; SL, specific lysis; TIL, tumor infiltrating lymphocytes.

Materials and Methods

Mice. 5–8-wk-old C.B-17 *scid/scid* mice, purchased from Taconic Farms, Inc. (Germantown, NY), were maintained in microisolater cages (three to five per cage) under specific pathogen–free conditions. Mice received autoclaved food and water. Investigators handling the mice wore gloves, mask, cap, and gown, and all animal manipulations were performed in a laminar flow hood in the same room. Procedures were reviewed and approved by the Institutional Animal Care and Use Committee.

Tumor Cell Lines. EBV-LCL were established by infection of PBL or purified B cells from EBV-seropositive donors with supernatants from the EBV-secreting marmoset cell line, B95-8, and were propagated in RPMI 1640 supplemented with 15% heat-inactivated fetal bovine serum (FBS), L-glutamine, sodium pyruvate, penicillin and streptomycin, essential and nonessential amino acids, and anti-PPLO agent (all purchased from Gibco Laboratories, Grand Island, NY). All cell lines were mycoplasma free.

Effector Cells. PBMC, purified B and T cells, were isolated from heparinized peripheral blood of healthy EBV-seropositive individuals, following standard techniques. Briefly, after gradient centrifugation over Ficoll–Hypaque (density 1.077 g/ml) (Nycomed, Oslo, Norway), mononuclear cells were suspended in RPMI 1640 supplemented with 10% heat-inactivated human serum (Whittaker Bioproducts, Walkersville, MD), L-glutamine, penicillin and streptomycin, essential and nonessential amino acids, sodium pyruvate, and anti-PPLO agent (hereafter referred to as complete medium [CM]) and allowed to adhere over plastic surfaces for 2 h at 37°C and 5% CO_2. Nonadherent cells (PBL) were thereafter separated by differential rosette formation with SRBC (Colorado Serum Co., Denver, CO) into E^+ (T cell fraction) and E^- cells.

For the generation of IL-2–activated PBMC, 2.5×10^6 cells/ml were incubated for 72 h with 500 IU/ml of hrIL-2 (Becton Dickinson, Collaborative Biomedical Products, Bedford, MA). To obtain nonspecifically activated T cells, aliquots of 10^6/ml purified T cells were stimulated with 20 μg/ml biotin-conjugated anti-CD3 mAb (Becton Dickinson & Co., San Jose, CA) at room temperature for 30 min, washed once in RPMI 1640 to remove unbound antibody, and incubated in CM with 10 μg/ml avidin (Pierce Chemical Co., Rockford, IL) to cross-link bound anti-CD3 mAb for 72 h, in the presence of 5×10^5/ml autologous irradiated (3,000 rad) PBMC.

For the generation of EBV-specific CTL, standard stimulation techniques were used (11). Briefly, purified T cells (E^+ cells) in CM were incubated in 24-well plates (Costar Corp., Cambridge, MA), at a final concentration of 2×10^6 cells/well, with 5×10^4 irradiated (9,000 rad) autologous EBV-LCL as stimulator cells and 10^6 irradiated (3,000 rad) autologous PBMC as feeder cells. On days 3, 6, and 9, each well received 1 ml of CM containing 10–20

IU of hrIL-2. On day 12, responder cells were replated in 24-well plates (1.5–2 × 10^6/well) in the presence of irradiated autologous EBV-LCL (responder/stimulator ratio = 5:1) and 10^6 irradiated autologous PBMC. Thereafter, cultures were fed every 3–4 d with CM containing 10 IU/ml IL-2 and restimulated weekly at the same cell concentration with irradiated autologous EBV-LCL and PBMC. Responding cells were used for in vivo experiments after 26 or 33 d in culture (i.e., inoculated the day after the third or fourth restimulation with autologous EBV-LCL, respectively).

In Vivo Studies. SCID mice were treated intraperitoneally with 30 μl rabbit anti-asialo GM1 antiserum (α-asialo GM1) (Wako Bioproducts, Richmond, VA) on days −1, 4, 8, and every 5–7 d thereafter for the duration of the study, for depletion of endogenous NK cell function (24), as described in detail elsewhere (25). The effector cells were administered either by the intraperitoneal or intravenous route at doses and times after EBV-LCL inoculation also specified in the presentation of results. Animals treated with IL-2 received 2,500 IU i.p. twice daily for 14 d, starting on the day of adoptive immunotherapy (first dose administered 4–6 h before CTL inoculation), unless otherwise specified. SCID mice were observed twice weekly for development of signs of disease (loss of activity, weight loss, ruffled hair, palpable tumors, ascites). Moribund animals were killed by cervical dislocation. Otherwise, SCID mice were followed for 30 wk and then killed.

Cytotoxicity Assays. Cytotoxicity of the responding T cells was assessed using standard 4-h chromium release assays. Target cells included the autologous EBV-LCL, EBV-LCL sharing one or more HLA class I and/or class II antigens with autologous EBV-LCL, fully HLA-mismatched EBV-LCL, autologous EBV^- activated B cells (stimulated with 10 ng/ml PMA and 1 μM/ml ionomycin), and the NK-sensitive erythroleukemia cell line K562. Target and effector cells were incubated in CM at various E/T ratios, as indicated, in round-bottom 96-well plates (Costar Corp.), at 37°C and 5% CO_2. After 4 h, supernatants were harvested and counted for 1 min. Spontaneous release was <15% of maximal ^{51}Cr release. The percentage of specific lysis (SL) was determined by the following equation: [experimental ^{51}Cr release (cpm) − spontaneous ^{51}Cr release (cpm)]/[maximal ^{51}Cr release (cpm) − spontaneous ^{51}Cr release (cpm)] × 100. In all cytotoxicity experiments, data are presented as mean SL of triplicate cultures performed for each E/T ratio.

For the experiments shown in Fig. 3, the indicated concentrations of anti–HLA-A,B,C (clone G46-2.6), anti–HLA-DR,DP,DQ (clone Tü36) (both purchased from PharMingen, San Diego, CA), anti-CD3 (clone SK-7) (Becton Dickinson & Co.), and anti–TCR α/β (clone WT-31) (Collaborative Biomedical Products) mAb were incubated for 1 h at 37°C in 5% CO_2 either with the target cells (anti-HLA class I and II mAb) or with the effector cells (anti-CD3 and TCR-α/β mAb) before the 4-h coincubation period.

The frequency of donor EBV–specific CTLp was assessed by limiting dilution analysis using a modification of techniques previously described (11, 12). Briefly, purified T cells were plated in limiting twofold dilutions in the presence of 2.5 × 10^4 autologous EBV-LCL and 2 × 10^3 autologous irradiated PBMC in 96-well round-bottom plates (Costar Corp.) and fed on days 3, 6, and 9 with 50 μl CM containing 10 IU/ml IL-2. 24 replicates for each dilution were used. At days 11–13, each CTL culture was split into four replicates and evaluated as effectors in standard 4-h ^{51}Cr release assays against autologous EBV^- activated B cells, autologous EBV-LCL, a mismatched EBV-LCL, and K562. Wells were scored positive when the percentage of SL exceeded 8–10% (11). 95% confidence limits are presented.

Histopathology and Immunohistochemistry. Samples of tumors and organs to be analyzed were fixed in 10% formalin or a 3% mercuric chloride in 4% formaldehyde solution, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Snap frozen sections in OCT medium (Diagnostics Division, Elkhart, Indiana) were also stained with hematoxylin and eosin. Antibodies used in immunohistochemistry studies included mouse anti–human CD20 mAb (L26 clone), mouse anti–human κ (R10-21-F3 clone) and λ (N10/2 clone) Ig light chain mAb, and mouse anti–human CD45RO mAb (UCHL1 clone), all purchased from Dako Corporation (Carpinteria, CA).

Flow Cytometry. EBV-LCL, purified T cells, in vitro–cultured EBV-specific T cells, and single-cell suspensions obtained from tumor-bearing animals were stained with FITC- or PE-conjugated mAb directed against the following human antigens: CD19, CD20, CD11a, CD18, CD54, CD11b, CD11c, CD44, CD49d, CD80, CD3, CD4, CD8, CD25, HLA-DR, CD16/CD56, CD45RO, CD45RA, and CD45 (all purchased from Becton Dickinson & Co.), and CD58 (Amac Inc., Westbrook, ME). Isotype controls were purchased from Becton Dickinson & Co. Mouse cells were detected by anti–mouse CD45 FITC-conjugated mAb (Boehringer Mannheim Corp., Indianapolis, IN). Viable cells in single-cell suspensions derived from tumors were isolated after gradient centrifugation over Ficoll–Hypaque. Before the addition of staining antibodies, single cell suspensions of tumors were incubated with 1 μg purified rat anti–mouse CD32/CD16 mAb (PharMingen) at room temperature for 10 min, to prevent nonspecific Fc receptor–mediated binding of staining antibodies to mouse cells. Samples of both the in vitro–cultured EBV-LCL and EBV-specific CTL inoculated into SCID mice were used for instrument setup before analysis of tumor single-cell suspensions.

A FACScan® flow cytometer using LYSIS II software (Becton Dickinson & Co.) in the Institution's Flow Cytometry Core Facility was used for analysis of surface antigen expression.

In Situ Hybridization. Fluorescence in situ hybridization of single-cell suspensions from tumors and mouse organs to detect human X and Y chromosomes (probes purchased from Oncor Inc., Gaithersburg, MD), was performed using standard techniques, described in detail elsewhere (25). For the detection of small EBV-encoded RNAs (EBER-1 and EBER-2), complementary deoxyribooligonucleotide probes (Dako Corp.) were used according to the manufacturer's protocols (26).

Southern Blotting. Southern blots of genomic DNA digested with EcoRI, HindIII, or BamHI were performed on a semiautomated blotting system (Probe Tech II; Oncor Corp.), as previously described (10). Placental DNA was used as germline, normal control. The probes used included a 5.6-kb HindIII–BamHI fragment spanning the entire J region of the Ig H chain gene and a 1.9-kb XhoI fragment adjacent to the terminal repeats of EBV DNA for detection of clonality and linearization of EBV DNA. For the *MYC* gene, DNA was digested with EcoRI or HindIII and probed with the XhoI–XbaI fragment of the first exon.

In Vivo Tracking with PKH26-labeled EBV-specific CTL. EBV-specific CTL were harvested, washed twice in HBSS without calcium or magnesium, counted, and stained with PKH26 (Sigma Chemical Co., St. Louis, MO) according to the manufacturer's instructions (27). Briefly, washed cells were resuspended in diluent C, after which they were immediately added to an equal volume of a 10-μM PKH26 solution in diluent C. Final cell concentration was 1.6 × 10^6 cells/ml. The cells were incubated at room temperature for 2 min, after which the staining reaction was stopped by the addition of an equal volume of pure FBS. Thereafter, the cells were washed three times in CM (10% FBS

instead of human serum) and adoptively transferred intravenously into animals bearing subcutaneous EBV$^+$ lymphomas. After 24 h, the animals were killed, tumor sections were snap frozen in OCT, and cryostat sections were analyzed by fluorescence microscopy in a fluorescence microscope (HEX-II; Nikon Inc., Garden City, NY) with filter combinations for rhodamine.

Statistical Analysis. The Wilcoxon rank-sum test was used to compare the survival of SCID mice treated with CTL alone or CTL plus IL-2, relative to untreated animals or animals treated with IL-2 alone, respectively.

Results

Features of Human EBV$^+$ Tumors in SCID Mice. We characterized the lymphomas developing in SCID mice after

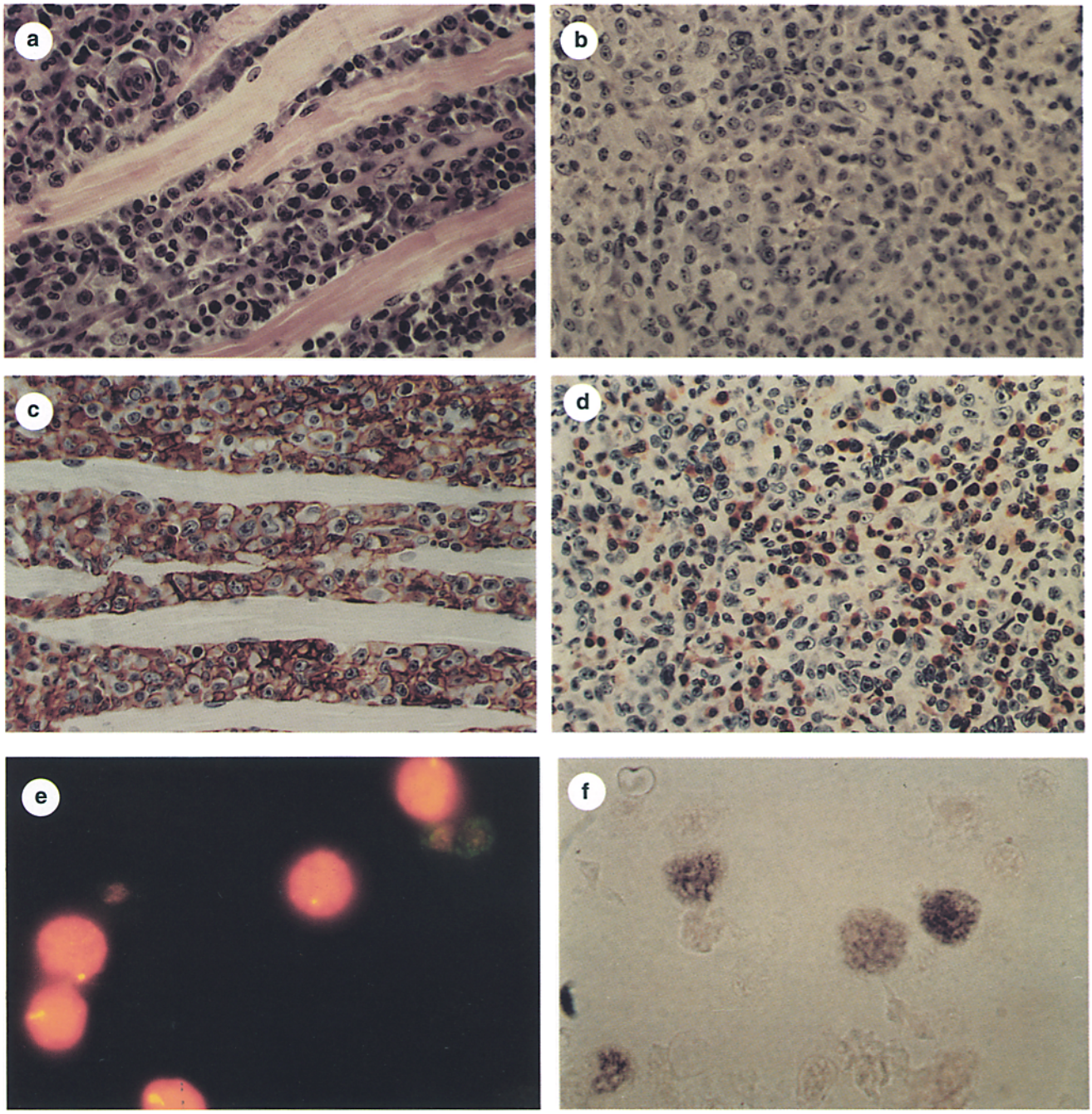

Figure 1. Characteristics of human EBV-LPD in the SCID mouse. (*a*) Subcutaneous human EBV-LPD infiltrating skeletal muscle in the SCID mouse. These lymphomas were classified as immunoblastic lymphomas, often with plasmacytoid features (hematoxylin and eosin) ×90. (*b*) The lymphoma cells were CD20$^+$ ×90. (*c*) The arising tumors were clonal, denoted in this case by absence of human Ig κ L chain expression, and (*d*) uniform positivity for human Ig λ L chain mAb. ×90. (*e*) Fluorescence in situ hybridization detected human X chromosome in the infiltrating tumor cells (e.g., male cells, one signal/cell). ×250. (*f*) In situ hybridization detected small EBV-encoded RNAs (EBER-1 and EBER-2) within the infiltrating cells (*brown inclusions*). ×250.

intraperitoneal or subcutaneous inoculation of EBV-LCL as to their pathologic and immunophenotypic features, pattern of dissemination, and molecular characteristics. Histologically, these EBV-LPD were classified as immunoblastic lymphomas (28) (Fig. 1 *A*). As depicted in Fig. 1 *B*, these lymphomas were positive for human CD20. Immunohistochemistry staining with mAb directed against human κ and λ Ig L chains suggested tumor clonality (e.g., tumor sample with κ Ig L chain negativity [Fig. 1 *C*], and λ L chain positivity [Fig. 1 *D*]). Organ infiltration of SCID mice bearing EBV-LPD by human cells was also confirmed by fluorescence in situ hybridization using probes specific for human X and Y chromosomes (e.g., human male cells infiltrating a mouse spleen, [Fig. 1 *E*]). These tumor- and organ infiltrating cells had a high expression of small EBV-encoded RNAs (EBER-1 and EBER-2), also detected by in situ hybridization (e.g., EBV^+ cells infiltrating mouse liver Fig. 1 *F*).

Animals inoculated intraperitoneally developed tumors in the abdominal cavity involving LN in the mesentery and porta hepatis, the liver, and spleen, as well as the thymus and mediastinal LN. Mice inoculated subcutaneously on the flank developed local tumors with subsequent extension of disease to the retroperitoneal space involving the kidneys and then the liver and spleen. Occasionally, tumors metastasized to axillary LN. The lymphomas detected in these organs were histologically and immunophenotipically identical to those developing at the primary site of inoculation.

Southern blot analysis of the fused termini of EBV revealed emergence of the inoculated EBV DNA clones in tumors recovered from SCID mice bearing human EBV-LPD (Table 1). In the case of donor 1, inoculation of EBV-LCL displaying two EBV clones by analysis of fused termini resulted in the development of tumors bearing the predominant or both EBV DNA clones. Southern blot analysis of the human Ig H chain gene showed that these tumors were clonal. In the case of donor 2 EBV-LCL, EBV termini analysis in BamHI and EcoRI digests also showed a ladder of fragments in the 4.5–7-kb size range, consistent with linear replicating EBV DNA (29). These fragments were present in tumors from only two mice inoculated with this cell line. A *MYC* gene rearrangement was not detected in any of the tumors.

Effect of Unstimulated or Nonspecifically Activated Effector Cells from EBV-seropositive Donors on the Growth of Autologous EBV^+ Tumors in SCID Mice. Our previous demonstration that infusions of PBMC from EBV-seropositive marrow donors could induce durable complete regressions of EBV-LPD in bone marrow graft recipients prompted us to explore the same strategy in the SCID mouse model. In these experiments, PBMC, IL-2–activated PBMC, purified T cells, and anti-CD3–activated T cells, derived from the same EBV-seropositive donors with high EBV-specific CTLp used in subsequent experiments, failed to alter either the proportion of animals developing lethal autologous tumors or the time to death of affected animals (in vivo E/T ratio 2:1 to 1:1) (data not shown). These cell populations were predominantly $CD3^+CD4^+$ and displayed minimal in vitro cytotoxicity against the autologous EBV-LCL (<12% SL, E/T ratio 25:1). The anti-CD3–activated T cells had a high expression of T cell activation antigens. The IL-2–activated PBMC showed high cytotoxicity against K562 (>45% SL, E/T ratio of 25:1). Because we could not find any significant in vivo antilymphoma activity of unprimed or nonspecifically activated cell populations, we then investigated whether T cells specifically stimulated with autolo-

Table 1. *Molecular Characteristics of Human EBV^+ Lymphomas in SCID Mice*

EBV-LCL inoculated			EBV-LPD in SCID mice			
EBV-LCL	EBV DNA	Ig H chain gene	Mouse	Tissue	EBV DNA	Ig H chain gene
Donor 1	Clonal*	Rearranged	N1	Tumor	Clonal	Rearranged
			N2	Tumor	Clonal	Rearranged
			N19	Node	Clonal*	Rearranged
			N19	Thymus	Clonal*	Rearranged
Donor 2	Clonal‡	Rearranged	R1	Tumor	Clonal	Rearranged
			R3	Tumor	Clonal	Rearranged
			R11	Tumor	Clonal	Rearranged
			S1	Tumor	Clonal	Rearranged
			S3	Tumor	Clonal	Rearranged
			S4	Tumor	Clonal	Rearranged
			S16	Tumor	Clonal‡	Rearranged
			S17	Tumor	Clonal‡	Rearranged
			S26	Tumor	Clonal	Rearranged

*Donor 1 EBV-LCL thymus, and LN from mouse N19 showed one major and one minor EBV DNA clone.
‡Evidence of EBV replication (both circular and linear forms detected).

Table 2. *Characteristics of Effector Cells Used in In Vivo Experiments*

	Donor 1		Donor 2	
	Purified T cells	EBV-CTL	Purified T cells	EBV-CTL
CD3 (%)	88.8–93.7	95.6–98.5	87.3–95.9	94.3–98.8
TCR-α/β (%)	73.2–77.4	95.2–97.1	82.2–86.8	95.0–97.4
TCR-γ/δ (%)	8.4–12.4	1.8–3.0	1.1–5.7	0.8–2.0
CD4 (%)	51.9–59.1	1.1–21.3	61.8–69.0	10.2–13.9
CD8 (%)	33.2–35.4	75.7–98.6	25.1–28.4	85.6–92.1
CD56 (%)	2.0–5.1	<1.5	1.5–3.2	<2.1
CD25 (%)	2.3–10.2	48.1–68.2	4.8–16.0	50.2–83.2
SL (%)				
(E/T ratio 6:1)*	ND	39.4–56.4‡	ND	34.4–44.9‡
EBV-specific CTLp*	1/1,689	1/125‡	1/4,752	1/25‡

*Cytotoxicity against autologous EBV-LCL
‡EBV-specific T cell cytotoxicity and CTLp performed after 26 d in culture with autologous EBV-LCL

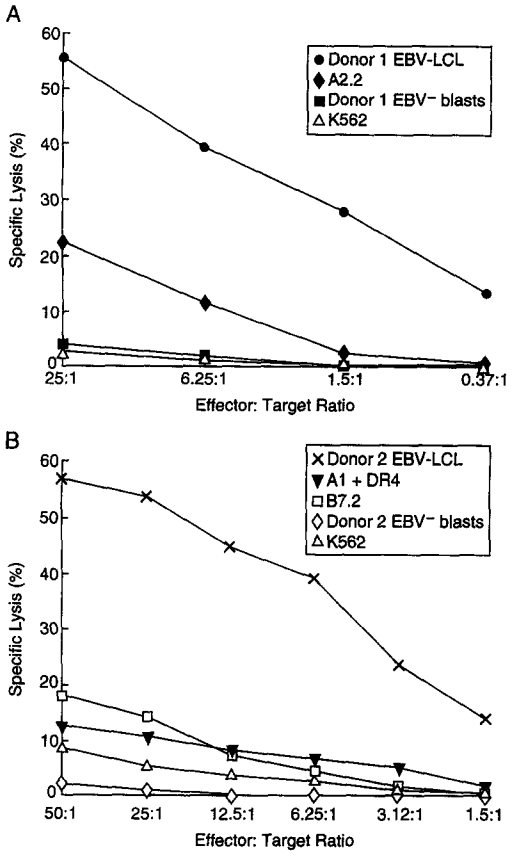

Figure 2. Cytotoxicity profile of EBV-specific T cells. (*A*) Cytotoxic activity of donor 1 EBV-specific T cells stimulated in vitro with the autologous EBV-LCL for 26 d. There was high SL of the autologous EBV-LCL (HLA type: A1,2.2; B44.1,62.3; DR4,7), intermediate cytotoxicity of an EBV-LCL sharing only A2.2 with the autologous EBV-LCL, and no SL of autologous EBV⁻ activated B cells or K562 cell line. (*B*) Cytotoxic activity of donor 2 EBV-specific T cells stimulated in vitro with the autologous EBV-LCL for 26 d. There was high cytotoxicity against the autologous EBV-LCL (HLA type: A1,3.2; B7.2,8; DR3,4), low cytotoxicity of target EBV-LCL sharing HLA A1 and DR4 (donor 1 EBV-LCL) and HLA B7.2 with the autologous EBV-LCL, and no SL of autologous EBV⁻ activated B cells or K562 cell line. Results are presented as mean SL of triplicate parallel cultures for each E/T ratio in standard 4-h ^{51}Cr release assays.

gous EBV-LCL could alter human EBV-induced lymphomagenesis in the SCID mouse model.

Characterization of EBV-specific Cytotoxic T Lymphocytes Generated In Vitro. To generate EBV-specific CTL, purified T cells from normal EBV-seropositive individuals were stimulated with autologous EBV-LCL. The characteristics of these T cells before and after 26 d of culture are presented in Table 2. For in vivo experiments, several sets of EBV-specific CTL derived from two EBV-seropositive donors were used. As shown in Table 2, the T cells generated in response to autologous EBV-LCL differed from the unstimulated population in that there was a predominance of CD8$^+$ cells, a high cytotoxicity against the autologous EBV-LCL, and a markedly higher frequency of EBV-specific CTLp. The responding cells were CD3$^+$, TCR-α/β$^+$, and CD16/CD56$^-$. These cells expressed CD11a/CD18 (LFA-1), CD58 (LFA-3), CD54 (ICAM-1), CD44, CD49d (VLA-α4), CD28, and CD45RO, and were negative for CD45RA, CD11b, CD11c, and CD80 (data not shown).

The cytotoxicity assays presented in Fig. 2 illustrate the selective cytotoxic profile of two EBV-specific CTL lines used in in vivo experiments after 26 d in culture. As shown in Fig. 2 *A*, CTL derived from donor 1 showed marked cytotoxicity against the autologous EBV-LCL, intermediate cytotoxicity against EBV-LCL sharing the allele A2.2 with the autologous EBV-LCL, and no SL against autologous EBV$^-$ activated B cells or K562. In Fig. 2 *B*, the cyto-

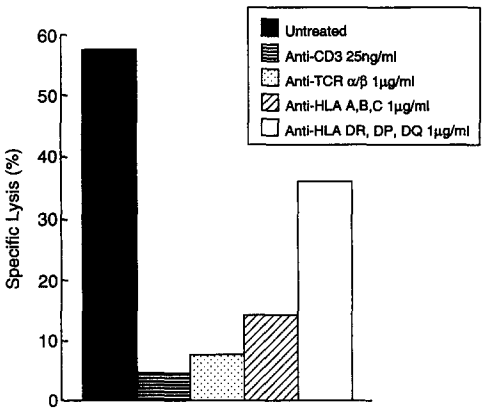

Figure 3. Inhibition of cytotoxicity of EBV specific T cells. Before the coincubation period in standard 4-h ^{51}Cr release assays, effector or target cells were incubated separately for 1 h with the indicated concentrations of anti-CD3 or anti-TCR-α/β mAb (EBV-specific T cells), or with anti-HLA-A,B,C or anti-HLA-DR,DP,DQ mAb (EBV-LCL). In the experiment shown, EBV-specific T cells from donor 2 stimulated in vitro for 26 d with the autologous EBV-LCL were used. The percentage of CD4 and CD8 T cells was, respectively, 12.5 and 85.2%. The addition of anti-CD3 or anti-TCR-α/β mAb inhibited almost completely the cytotoxicity against the autologous EBV-LCL. Addition of anti-HLA-A,B,C mAb inhibited SL to a higher degree than anti-HLA-DR,DP,DQ mAb, suggesting the predominance of HLA class I-restricted CTL. Results are presented as mean SL of triplicate parallel cultures for each antibody concentration at an E/T ratio of 10:1.

toxicity profile of EBV-specific CTL derived from donor 2 is depicted. Again, there was a high SL against the autologous EBV-LCL and intermediate lysis of two EBV-LCLs, one sharing the HLA alleles A1 and DR4 and the other HLA-B7.2, with the autologous EBV-LCL. Once again, there was virtually no cytotoxicity against autologous EBV^- activated B cells or K562 cell line.

The assay presented in Fig. 3 shows mAb-mediated inhibition of cytotoxicity of an EBV-specific CTL line derived from donor 2 after 26 d in culture with the autologous EBV-LCL (E/T ratio 10:1; $CD4^+$, 12.5%; $CD8^+$, 85.2%). As shown, SL of autologous EBV-LCL was almost totally

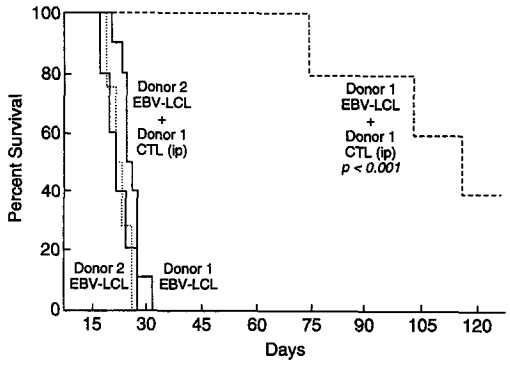

Figure 4. Survival of SCID mice inoculated intraperitoneally with 10^7 donor 1 or donor 2 EBV-LCL and randomized to be treated intraperitoneally on day 4 with 10^7 donor 1 EBV-specific T cells (26 d in culture). Mice were treated intraperitoneally with α-asialo GM1 on days −1, +4, +8, and every 5–7 d thereafter. Only mice inoculated with the autologous EBV-LCL responded to the infusion of EBV-specific T cells, and two to five treated animals never developed EBV-LPD.

inhibited by coincubation with anti-CD3 or anti-TCR-α/β mAb and largely abrogated by the addition of anti-HLA-A,B,C mAb. Anti-HLA-DR,DP,DQ mAb partially inhibited the cytotoxicity against autologous EBV-LCL, potentially reflecting the presence of EBV-specific $CD4^+$ CTL.

Effect of EBV-specific CTL Administered Intraperitoneally into SCID Mice Bearing Autologous or Allogeneic Intraperitoneal EBV^+ Lymphomas. In the experiment depicted in Fig. 4, SCID mice were inoculated intraperitoneally with 10^7 EBV-LCL derived from either of two normal EBV-seropositive donors and were randomized either not to be treated ($n = 10$) or to receive 10^7 EBV-specific CTL derived from donor 1 intraperitoneally 4 d later ($n = 5$). Although these donors shared HLA-A1 and DR4, there was little cytotoxicity against EBV-LCL from donor 2 by EBV-specific CTL derived from donor 1 (10.6% SL, E/T ratio 25:1). As shown in Fig. 4, SCID mice inoculated intraperitoneally with 10^7 EBV-LCL alone survived a median of 23–26 d, and all had died of lymphomas by day 32. SCID mice inoculated with EBV-LCL from donor 2 that were treated on day 4 with EBV-specific CTL derived from donor 1 also all died of EBV-LPD by day 27. In contrast, SCID mice inoculated with EBV-LCL from donor 1 and subsequently treated with autologous EBV-specific CTL survived a median of 112 d ($P < 0.001$). Of these animals, two of five survived long term without ever developing lymphomas. Nevertheless, despite the significantly improved survival, three of five mice receiving autologous CTL ultimately died of EBV-LPD after day 75, suggesting that, in these animals, inoculation of a single dose of 10^7 EBV-specific CTL (in vivo E/T ratio 1:1) delayed rather than prevented the development of EBV-LPD. Similar findings were obtained with EBV-specific CTL derived from donor 2 against autologous but not donor 1–derived EBV-LPD (data not shown).

Effect of EBV-specific CTL Administered Intravenously into SCID Mice Bearing Intraperitoneal or Subcutaneous EBV^+ Tumors. Thereafter, we investigated whether the infusion of these putative EBV-specific and HLA-restricted effectors by the intravenous route would also mediate a therapeutic effect. The intravenous inoculation of 10^7 EBV-specific CTL resulted in improved survival of SCID mice inoculated intraperitoneally with autologous EBV-LCL ($n = 5$) ($P < 0.001$), relative to untreated animals ($n = 10$) (Fig. 5 *A*). However, in contrast to animals treated intraperitoneally with EBV-specific CTL, all mice died of EBV-LPD.

Since the vast majority of the responding CTL were $CD3^+CD8^+TCR\text{-}\alpha/\beta^+$ and expressed CD25, we hypothesized that, in the intraperitoneal EBV^+ SCID mouse model, these cells might require IL-2 support for prolonged in vivo survival. Accordingly, in a subsequent experiment using a second set of EBV-specific CTL derived from the same EBV-seropositive donor (26 d in culture), SCID mice inoculated intraperitoneally with 10^7 EBV-LCL were divided into three groups. Mice in group 1 were left untreated ($n = 5$). The second group ($n = 5$) was treated with a low dose of IL-2 alone for 14 d. The third group ($n = 5$) was treated with the same dose of IL-2 and also received 10^7 autolo-

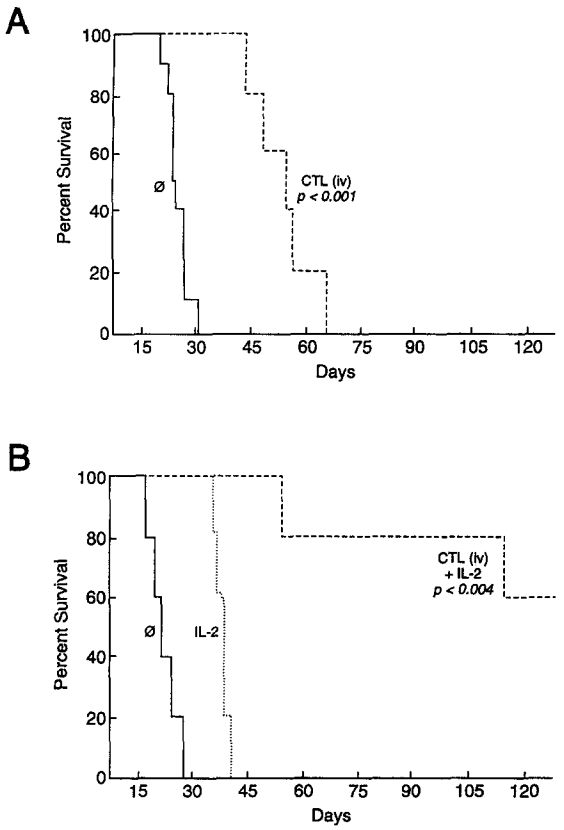

Figure 5. (*A*) Survival of SCID mice inoculated intraperitoneally with 10^7 donor 2 EBV-LCL and randomized not to be treated (∅) or to receive 10^7 autologous EBV-specific T cells (26 d in culture) intravenously on day 4. Mice were also treated with α-asialo GM1 intraperitoneally on days −1, +4, +8, and every 5–7 days thereafter. EBV-specific T cell–treated mice had a significantly improved survival relative to the group of untreated animals. (*B*) Survival of SCID mice inoculated intraperitoneally with 10^7 donor 2 EBV-LCL and randomized not to be treated (∅), to receive IL-2 alone (2,500 IU i.p. twice daily for 14 d), or IL-2 and 10^7 autologous EBV-specific T cells (26 d in culture) intravenously on day 4. Mice were treated intraperitoneally with α-asialo GM1 on days −1, +4, +8, and every 5–7 d thereafter. CTL plus IL-2–treated animals had a significantly prolonged survival relative to untreated animals or animals treated with IL-2 alone, of five animals treated with CTL and IL-2, two never developed EBV-LPD.

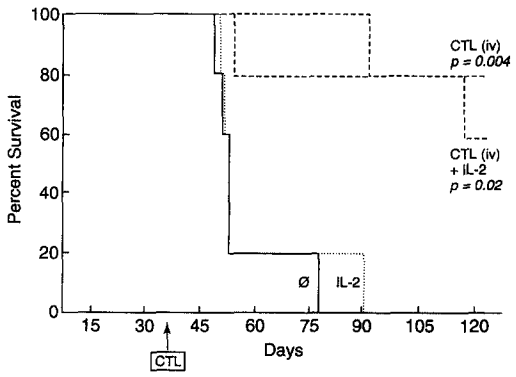

Figure 6. Survival of SCID mice inoculated subcutaneously with 20 × 10^6 EBV-LCL and randomized not to be treated (∅), or to receive IL-2 alone (2,500 IU i.p. twice daily for 14 d) on day 35, 10^7 autologous EBV-specific T cells (26 d in culture) intravenously, or the combination of IL-2 and CTL. Mice were treated intraperitoneally with α-asialo GM1 on days −1, +4, +8, and every 5 to 7 days thereafter. Both CTL- and CTL–plus–IL-2–treated animals had a significantly improved survival relative to untreated animals or SCID mice treated with IL-2 alone.

gous EBV-specific CTL intravenously on day 4 after EBV-LCL inoculation. As shown in Fig. 5 *B*, all animals in groups 1 and 2 died of lymphoma by days 31 and 40, respectively. Survival in group 3 was significantly prolonged relative to mice in group 2 ($P = 0.004$). Three of five animals in this group ultimately succumbed to lymphomas, but onset was delayed, with deaths at days 59, 115, and 130 after EBV-LCL inoculation. Two animals never developed EBV-LPD.

In subsequent experiments, we used an alternative animal model in which EBV-LCL were inoculated subcutaneously rather than intraperitoneally into SCID mice and investigated whether the intravenous administration of EBV-specific CTL could mediate regressions of well-established EBV-LPD. In the experiment depicted in Fig. 6, animals bearing large subcutaneous tumors (median mean surface area, 80 mm^2; equivalent in all groups of mice) were treated intravenously 35 d after EBV-LCL inoculation with autologous 10^7 EBV-specific CTL with or without IL-2, as above. There were tumor regressions over the following 12–24 d in the two groups of animals treated with CTL, whereas untreated mice and those treated with IL-2 alone had progressive tumor growth and extension of disease to the abdominal cavity, ultimately causing the death of the animals. CTL-treated mice had improved survival relative to control animals (CTL, $P = 0.004$; CTL + IL-2, $P = 0.02$). All mice treated with CTL and four of five mice treated with CTL plus IL-2 had regressions of subcutaneous tumors. Ultimately, three animals in each group died of EBV-LPD during the course of tumor regression. At necropsy, these mice had extensive abdominal disease. Still, two of five mice in each group had complete tumor regressions and survived >5 mo after CTL treatment. At day 200, the surviving four animals were killed, and full necropsies were performed. Although one mouse treated with CTL alone had complete regression of the subcutaneous tumor, kidney infiltration by tumor cells was still detected. Impressively, the tumor was infiltrated by human $CD45RO^+$ cells, demonstrating the persistence of human activated T cells infiltrating the EBV^+ tumor >5 mo after intravenous adoptive transfer of EBV-specific T cells (data not shown). The other three animals had no evidence of EBV-LPD.

In Vivo Tracking and Activity of EBV-specific CTL in SCID Mice Bearing Both Autologous and Allogeneic EBV^+ Subcutaneous Tumors. To evaluate homing of the CTL population to subcutaneous tumor sites, we labeled these EBV-specific effector cells with a lypophilic fluorescent dye, PKH26. In preliminary experiments, we determined the optimal concentration and time of incubation of PKH26 for maximal nontoxic membrane binding. Incubation with 10 μM PKH26 for 2 min resulted in a homogeneous bright staining of the EBV-specific CTL population, without loss of viability or cytotoxicity (data not shown). Also, in preliminary experiments, EBV^+ lymphoma-bearing mice were given either

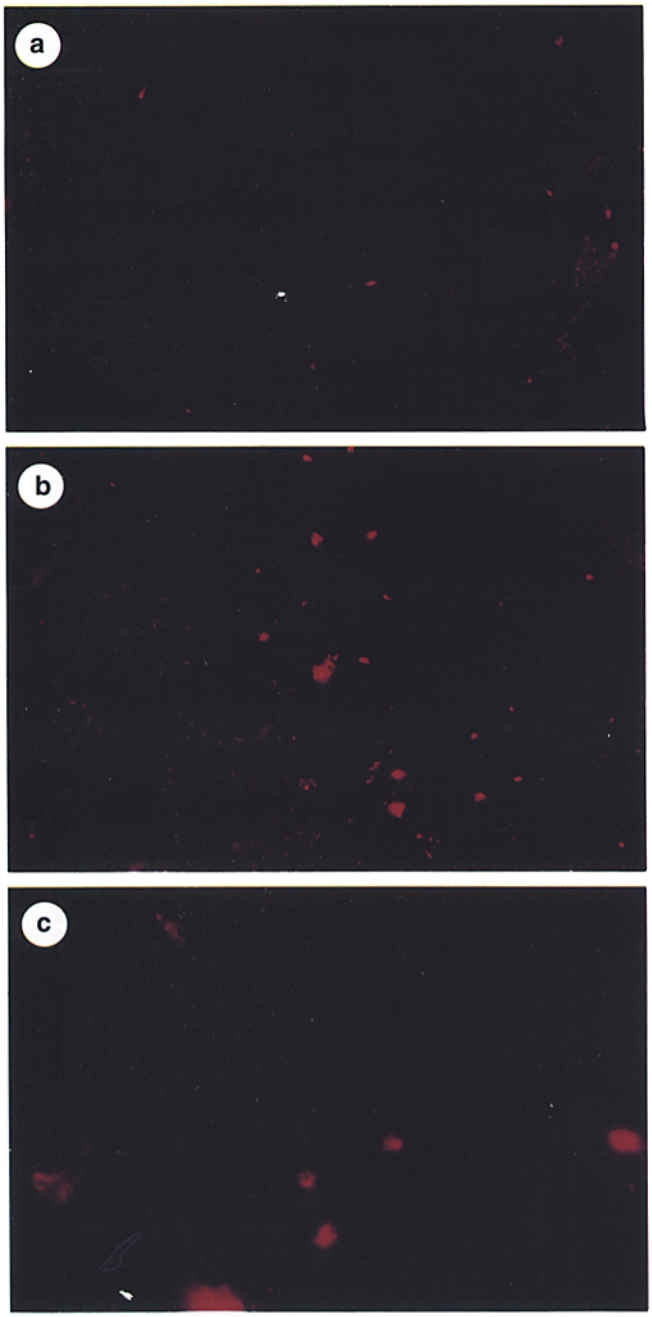

Figure 7. Early preferential localization of PKH26-labeled EBV-specific CTL to the autologous tumor in SCID mice bearing both the autologous and HLA-mismatched EBV$^+$ tumors. (*A*) HLA-mismatched EBV$^+$ tumor showing only background autofluorescence. ×25. (*B*) Autologous EBV$^+$ tumor on the same mouse infiltrated by PHK26-labeled EBV-specific T cells. ×25. (*C*) Higher magnification of *B* showing the morphology of the infiltrating cells. ×90.

no treatment or an intravenous infusion of PKH26-labeled autologous EBV-specific CTL. After 24 h, the animals were killed and sections of the tumors were analyzed by fluorescence microscopy. Infiltration of EBV$^+$ tumors by labeled CTL was documented, whereas tumors of untreated animals only showed background autofluorescence (data not shown). In subsequent experiments, 10^7 autologous PKH26-labeled CTL were adoptively transferred intravenously 33 d after inoculations of EBV-LCL into SCID mice bearing, on opposite flanks, both autologous and fully HLA-mismatched subcutaneous EBV$^+$ tumors against which there was no cytotoxicity in vitro. After 24 h, animals were sacrificed, and snap-frozen sections were analyzed by fluorescence microscopy. While HLA-mismatched EBV$^+$ tumors exhibited some background autofluorescence, very few bright signals could be discerned (Fig. 7 *A*). In contrast, the autologous tumors in the same animals exhibited numerous bright fluorescent signals (Fig. 7 *B*) which, on a higher magnification, could be discerned as infiltrating labeled cells (Fig. 7 *C*). Immunohistochemistry studies of the sample depicted in Fig. 7 confirmed the presence of human CD45RO$^+$ cells in the autologous but not in the HLA-mismatched EBV$^+$ lymphoma (data not shown). In separate identical experiments, EBV-specific CTL from each donor showed the same selective pattern of infiltration.

To further confirm the preferential infiltration of EBV-specific CTL into EBV$^+$ lymphomas bearing appropriate HLA determinants, six animals bearing large subcutaneous EBV$^+$ tumors in each flank, one derived from the autologous (donor 2) and the other from an HLA-mismatched donor, were randomized to be treated or not with EBV-specific CTL derived from donor 2 on day 33, which exhibited strong cytotoxicity in vitro against autologous and no SL of the HLA-mismatched EBV-LCL. Mice were also infused intraperitoneally with 5,000 IU IL-2 every other day, for a total of four doses. Only autologous tumors responded to the CTL infusion, whereas mismatched EBV$^+$ tumors of CTL-treated animals and both tumors of control animals continued to grow. 7–14 d after adoptive intravenous transfer of 10^7 EBV-specific CTL, the mean surface area of autologous EBV$^+$ tumors decreased by 40–60%. The autologous tumors remained confined to the original site of inoculation, whereas mismatched tumors were larger (140–200%) and infiltrated the abdominal cavity, eventually causing death of the animals. 7 d after adoptive CTL transfer, single-cell suspensions of autologous and HLA-mismatched tumors from two of the CTL-treated animals revealed preferential infiltration of the autologous subcutaneous tumor by human CD3$^+$ cells. Very few human T cells were found infiltrating the HLA-mismatched subcutaneous tumors or in peritoneal washes containing human CD45$^+$ cells, directly invading the peritoneal cavity from the HLA-mismatched subcutaneous tumor (necropsy finding) (data not shown). A similar experiment was performed using donor 1 EBV-specific T cells, and CD3$^+$CD8$^+$CD4$^-$ cells were detected infiltrating only the autologous EBV$^+$ tumor 14 d after adoptive transfer (data not shown).

To further analyze the in vivo selectivity of EBV-specific T cell homing, two SCID mice were inoculated in the right flank with donor 1 EBV-LCL (HLA type: A1,2.2; B44.1,62.3; DR4,7) and in the left flank with an EBV-LCL derived from a genotypically HLA haplotype–sharing related donor (HLA type: A1,31; B27.5,44.1; DR4,7). These EBV-LCL also shared an additional HLA-DR antigen on the unshared haplotype (4/6 HLA match by IEF).

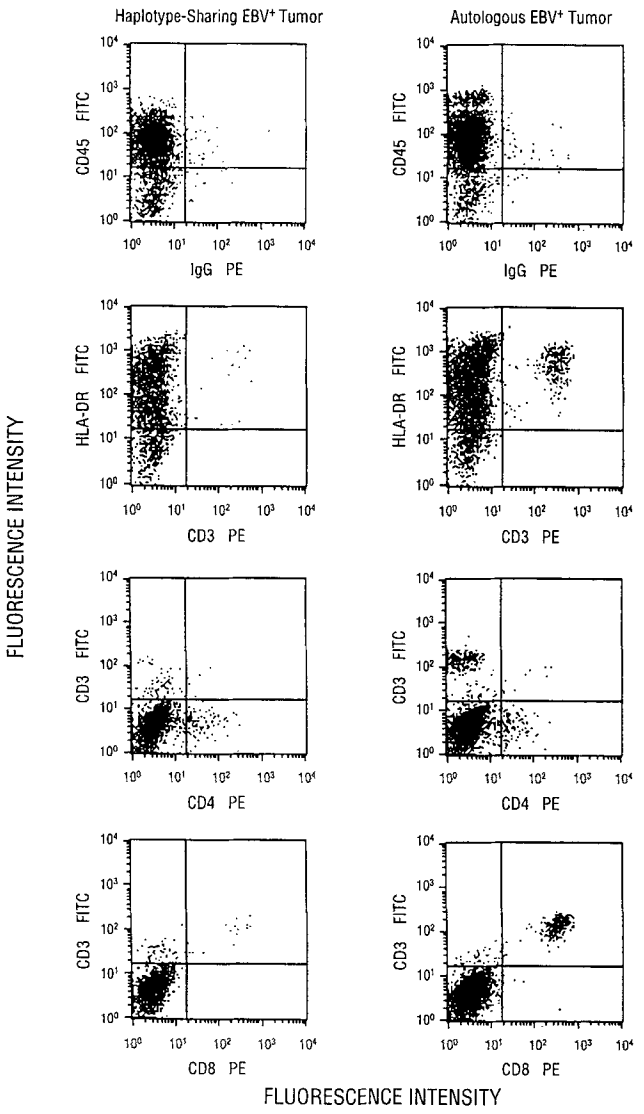

Figure 8. Preferential infiltration of the subcutaneous autologous EBV$^+$ tumor in mice bearing both the autologous (HLA-A1,2.2; B44.1,62.3; DR4,7) and an HLA haplotype–sharing genotipically related EBV$^+$ tumor (HLA-A1,A31; B27.5,44.1; DR4,7). 14 d after adoptive transfer of EBV-specific T cells, human CD45^{bright}CD3$^+$HLA-DR$^+$ CD4$^-$CD8$^+$ cells were detected infiltrating predominantly the autologous subcutaneous EBV$^+$ tumor (*right*) (6.4%), whereas very few T cells (0.6%) were detected infiltrating the haplotype-sharing subcutaneous EBV$^+$ tumor (*left*). The lymphoma cells were HLA-DR$^+$CD3$^-$. Each dot blot represents the fluorescence intensity of 5,000 gated cells.

At day 45, the animals were treated intravenously with 10^7 and intraperitoneally with 2×10^6 donor 1 EBV-specific T cells cultured in the presence of the autologous EBV-LCL for 33 d. In vitro, the cytotoxicity of these effector cells against the autologous EBV-LCL, the haplotype-sharing EBV-LCL, an HLA-mismatched EBV-LCL, and K562 was, respectively, 44, 28, 3, and 0% (E/T ratio 5:1). Mice also received 5,000 IU IL-2 every other day for a total of four doses. Over the next 10 d, regressions were observed only in donor 1 tumors (20–30% decrease in mean surface area). Flow cytometry analysis of both tumors 14 d after adoptive T cell transfer revealed an ~10-fold higher infiltration of the autologous tumor by human T cells, relative to the haplotype-sharing EBV$^+$ tumor. The human lymphocytes infiltrating the tumor were CD45^{bright}CD3$^+$HLA-DR$^+$ and, predominantly, CD4$^-$CD8$^+$. The EBV$^+$ lymphoma cells were HLA-DR$^+$CD3$^-$. Data from one of these animals are represented in Fig. 8. In this case, 6.4% human CD3$^+$ cells were detected infiltrating the autologous EBV$^+$ tumor versus 0.6% infiltrating the haplotype-matched EBV$^+$ tumor.

Discussion

As originally demonstrated by Mosier et al. (20), and confirmed in this and other reports (22, 23, 25), SCID mice inoculated with PBL from EBV-seropositive donors or with human EBV-transformed B cell lines rapidly develop lethal human EBV–induced lymphoproliferations with characteristics similar to those arising in immunocompromised patients. Recently, several groups have used this preclinical model to explore the potential of gamma globulin (30), mAbs directed against human CD21, CD23, CD24, and CD40 B cell antigens (31–33), low dose IL-2 treatment (34), and imexon (35) in the treatment of human EBV–induced lymphoproliferations developing in the SCID mouse.

To date, only two studies have examined the cellular effects of adoptively transferred human lymphocytes in this xenograft model. In 1993, Boyle et al. (36) reported that adoptive intraperitoneal transfer of autologous EBV-specific CTL prolongs the survival of SCID mice inoculated intraperitoneally with EBV-LCL. Rencher et al. (37) then reported that concomitant intraperitoneal administration of CD8$^+$ but not CD4$^+$ EBV-specific T cells could decrease the incidence of abdominal EBV$^+$ tumors after intraperitoneal inoculation of a small number of EBV-LCL. Neither of these studies used SCID mice pretreated to abrogate their NK cell function. Furthermore, Rencher et al. (37) did not show whether treatment with T lymphocytes resulted in a survival advantage.

This report confirms these observations and documents several new findings of potential significance to our understanding of the response of EBV-induced lymphoproliferations to adoptive immunotherapeutic approaches. For our experiments, C.B-17 *scid/scid* mice treated with α-asialo GM1 were used to ensure suppression of endogenous asialo GM1–expressing NK cells. Several investigators have shown that BALB/c *nu/nu* (nude) and SCID mice treated with α-asialo GM1 have lower NK cell function and higher engraftment of murine tumors, human PBL, and human tumors (24, 38, 39). In a separate series of experiments, we demonstrated that inoculation of as few as 5×10^4 EBV-LCL induced lethal tumors in 40–100% of α-asialo GM1–treated SCID mice (depending on the EBV-LCL tested) (25). At higher cell doses, all α-asialo GM1–treated animals died of EBV$^+$ lymphomas, with duration of survival inversely proportional to the dose of EBV-LCL administered (25). At higher doses than were used in vivo, α-asialo GM1

did not bind in vitro to human EBV–specific CTL or inhibit CTL cytotoxicity against the autologous EBV-LCL (data not shown). Accordingly, we adopted this model in our studies of adoptive cell therapy to abrogate endogenous NK cell function, which might otherwise contribute to the therapeutic responses observed.

Taken together, our studies failed to demonstrate any significant in vivo activity of adoptively transferred unprimed or nonspecifically activated cell populations against EBV-LPD developing in xenografted SCID mice. Murphy et al. (38) found a higher prevalence of EBV-LPD in SCID mice inoculated with a human colon carcinoma cell line and treated with anti-CD3–activated PBL from EBV-seropositive donors, which may suggest that nonspecifically activated T cells might contribute to rather than act against the development of EBV-LPD in the hu-PBMC-SCID mouse model. In our marrow allograft recipients, a striking increase in the number of circulating EBV-specific CTLp within 2 wk after their administration has been documented (12), reflecting either a massive proliferation of the EBV-specific CTL infused or their recruitment from the donor bone marrow–derived T cells developing within the transformed host's environment. In our SCID mouse model, recruitment of additional effectors is unlikely. In addition, whereas it remains to be determined whether the small number of EBV-specific CTLp infused in the unprimed or nonspecifically activated cell populations can expand in the SCID mouse microenvironment, any expansion would likely be limited.

In subsequent experiments, we investigated whether T cells specifically stimulated with autologous EBV-LCL could alter EBV^+ lymphomagenesis in the SCID mouse model. The T cells generated in vitro were EBV specific, HLA restricted, and predominantly of CD8 phenotype. The addition of anti–HLA class I mAb markedly inhibited cytotoxicity against autologous EBV-LCL, suggesting the predominance of HLA class I–restricted $CD8^+$ T cell–mediated cytotoxicity in the responding T cell population. In vivo, only $CD8^+$ cells were found infiltrating the autologous EBV^+ tumors by cytofluorometry (Fig. 8), suggesting that they were important effectors in the observed responses. In SCID mice inoculated intraperitoneally with EBV-LCL, adoptive transfer of EBV-specific CTL intraperitoneally at the same cell dose used in previous in vivo studies with unsensitized effectors (10^7 cells) resulted in improved survival only of animals bearing autologous intraperitoneal tumors. Mice bearing partially mismatched EBV^+ tumors, against which there was virtually no in vitro cytotoxicity, did not respond, suggesting also an HLA-restricted activity in vivo. Similar results were obtained with CTL propagated from the blood of each of the donors used.

We then investigated whether EBV-specific CTL given intravenously could also induce tumor regression in SCID mice bearing intraperitoneal EBV-LPD, since this route of effector cell administration would be more comparable to that used clinically (10, 13, 14). In these experiments, treated mice had improved survival relative to untreated animals. Nonetheless, all animals ultimately died of EBV-LPD and after a shorter interval than those treated intraperitoneally. In interpreting these results, we considered two possibilities: first, that the intravenously administered effectors did not survive in sufficient numbers to protect the animals or, alternatively, that their homing to intraperitoneal tumor sites was impaired. To address the first possibility, we examined the effects of administering IL-2 at low doses for 14 d after infusions of effector cells. As shown in Fig. 5 *B*, IL-2 alone had little effect on the survival of α-asialo GM1–treated mice inoculated intraperitoneally with EBV-LCL. This result is similar to that reported by Baiocchi and Caligiuri (34), who observed that IL-2 treatment of SCID mice inoculated intraperitoneally with PBL from EBV-seropositive individuals did not prevent lymphomagenesis if mice were also treated with α-asialo GM1. In contrast, the survival of animals that received an intravenous infusion of EBV-specific CTL and IL-2 was comparable to that of animals given an intraperitoneal injection of effectors without IL-2. In both groups, two of five animals never developed EBV-LPD. Thus, in this intraperitoneal EBV^+ tumor SCID mouse model, concomitant administration of IL-2 appears to have resulted in a survival advantage relative to mice treated with EBV-specific T cells alone.

In subsequent experiments, we also demonstrated that intravenous administration of 10^7 EBV-specific CTL induced regressions of large EBV^+ subcutaneous tumors, at an in vivo E/T ratio no greater than 1:100. In contrast to animals bearing intraperitoneal EBV^+ tumors and treated intravenously with EBV-specific CTL alone, mice bearing subcutaneous tumors had complete regressions of EBV-LPD after one infusion of EBV-specific CTL and survived long term, even in the absence of exogenous IL-2 support.

In vitro, the EBV-specific T cells used for adoptive immunotherapy exhibited HLA-restricted cytotoxicity. In vivo, this restricted activity was manifested not only by preferential regression of autologous EBV^+ tumors, but also by preferential homing and selective CTL infiltration of these tumors. In animals bearing both the autologous and HLA-mismatched EBV^+ tumors, PKH26-labeled CTL homed within 24 h of adoptive intravenous transfer to the autologous but not to HLA-mismatched tumors. Immunohistochemistry and cytofluorometry studies also detected EBV-specific CTL infiltrating the autologous but not HLA-mismatched EBV^+ tumors up to 14 d after their infusion (data not shown).

In the experiment shown in Fig. 8, although the autologous EBV-specific T cells lysed in vitro the HLA genotypically haplotype-identical related EBV-LCL, albeit to a lesser degree than the autologous EBV-LCL (12–17% less SL for all the dilutions tested), very few human T cells were detected infiltrating the haplotype-identical EBV^+ tumor. This degree of preferential infiltration of the autologous tumors by autologous EBV-specific T cells may reflect differences in the frequencies of EBV-specific CTLp recognizing specific EBV antigens in the context of HLA class I determinants uniquely expressed on the autologous rather than on the haplotype-matched EBV^+ tumor cells. Murray et al. (40) and Khanna et al. (41) have shown that whereas certain EBV antigens such as EBV nuclear antigen

(EBNA) 3A, 3B, and 3C may be recognized by EBV-specific T cells upon presentation by a variety of HLA class I antigens, others, such as latent membrane protein 2 and EBNA 2, are preferentially associated with specific HLA class I molecules. Further studies using EBV-specific T cell clones of defined HLA restriction will be required to clarify this issue.

Studies investigating the specific activity of tumor infiltrating lymphocytes (TIL), CTL, and LAK cells in murine models have also shown that these cells can selectively migrate to and infiltrate a sensitizing tumor, as opposed to normal surrounding tissue (8, 42, 43). However, Wallace et al. (44) did not find preferential homing of PKH-labeled TIL to autologous versus nonautologous tumors or normal lung tissue, despite having a specific in vivo activity against the autologous tumor. Kast et al. (45) have also shown that adenovirus antigen–specific murine T cells from C57Bl/6 (B6) mice will selectively induce regressions of adenovirus (Ad) type 5 E1A/E1B–induced B6 tumors, but not of Ad 5 E1A/1B-induced BALB/c tumors in B6 mice bearing both the B6 and BALB/c tumor, indicating their H-2 restriction. Similarly, when Crowley et al. (46) tested the in vivo activity of human CTL in a xenograft nude mouse model of human melanoma, mice bearing HLA-A2 hepatic melanoma metastases had a reduction in the number of metastases only after treatment with HLA-A2–restricted melanoma-specific CTL but not with infusions of HLA-A11–restricted melanoma reactive CTL. In neither of these studies, however, were the homing characteristics of the infused effectors examined.

The mechanisms by which small numbers of T cells induce regressions of large tumors, as observed in this model, and more dramatically in initial clinical reports (10), are currently unknown. In murine models, both cytotoxic $CD8^+$ and noncytotoxic $CD4^+$ and $CD8^+$ TIL that generate cytokines, particularly IFN-γ and TNF, have been implicated in tumor regression (47). These regressions were T cell dependent and tumor specific. In our studies, the selective regression of autologous tumors in animals bearing both autologous and allogeneic tumors does not preclude the possibility that EBV-specific T cells selectively infiltrating the autologous tumors induce regressions by the release of locally active cytokines, but do argue against the induction of tumor regression by the nonspecific activity of cytokines released into the general circulation. Experiments to address these questions in the informative SCID mouse models described in this study are currently in progress.

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Address correspondence to Dr. Richard J. O'Reilly, MSKCC, 1275 York Avenue, New York, NY 10021.

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