Generation of Epstein-Barr virus–specific cytotoxic T lymphocytes resistant to the immunosuppressive drug tacrolimus (FK506)

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Adoptive transfer of autologous Epstein-Barr virus–specific cytotoxic T lymphocytes (EBV-CTLs) to solid organ transplant (SOT) recipients has been shown safe and effective for the treatment of EBV-associated posttransplantation lymphoproliferative disorders (PTLDs). SOT recipients, however, require the continuous administration of immunosuppressive drugs to prevent graft rejection, and these agents may significantly limit the long-term persistence of transferred EBV-CTLs, precluding their use as prophylaxis. Tacrolimus (FK506) is one of the most widely used immunosuppressive agents in SOT recipients, and its immunosuppressive effects are largely dependent on its interaction with the 12-kDa FK506-binding protein (FKBP12). We have knocked down the expression of FKBP12 in EBV-CTLs using a specific small interfering RNA (siRNA) stably expressed from a retroviral vector and found that FKBP12-silenced EBV-CTLs are FK506 resistant. These cells continue to expand in the presence of the drug without measurable impairment of their antigen specificity or cytotoxic activity. We confirmed their FK506 resistance and anti-PTLD activity in vivo using a xenogenic mouse model, suggesting that the proposed strategy may be of value to enhance EBV-specific immune surveillance in patients at high risk of PTLD after transplantation. (Blood. 2009;114:4784-4791)

Introduction

Adoptive transfer of Epstein-Barr virus–specific cytotoxic T lymphocytes (EBV-CTLs) can induce the regression of EBV-associated posttransplantation lymphoproliferative disorders (EBV-PTLDs) in recipients of allogeneic hematopoietic stem cell (HSC) grafts.1,2 Moreover, prophylactic administration of EBV-CTLs can efficiently prevent the occurrence of EBV-PTLDs in these patients due to the long-term in vivo persistence of these cells after adoptive transfer.1,2

Although EBV-CTLs can also be effective to treat PTLDs occurring after solid organ transplantation,1,9 it has proven difficult to obtain reduction in morbidity in these patients using a prophylactic approach.10 Indeed, in contrast to allogeneic HSC transplantation, solid organ transplant (SOT) recipients require continued administration of immunosuppressive drugs to avoid graft rejection,11,12 and these agents may significantly impair the growth, expansion, and long-term persistence of adoptively transferred EBV-CTLs.13

Although this inhibition evidently does not preclude the short-term in vivo cytotoxic activity of adoptively transferred EBV-CTLs and hence benefit in the case of established disease,3,9 it effectively precludes their cost-effective use as prophylactic treatment for patients at high risk for PTLD development after solid organ transplantation. To overcome this obstacle and improve CTL proliferation and persistence despite continued immunosuppression, we have made EBV-CTLs resistant to the effects of tacrolimus (FK506), a commonly used immunosuppressive drug.

FK506 is widely used as an immunosuppressive drug to prevent rejection of renal, liver, and heart-lung transplant grafts.14-17 FK506 inhibits host immune responses by binding to immunophilin FK506-binding proteins (FKBPs).18 Of the 10 known mammalian FKBPs, the 12-kDa FK506-binding protein (FKBP12) appears to play a key role in mediating FK506 immunosuppression,19 forming a complex with the drug that inhibits the Ca2+–activated serine/threonine phosphatase calcineurin, and thereby preventing cytoplasmic nuclear factor of activated T cell (NFATc) dephosphorylation, which is required for interleukin-2 (IL-2) production and T-cell activation.20,21

We hypothesized that EBV-CTLs in which we prevented binding of FK506 to FKBP12 would retain their ability to proliferate even in the presence of the drug. To achieve this effect we selectively down modulated FKBP12 in EBV-CTLs using a specific small interference RNA (siRNA) expressed by a retroviral vector. We show that stable knockdown of FKBP12 in EBV-CTLs allows them to expand in the presence of therapeutic doses of FK506 without affecting their ex vivo or in vivo antigen specificity and function.

Methods

Plasmid construction and retrovirus production

We obtained the full-length human FKBP12 cDNA, by reverse-transcription polymerase chain reaction from peripheral blood mononuclear cells (PBMCs) and cloned it into the expression plasmid p.eGFP-C1


An Inside Blood analysis of this article appears at the front of this issue.

The online version of this article contains a data supplement.
supernatant and IL-2, as previously described. T cells were then retrovirally transduced on day 3 in 24-well plates precoated with recombinant fibronectin (FN CH-296; Retronectin; Takara Shuzo) using specific retroviral vectors, containing the puromycin resistance gene, or pSUPER vector (OligoEngine), containing a GFP Selectable marker, as previously described. Control vectors encoding an irrelevant siRNA were also used. The vector encoding a firefly luciferase (FFLuc) was constructed and used as previously described. We prepared the retroviral supernatant using 293T cells cotransfected with 3 plasmids (the retroviral construct, Peg-Pam-e encoding gag-pol, and RDF encoding the RD114 envelope) using the Gene Juice transfection reagent (Novagen Brand). Supernatants were collected 48 and 72 hours later.

**Screening of FKB12-siRNAs**

We cloned 17 different siRNAs that targeted FKB12 mRNA into the pSUPER retroviral vector. To evaluate the silencing activity of these siRNAs, we cotransfected 293T cells with the reporter plasmid (p-eGFP-pSUPER) and pSUPER vectors encoding siRNAs (ratio of 1:2). Silencing mediated by the siRNAs was measured by quantifying the expression of GFP in 293T cells using a BD FACS Calibur Flow Cytometer (BD Biosciences) 48 hours after plasmid transfection.

**Western blot analysis**

Cell lysates were resolved on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. FKB12 was detected using a rabbit polyclonal antibody (AbCAM Inc.). Immunoblots were developed using enhanced chemiluminescence detection reagents (Amersham Biosciences). To evaluate the equal loading of the proteins, membranes were reprobed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (mAb; Santa Cruz Biotechnology). The interferon-γ enzyme-linked immunospot (ELIspot) assay (Mabtech, Inc) was performed as previously described. Briefly, EBV-CTLs were plated in triplicate at 10^5 in the presence of the appropriate peptides (5 μM). Negative controls included EBV-CTLs alone and EBV-CTLs plated with irrelevant peptides. As positive controls, EBV-CTLs were stimulated with autologous EBV-LCLs.

**Chromium release assay**

The cytotoxic activity of EBV-CTLs was evaluated in a standard 4-hour 51Cr release assay, as previously described. Target cells included autologous and HLA class I and II mismatched EBV-LCLs, and the K562 cell line (as a natural killer cell target). Cells were incubated in media alone or in the presence of temsirolimus (from 5 ng/mL to 100 ng/mL). Transduced T cells were stimulated with bound-OKT3 (Ortho Biotech) and CD28 (Pharmingen) mAbs in the presence of low doses of FK506 (from 5 ng/mL to 25 ng/mL). After 72 hours, CTLs or T cells were pulsed with 1 μCi (0.037 MBq) methyl-3[H]thymidine (Amersham Pharmacia Biotech), cultured for an additional 16 hours, and then harvested onto filters and dried. Thymidine uptake was measured as counts per minute in a scintillation counter (liquid scintillation analyzer TRI-CARB 2910 TR; Perkin Elmer). The percentage suppression was calculated using the following formula: 100 × [1 - (experimental cpm – control cpm)/(without drug cpm – negative control cpm)], as previously reported.

**Transduction of tumor cells with the luciferase vector**

EBV-LCLs were transduced with the FFLuc vector on a Retronectin-coated plate and then selected using puromycin (Sigma-Aldrich) as previously reported. The antigen specificity of EBV-CTLs was evaluated with EBV-specific multimers, as previously described. We chose multimers based on available donor human leukocyte antigen (HLA) types, which recognized the following EBV peptides: EBNA3B, HLA-A11: IVTDFSVIK, and EBNA3B, HLA-A11: AVFDRKSDKAK; EBNA3A, HLA-B8: QAKWRLQTTL; BZLF1, HLA-B8: RAKFKQDLL (listed in Khanna and Burrows; Houssaint et al). Multimers were prepared by Proimmune. Samples were costained with CD8-allophycocyanin and CD3–peridinin chlorophyll protein mAbs. Isotype controls were included. For each sample, a minimum of 10 000 events were analyzed using a FACSS Calibur with a filter set for 4 fluorescence signals and CellQuest software.
To confirm transient expression, 5 × 10^6 tumor cells were lysed and aliquots diluted in 100 μL of d-luciferin according to the manufacturer’s instructions (Promega). Bioluminescence was measured using a luminometer (Monolight; BD Biosciences).

In vivo experiments

All mouse experiments were performed in accordance with Baylor College of Medicine Animal Husbandry and Institutional Animal Care and Use Committee guidelines and were approved by the Baylor College of Medicine’s institutional review board.

Antitumor activity

To assess persistence and antitumor activity of control and genetically modified human EBV-CTLs in the presence of FK506 in vivo, we used a xenograft lymphoma severe combined immunodeficient (SCID) mouse model and the IVIS imaging system (Xenogen; Caliper Life Sciences) as previously described. 30 Six- to 8-week-old CB17/SCID mice (Harlan-Sprague) were sublethally irradiated (230 cGy) and engrafted intraperitoneally with 3 × 10^6 FFLuc + EBV-LCLs resuspended in Matrigel (BD Biosciences). 30 Three or 4 days after engraftment (when the light emission of the tumor was consistently measurable) and again 1 week later, mice received 10 × 10^6 autologous EBV-CTLs intraperitoneally. In addition, mice also received 3 times a week intraperitoneal injections of 1000 U/mouse IL-2 (Teceleukin; Fisher Bioservices) and 10 mg/kg body weight of FK506. 31 Control animals received only IL-2. Tumor growth was evaluated over time using the IVIS imaging system. Briefly, a constant region of the tumor was drawn over the tumors and the intensity of the signal measured as total photon/sec/cm^2/steradian (p/s/cm^2/sr) as previously described. 27,30

Statistical analysis

All in vitro experiments were summarized as mean plus or minus SD. Student t test was used to determine the statistical significant differences between samples, with P value less than .05 indicating a significant difference. Survival was evaluated by Kaplan-Meier analysis (SPSS software) and the statistical significance of observed differences assessed by log-rank and Breslow testing.

Results

Selection of siRNAs targeting the human FKBP12 mRNA

To rapidly select functional siRNAs, the 17 predicted siRNA sequences targeting the human FKBP12 mRNA were cloned in the pSUPER vector. 22 FKBP12-siRNA-pSUPER or control siRNA-pSUPER (irr-siRNA) vectors were then cotransfected into 293T cells with the reporter plasmid eGFP-hFKBP12 (encoding the fusion protein eGFP-hFKBP12), GFP expression, measured by fluorescence-activated cell sorting (FACS) analysis 48 hours after transfection, was used as a surrogate marker of FKBP12 mRNA silencing by specific siRNAs. As shown in Figure 1A, the GFP mean fluorescence intensity (MFI) of eGFP-hFKBP12 293T cells was 1067 ± 80 and remained stable in 293T cells cotransfected with the irr-siRNA (1097 ± 89). Variable decreases of the GFP MFI were observed in 293T cells after transfecting each FKBP12-siRNA-pSUPER vector. The siRNA designated siRNA4, which targets the GATGGAAAGAAATTTGATT mRNA sequence, significantly knocked down the GFP MFI to 163 (P < .001; Figure 1A-B). The combination of siRNA4 with other siRNAs had no further effect on GFP expression or MFI (data not shown). Hence, siRNA4 was selected for all subsequent experiments.

siRNA4 significantly knocked down expression of endogenous FKBP12 in EBV-CTLs

We measured the effects of siRNA4 on the expression of endogenous FKBP12 in EBV-CTL lines generated from 6 EBV-seropositive donors. These CTLs were transduced after the third antigen stimulation either with the retroviral vector pSUPER encoding the siRNA4 or with the control irr-siRNA vector. After transduction, CTLs were maintained in culture and selected with puromycin (0.1-0.5 μg/mL). As shown in Figure 1C, FKBP12 expression, assessed by Western blot (WB) analysis, was reduced by 42% in EBV-CTLs transduced with siRNA4, but not in CTLs expressing the irr-siRNA. This effect was further increased when CTLs were selected in the presence of puromycin because we observed a 94% reduction of FKBP12 in siRNA4 + EBV-CTLs (Figure 1C). FKBP12 knockdown was stable in EBV-CTLs maintained in culture for more than 2 months (data not shown).

Enrichment of FKBP12-silenced EBV-CTLs in the presence of FK506

We next measured the effects of reduced FKBP12 expression on the resistance of EBV-CTLs to FK506. We transduced EBV-CTLs...
with either pSUPER.eGFP encoding siRNA4 or pSUPER.eGFP encoding control siRNA and determined transduction efficiency by measuring GFP expression using FACS analysis. Transduction efficiency was 46% (±22%) for siRNA4-CTLs and 56% (±27%) for irr-siRNA-CTLs. After transduction, EBV-CTLs were maintained in culture by weekly stimulation with irradiated autologous EBV-LCLs and suboptimal doses of IL-2 (20 U/mL added twice a week) because ex vivo cultured EBV-CTLs become highly dependent on IL-2 for their proliferation and no expansion is observed in the absence of exogenous cytokines (data not shown and Quintarelli et al32), with or without the addition of FK506 (5 ng/mL added twice a week) starting 1 week after transduction (fourth stimulation). We determined whether CTL lines were selected/enriched over time in FK506-resistant cells by monitoring changes in the percentage of GFP+ cells. As shown in Figure 2A and B, the percentage of GFP+ cells significantly increased over the following 7 weeks of culture for siRNA4-EBV-CTLs cultured in the presence of FK506 (from 46% ±22% at the fourth stimulation to 89% ±5% at the 11th stimulation; P = .001). In contrast, the percentage of GFP+ cells remained stable for irr-siRNA-EBV-CTLs cultured in the presence of FK506 (from 56% ±27% at the 4th stimulation to 57% ±23% at the 11th stimulation; P = not significant). In parallel with the increasing percentages of GFP+ cells, the GFP MFI significantly increased in siRNA4-EBV-CTLs (from 692 ±263 to 2625 ±318; P = .002) when these cells were maintained in culture in the presence of FK506 (Figure 2C). These data show a selective enrichment of FKBP12-silenced EBV-CTLs, which suggests they had indeed become FK506 resistant. As expected, the percentage of GFP+ cells did not increase when siRNA4-EBV-CTLs were cultured without FK506, confirming the lack of any intrinsic selective growth advantage for CTLs in which FKBP12 was down-regulated (Figure 2B-C). The selection of siRNA4+ EBV-CTLs was also maintained when a higher dose of FK506 (10 ng/mL) was added to the culture (supplemental Figure 1, available on the Blood website; see the Supplemental Materials link at the top of the online article).

Finally, we confirmed that the positive selection of siRNA4+ EBV-CTLs in the presence of FK506 corresponded to an enrichment of EBV-CTLs with a significant down-regulation of FKBP12. As shown in Figure 2D, FKBP12 was detectable by WB in nontransduced (NT) EBV-CTLs and in those transduced with irr-siRNA cultured without FK506. In contrast, FKBP12 was completely undetectable in EBV-CTLs transduced with siRNA4 and maintained in culture for 5 weeks with antigen stimulation and in the presence of FK506 (Figure 2D). We have further supported the significance of this finding by exploring the NFAT pathway, which was partially preserved only in T cells expressing siRNA4 when stimulated in the presence of FK506 (supplemental Figure 1D).

siRNA4+ EBV-CTLs retain antigen-specific expansion in the presence of FK506

To discover whether enrichment of CTLs with knocked-down FKBP12 observed in the previous experiments was determined by a preserved proliferation of siRNA4+ EBV-CTLs in the presence of FK506, we measured EBV-CTL proliferation and numeric increase after antigen stimulation by a thymidine uptake assay and counting viable cells using trypan blue exclusion, respectively. Figure 3A shows the thymidine uptake of EBV-CTLs expressing either irr-siRNA or siRNA4 and cultured in the presence of increasing concentration of FK506. We observed that concentrations of FK506 as low as 5 ng/mL significantly reduced the proliferative capacity of NT and irr-siRNA+ EBV-CTLs. In contrast, siRNA4+ CTLs retained most of their proliferative capacity when FK506 was added, irrespective of the dose. Figure 3B shows data for 6 EBV-CTL lines cultured in the presence of 5 ng/mL FK506. Proliferation of both control NT and irr-siRNA+ EBV-CTLs (3.2 × 105 ± 0.7 × 104 cpm and 3.6 × 104 ± 1.4 × 104 cpm, respectively) was significantly reduced in the presence of 5 ng/mL FK506 (0.9 × 105 ± 0.5 × 104 cpm and 1.5 × 104 ± 0.9 × 104 cpm, respectively; P = .003), which translates to 70% (±14%) inhibition for NT EBV-CTLs and 60% (±20%) inhibition for irr-siRNA+ EBV-CTLs. By contrast, proliferation of siRNA4+ EBV-CTLs in the presence of FK506 was substantially preserved. Thymidine incorporation in presence of FK506 was 3.3 × 105 (±1.3 × 104) cpm, which was only slightly reduced from that of siRNA4+ EBV-CTLs cultured in the absence of FK506 (4.4 × 105 ± 1.5 × 105 cpm; P = .06). Indeed, the percentage of inhibition of siRNA4+ EBV-CTLs was 29% (±7%) in
the presence of FK506 (Figure 3B). This maintenance of thymidine uptake was significantly greater than that observed in NT or irr-siRNA+ EBV-CTLs cultured in the presence of FK506 (P < .01).

When control EBV-CTLs and siRNA4+ EBV-CTLs were maintained in culture for more than 7 weeks using weekly stimulation with EBV-LCLs, suboptimal doses of IL-2 (20 U/mL twice a week), with or without the addition of FK506 (5 ng/mL twice a week), only EBV-CTLs expressing siRNA4 numerically expanded in the presence of FK506 (median fold increase, 24; range, 3-70; Figure 3C). By contrast, the expansion of NT and irr-siRNA+ EBV-CTLs was almost completely inhibited in the presence of FK506 (median fold expansion, 1; range 0-2; Figure 3C). Similar results were obtained when a dose of 10 ng/mL FK506 was added to the culture (supplemental Figure 2).

Silencing of FKBP12 does not affect EBV-CTL functionality

We evaluated the immunophenotype, cytotoxic activity, and EBV-antigen specificity of FKBP12-silenced EBV-CTLs using FACS analysis, 51Cr release assay, and multimers and IFNγ ELISPOT assay, respectively. As shown in Figure 4A, NT and irr-siRNA+ EBV-CTLs were predominantly CD3+ CD8+ T lymphocytes (88% ± 8% and 87% ± 6%, respectively). The majority of CTLs expressed the αβ TCR (90% ± 1% and 88% ± 11%), with less than 2% of natural killer cells (CD3- CD56+). Selective expansion of siRNA4+ EBV-CTLs in the presence of FK506 did not alter their phenotype (CD3+ CD8+ = 83% ± 12%; αβ TCR+ = 82% ± 14%; and CD3- CD56+ = 0.3% ± 0.2%; Figure 4A). We evaluated the cytotoxic activity of EBV-CTLs using a standard 4-hour 51Cr

Figure 3. siRNA4+ EBV-CTLs retain proliferative activity in the presence of FK506. (A) The thymidine uptake of NT, irr-siRNA-, and siRNA4+ EBV-CTLs after stimulation with autologous EBV-LCLs in the presence of increasing concentration of FK506. In the presence of FK506, proliferation of both NT and irr-siRNA- EBV-CTLs is significantly reduced compared with siRNA4+ EBV-CTLs. (B) The percentage inhibition for NT, irr-siRNA-, and siRNA4+ EBV-CTLs grown in the presence of FK506. Bars represent the mean ± SD of 4 CTL lines. (C) The expansion in cell numbers of NT, irr-siRNA-, and siRNA4+ EBV-CTLs stimulated weekly with EBV-LCLs and IL-2 (20 U/mL) with or without the addition of FK506 (5 ng/mL). T-cell numbers increased for all CTLs in the absence of FK506, but in the presence of FK506 increased only for siRNA4+ CTLs. Shown are median ± SEM for 6 CTL lines.
Table 1. Analysis of EBV specificity within the healthy donor CTL lines

<table>
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<tr>
<th>Peptide</th>
<th>Lytic</th>
<th>EBNA1</th>
<th>EBNA3</th>
<th>Irrelevant</th>
</tr>
</thead>
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<tr>
<td>Donor 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NT CTLs</td>
<td>243 ± 47</td>
<td>1202 ± 154</td>
<td>257 ± 47</td>
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<td>1050 ± 77</td>
<td>248 ± 61</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NT CTLs</td>
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<tr>
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<td></td>
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</tr>
<tr>
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<tr>
<td>siRNA4 + CTLs + FK506</td>
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<td>611 ± 95</td>
<td>0 ± 0</td>
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</table>

Numbers are mean ± SD of interferon-γ (IFN-γ) spot-forming cells/10⁵ cytotoxic T lymphocytes (CTLs).

EBV indicates Epstein-Barr virus; NT-CTLs, nontransduced cytotoxic T lymphocytes; siRNA, small interfering RNA; irr-siRNA, irrelevant siRNA; and ND, not determined.

release assay. As shown in Figure 4B, EBV-CTLs expressing siRNA4 and cultured for 4 weeks in the presence of FK506 still retained major histocompatibility complex–restricted cytotoxic activity against EBV⁺ targets (66% ± 22% lysis of autologous EBV-LCLs and 16% ± 12% of allogeneic EBV-LCLs, at a 20:1 E/T ratio). This was similar to irr-siRNA⁺ EBV-CTLs (61% ± 12% lysis of autologous EBV-LCLs and 15% ± 10% of allogeneic EBV-LCLs, at a 20:1 E/T ratio). As expected, addition of FK506 during the 4-hour incubation did not impair CTL cytotoxic activity (data not shown). To ensure that EBV-CTLs retained the broad EBV-specific reactivity necessary for effective control of EBV-PTLDs, we analyzed the specificities of 4 CTL lines with informative HLA typing, using their binding of EBV-specific HLA multimers and their IFNγ production in response to EBV-derived peptides in an ELISpot assay. Figure 4C shows a representative donor in whom siRNA4⁺ EBV-CTLs grown in the presence of FK506 for 3 weeks maintained the same frequency of T cells recognizing EBV-associated antigen. EBV-specific T cells were also functional as they specifically released IFNγ in response to EBV-derived peptides (Figure 4D). In all 4 CTL lines, the broad repertoire of the EBV-CTLs was retained with detection of specific IFNγ production against lytic and latent EBV-associated proteins (Table 1).

siRNA4⁺ EBV-CTLs have improved control of tumor growth in vivo in the presence of FK506

To assess whether siRNA4⁺ EBV-CTLs could control the growth of EBV⁺ lymphomas in the presence of FK506 in vivo, we used a SCID mouse xenograft model. Eight-week-old sublethally irradiated SCID mice (8 mice/group) were implanted with FFLuc-labeled EBV⁺ lymphomas in the peritoneal cavity. Light emission by tumor cells was monitored as an indication of tumor growth. Once progressive increase of bioluminescence occurred (usually 3-4 days after tumor injection) mice were treated intraperitoneally either with 2 doses of irr-siRNA⁺ EBV-CTLs or siRNA4⁺ EBV-CTLs, followed by intraperitoneal injection of IL-2 (1000 U/mice) and FK506 (10 mg/kg) 3 times a week. Control groups received an equivalent amount of IL-2 but not FK506. As shown in Figure 5A and B, the tumor bioluminescence of mice treated with irr-siRNA⁺ CTLs and FK506 progressively increased (from 6 × 10⁷ ± 4 × 10⁷ to 1.9 × 10⁹ ± 1.6 × 10⁹ p/s/cm²/sr by day 33) compared with mice receiving siRNA4⁺ CTLs and FK506 (from 5.3 × 10⁷ ± 2 × 10⁷ to 3.2 × 10⁸ ± 3.6 × 10⁸ p/s/cm²/sr by day 33; P = .02). Control of tumor growth by siRNA4⁺ CTLs in presence of FK506 was similar to that obtained in mice treated with irr-siRNA⁺ EBV-CTLs or siRNA4⁺ EBV-CTLs without FK506 (tumor bioluminescence 3.3 ± 10⁸ ± 2.8 × 10⁸ and 4.4 ± 10⁸ ± 1.9 × 10⁸, respectively, by day 33). The control of tumor growth in mice receiving siRNA4⁺ EBV-CTLs in the presence of FK506 translated into an improved survival of these mice by day 40 after tumor injection (P = .04) compared with mice receiving irr-siRNA⁺ CTLs and FK506 (Figure 5C). Hence, siRNA4⁺ EBV-CTLs can control tumor growth in vivo in the presence of FK506.
Discussion

We have shown that EBV-CTLs can be made resistant to FK506, the most common immunosuppressive drug used for liver, heart, and renal transplant recipients. This effect is obtained by a selective siRNA-mediated knockdown of FKBP12, a key protein that, after FK506 binding, blocks the activation of the calcineurin pathway, which in turn inhibits NFAT activation. As a consequence, even at doses of FK506 that inhibited EBV-CTLs with unmodified levels of FKBP12, gene-modified CTLs continue to proliferate and function as antitumor effector cells in vitro and in vivo in a xenograft model. This strategy can therefore preserve the function of desirable T cells, while maintaining suppression of alloreactive T cells. The approach could be adapted for the control of PTLDs or for other viral infections that cause morbidity and mortality in patients receiving solid organ transplantation, and in particular EBV infection can induce the occurrence of often fatal lymphomas. Although adoptive transfer of virus-specific T cells have proven safe in SOT as well as HSC transplant recipients, the continuous immunosuppressive environment in SOT recipients limits proliferation and persistence of these cells in distinction to their expansion after HSC transplantation. Although the short-term effects of adoptively transferred EBV-CTLs may control lymphoproliferative diseases in SOT recipients, they act imperfectly to control the high virus load in these patients, and their lack of persistence means they cannot readily be used as prophylaxis to prevent rejection of the transplanted organ. As a result, they have increased susceptibility to a range of viral infections, and in particular EBV infection can induce the occurrence of often fatal lymphomas. Recipients of SOT receive life-long immunosuppression to prevent rejection of the transplanted organ. As a result, they have increased susceptibility to a range of viral infections, and in particular EBV infection can induce the occurrence of often fatal lymphomas. Although adoptive transfer of virus-specific T cells have proven safe in SOT as well as HSC transplant recipients, the continuous immunosuppressive environment in SOT recipients limits proliferation and persistence of these cells in distinction to their expansion after HSC transplantation. Although the short-term effects of adoptively transferred EBV-CTLs may control lymphoproliferative diseases in SOT recipients, they act imperfectly to control the high virus load in these patients, and their lack of persistence means they cannot readily be used as prophylaxis to provide long-term control of EBV reactivation. The immunosuppressive drugs received by subject after SOT are designed specifically to inhibit T cells and reduce their ability to secrete and respond to cytokines upon stimulation.

We chose to block the function of FK506 by stably silencing the expression of FKBP12 using retroviral transduction with a specific siRNA vector, based on previous work in knockout mice showing that FK506-induced growth inhibition was abolished in FKBP12-deficient T cells and that these cells had normal function in response to antigen stimulation. FK506 is an immunophilin-binding drug that specifically targets FKBP12. Down-regulation of FKBP12 should prevent the formation of the FK506-FKBP12 complex that binds to calcineurins A and B and inhibits the dephosphorylation of cytoplasmic nuclear factor of activated T cells (NFAT), which translocates to the nucleus to initiate IL-2 transcription in activated T cells. FKBP12, however, is an abundant protein and therefore it was not clear whether it would be susceptible to knockdown. Indeed of 17 siRNAs only 5 produced more than 50% inhibition and only 1 induced more than 80% inhibition, and was chosen for further studies. Our data demonstrate that silencing of FKBP12 in EBV-CTLs allows them to proliferate in the presence of the drug, so that their functionality should not be impaired in vivo when patients continue on their immunosuppressive regimen. Although other FKBP (such as FKBP12.6 and FKBP51) are present in T cells, their role in mediating FK506-induced immunosuppression is dispensable, likely due to the their limited accessibility or lower binding affinity to the drug. The substantial retained proliferation of our FKBP12-silenced CTLs in the presence of FK506 supports this interpretation.

Although previous studies showed that FKBP12-deficient murine T cells were not dysfunctional, down-regulation of this protein in human EBV-CTLs may alter critical effector T-cell functions, such as cytotoxic activity, activation, and expansion after antigen-specific stimulation, or may alter phenotype or antigen specificity. Our data show that FKBP12-silenced EBV-CTLs have no such alterations. Hence, siRNA EBV-CTLs retain their major histocompatibility complex–restricted cytotoxic activity, produce IFNγ on exposure to EBV-associated antigens, and proliferate in the presence of FK506. As a result, the control of lymphoma growth in our in vivo xenogenic mouse model was significantly prolonged in mice receiving FK506.

FK506 is not an exclusive ligand for FKBP12. Rapamycin, another immunosuppressive agent used in SOT recipients, also binds to the FKBP12 to produce T-cell inhibition. Although rapamycin acts through the mammalian target of rapamycin rather than the calcineurin pathway, the beneficial effects of FKBP12-silenced T cells may also be extended to patients receiving rapamycin immunosuppression (supplemental Figure 3).

We conclude that the down-regulation of FKBP12 in EBV-CTLs can be used to sustain their proliferative activity even in the presence of FK506, and provide efficient control of EBV-related lymphomas. A similar approach may be of value for protecting other pathogen-specific T cells such as cytomegalovirus- and adenovirus-specific CTLs that could be infused in patients after solid organ transplantation, thereby helping reduce morbidity and mortality from infection in these patients while retaining organ engraftment. Combinations of pharmacologic immunosuppression, including mycophenolate and steroids in addition to calcineurin inhibitors, are often used and resistance to multiple immunosuppressive drugs may be explored. Obviously other obstacles to adoptive EBV-CTL therapy in solid organ transplantation would remain, including cytokine dependency of CTLs and the role and direct immunosuppressive effects of the tumor environment, and will need further elucidation. The combination of different strategies will help in designing the most successful approach.

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Authorship

Contribution: B.D.A. designed the research, performed the majority of the experiments, analyzed the data, and wrote the manuscript; G.D. designed the research, supervised the generation of the retroviral vectors, analyzed the data, and wrote the manuscript; C.Q. and L.E.H. performed some in vitro experiments; L.Z. and M.Z. provided technical assistance for some of the in vitro experiments; F.P., H.E.H., M.K.B., and C.M.R. provided assistance in the design of the research and critically reviewed the manuscript; B.S. designed the research, analyzed the data, and wrote the
manuscript; and all authors approved the final version of the manuscript.

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References

Generation of Epstein-Barr virus–specific cytotoxic T lymphocytes resistant to the immunosuppressive drug tacrolimus (FK506)

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