Co-expression of cytokine and suicide genes to enhance the activity and safety of tumor specific cytotoxic T lymphocytes

Concetta Quintarelli, Juan F. Vera, Barbara Savoldo, Greta MP. Giordano Attianese, Martin Pule, Aaron Foster, Helen E. Heslop, Cliona M. Rooney Malcolm K. Brenner and Gianpietro Dotti

Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, USA

Corresponding author: Gianpietro Dotti, MD, Center for Cell and Gene Therapy, Baylor College of Medicine, 6621 Fannin Street, MC 3-3320, Houston, Texas 77030, USA; Phone: 832 824 4725; Fax:: 832 825 4732; E-mail: gdotti@bcm.tmc.edu
Abstract

The anti-tumor effect of adoptively transferred tumor-specific cytotoxic T lymphocytes (CTLs) is impaired by the limited capacity of these cells to expand within the tumor microenvironment. Administration of IL-2 has been used to overcome this limitation, but the systemic toxicity and the expansion of unwanted cells, including regulatory T cells, limit the clinical value of this strategy. To discover whether transgenic expression of lymphokines by the CTLs themselves might overcome these limitations, we evaluated the effects of transgenic expression of IL-2 and IL-15 in our model of Epstein Barr Virus-specific CTLs (EBV-CTLs). We found that transgenic expression of IL-2 or IL-15 increased the expansion of EBV-CTLs both in vitro and in vivo in a SCID mouse model, and enhanced anti-tumor activity. Although the proliferation of these cytokine genes transduced CTLs remained strictly antigen dependent, clinical application of this approach likely requires the inclusion of a suicide gene to deal with the potential development of T-cell mutants with autonomous growth. We found that the incorporation of an inducible caspase-9 suicide gene allowed efficient elimination of transgenic CTLs after exposure to a chemical inducer of dimerization, thereby increasing the safety and feasibility of the approach.
Introduction

Adoptive transfer of antigen specific cytotoxic T lymphocytes (CTLs) has shown efficacy in some patients with melanoma\(^1,2\), Hodgkin’s lymphoma\(^3,4\) and nasopharyngeal carcinoma\(^5,6\). However, the anti-tumor activity of adoptively transferred CTLs is hampered by the limited capacity of these cells to significantly expand within the tumor microenvironment\(^7\). The development of strategies to overcome this restriction could significantly improve the clinical outcome of patients receiving adoptive T-cell therapy.

Since adoptively transferred antigen specific CTLs are highly dependent on exogenous cytokines for their continued growth and survival\(^8\) systemic administration of IL-2 has been used to enhance their in vivo expansion and persistence\(^1\). However, the prolonged administration of IL-2 is often associated with serious side effects, limiting the amount and duration of cytokine administration\(^1\). Moreover, the effects of systemically administered cytokines are non-selective. IL-2 may favor the expansion of unwanted cell subsets, such as regulatory T cells\(^9\), that constitutively express the IL-2 receptor and adversely affect the function of anti-tumor CTLs\(^10-12\).

Genetic manipulation of CTLs to express growth cytokines such as IL-2 and IL-15, could make them less helper-cell dependent and better able to sustain their proliferation and activation after antigenic stimulation\(^13,14\). However, the constitutive expression of transgenes that enhance growth raises the concern that the T cells may lose antigen specificity and growth-dependence and become growth-autonomous\(^15\). This is a particular concern where retroviruses are used to ensure transgene integration and thereby obtain cytokine secretion by the progeny of the modified cells\(^16,17\). We therefore used our EBV+ tumor model and EBV-specific CTLs (EBV-CTLs) to evaluate the biological effects of transgenic expression of IL-2 or IL-15, in association with transfer of a suicide gene based on an inducible caspase-9 (iCasp-9) protein\(^18\) that can be activated using a specific chemical inducer of dimerization (CID), analogs of which have been safely tested in a Phase I study\(^19\).

We found that both transgenic IL-2 and IL-15 sustained CTL expansion and function in vivo, and that these cytokine-gene modified cells retained antigen specificity and dependence. Activation of the iCasp-9 gene with CID efficiently ablated cytokine production and eliminated adoptively transferred T cells, suggesting that this combined
approach could safely augment the efficacy of adoptively transferred tumor-specific CTLs.

**Materials and methods**

*Human and animal studies were approved by the Institutional Review Board of Baylor College of Medicine.*

**Plasmid construction and retrovirus production.** CD34 was used as a selectable marker of transduced cells. Full length human CD34 (NCBI AF523361) was cloned by RT-PCR using Clone ID: 4746591 (Invitrogen, Carlsbad, CA). We truncated the cytoplasmic tail of CD34, 24 amino acids (NGG) downstream from the last amino acid of the putative transmembrane domain. This truncation was based on preliminary experiments showing stable expression of ΔCD34 in Jurkat cells (>1 month; data not shown), and the efficient selection of transduced T cells using anti-CD34 microbeads (Miltenyi, Bergisch Gladbach, Germany), a method approved for clinical use (CliniMacs). The full length human IL-2 and IL-15 genes were cloned by PCR from plasmids obtained from InVivogene (San Diego, CA). The human IL-15 gene encodes the isoform with the long 48 AA signal peptide20. The construction of the iCasp-9 suicide gene was previously reported18. Briefly, caspase-9 gene is deleted for the CARD domain and fused in frame with a 12-kDa human FK506 binding domain (FKBP12; GenBank AH002818) that contain an F36V mutation, allowing dimerization of the caspase-9 and activation of the apoptotic pathway after exposure to the FK506 analog AP2018718,21. The 3 genes (iCasp-9, ΔCD34, IL-2 or IL-15) were linked using 2A-like peptides derived from foot-and-mouth disease virus, to allow transcription and expression of one single mRNA molecule. The sequences of the 2A-like peptides were: pSTA1-TaVR AEGRGSSLTCGDVEENPGP and pSTA1-ERAV QCTNYALLKLAGDVESNPGP22,23. The entire cassette was cloned into the SFG retroviral vector and schema of SFG.iCasp-9.2A.ΔCD34.2A.IL-2 (iC.ΔCD34/IL-2v), SFG.iCasp-9.2A.ΔCD34.2A.IL-15 (iC.ΔCD34/IL-15v) and SFG.ΔCD34 (ΔCD34v) are illustrated in Fig. 1A. These vectors were used for all the *in vitro* and *in vivo* experiments. The vector encoding the fusion protein eGFP-Fireflyluciferase (eGFP-FFLuc) was previously described24. The retroviral supernatant was prepared as previously described25. Briefly, 293T cells were co-transfected with three plasmids (the retroviral construct, Peg-Pam-e encoding for *gag-pol*, and DRF
encoding for the RD114 envelop\textsuperscript{26}, using the Fugene6 transfection reagent (Roche, Indianapolis, IN), and supernatants were collected 48 and 72 hours later.

**Generation and transduction of EBV-CTLs.** EBV-CTLs were prepared by stimulating peripheral blood mononuclear cells (PBMCs) with gamma-irradiated (40 Gy) autologous EBV-transformed lymphoblastoid cell lines (LCLs) on day 0 and +9, then weekly thereafter. Recombinant human interleukin-2 (rhIL-2) (50 U/mL) (Proleukin; Chiron, Emeryville, CA), was added twice a week from day 14 as previously described\textsuperscript{27}. For transduction, EBV-CTLs obtained after at least 3 stimulations were plated at 0.5 x 10\textsuperscript{6} cells/well in 24-well plates precoated with recombinant fibronectin fragment (FN CH-296; Retronectin; Takara Shuzo, Otsu, Japan) and incubated with the retroviral supernatant\textsuperscript{25}. Three days after transduction, EBV-CTLs were collected, and then stimulated weekly with autologous LCLs, with or without the addition of exogenous cytokines rhIL-2 (50 U/mL) or recombinant human IL-15 (rhIL-15) (10 ng/mL) (R&D System, Minneapolis, MN). The release of IL-2 and IL-15 by control and transgenic CTLs was measured in the culture supernatant by specific ELISAs from R&D system (Minneapolis, MN).

**Immunophenotyping.** Cells were stained with PE-, FITC- or PerCP-conjugated monoclonal antibodies (MAbs). We used CD3, CD4, CD8, CD56, CD34, CD45RA, CD45RO and CD62L from Becton-Dickinson (Mountain View, CA, USA) and MAbs specific for the TCR-Vß repertoire (IOTest ßMark kit; Immunotech, Emeryville, CA). Tetramers targeting known MHC class I epitopes of EBV-related antigens were also used\textsuperscript{25,28}. The induction of apoptosis of EBV-CTLs was evaluated using the Annexin-V/7-AAD staining (Becton-Dickinson). In some experiments Annexin-V+ CTLs were selected using Annexin-V-FITC antibody and anti-FITC magnetic beads (Miltenyi). Cells were analyzed by a FACScan (Becton Dickinson) equipped with the filter set for triple fluorescence signals.

**Activation of the suicide gene.** The chemical inducer of dimerization (CID) (AP20187; ARIAD Pharmaceuticals, Cambridge, MA) was kindly provided by Dr. Spencer (Baylor College of Medicine) and added at the indicated concentrations to EBV-CTLs transduced either with ΔCD34\textsubscript{v}, or iC.ΔCD34/IL-2\textsubscript{v} or iC.ΔCD34/IL-15\textsubscript{v} vectors. The elimination of transgenic cells co-expressing the iCasp-9 suicide gene was evaluated 24-48 hours later by FACS analysis, enumerating the percentage of ΔCD34+ cells in the culture. In parallel, we measured IL-2 and IL-15 cytokines in the culture supernatant.
using specific ELISAs (R&D System). For long-term experiments, IL-2 and IL-15 transgenic CTLs were selected with magnetic beads (Miltenyi) based on their expression of ΔCD34, exposed to one single dose of CID (50nM) and then maintained in culture by weekly stimulation with autologous LCLs, without addition of exogenous cytokines and without further addition of the CID. Viable cells were enumerated each week using trypan blue exclusion. Control CTLs were maintained in culture without exposure to CID.

**Chromium release assay.** We evaluated the cytotoxic activity of EBV-CTLs by using a standard 4-hour $^{51}$Cr release assay, as previously described$^{29}$. As target cells we used autologous LCLs, HLA class I and II mismatched LCLs, as well as the HSB-2 and K562 cell lines that measure lymphokine-activated and natural killer activity, respectively. Target cells incubated in media alone or in 1% Triton X-100 were used to determine spontaneous and maximum $^{51}$Cr release, respectively. The mean percentage of specific lysis of triplicate wells was calculated as follows: $\frac{\text{[test counts} - \text{spontaneous counts]}}{\text{[maximum counts} - \text{spontaneous counts}] \times 100}$.

**Enzyme-Linked Immunospot (ELISPOT) assay.** The IFN$\gamma$ ELISPOT assay was performed as previously described$^{25,28,30}$. We plated EBV-CTLs in triplicate, serially diluted from $1 \times 10^5$ to $1 \times 10^4$ cells/well and stimulated with $100 \mu$L of autologous, irradiated LCLs ($1 \times 10^5$ cells) or EBV-derived peptides (5 $\mu$M). Negative controls included EBV-CTLs alone and EBV-CTLs loaded with irrelevant peptides.

**Western blot analysis.** Cell lysates were resolved on SDS-PAGE. Caspase-9 and CD34 proteins were detected by immunoblot using specific monoclonal Abs (Abcam, Inc, Cambridge, MA). Immunoblots were developed using enhanced chemiluminescence detection reagents (Amersham, Biosciences). Membranes were reprobed using the monoclonal anti-GAPDH Ab (Santa Cruz Biotechnologies, Santa Cruz, CA).

**In vivo study using a xenogenic SCID mouse model.** To assess the expansion, persistence and anti-tumor effect of transgenic EBV-CTLs in vivo, we used a SCID mouse model and an in vivo imaging system. CB17 SCID mice 8-10 weeks old were purchased from Harland Sprague Dawley, Indianapolis, Indiana. Mouse experiments were performed in accordance with Baylor College of Medicine Animal Husbandry guidelines.

In vivo expansion and anti-tumor effects of transgenic CTLs. To evaluate the in vivo expansion of transgenic CTLs, EBV-CTLs transduced either with iC.ΔCD34/IL-2v,
iC.ΔCD34/IL-15v or ΔCD34v control vector were transduced a second time with the vector encoding the eGFP-FFLuc gene. A total of 4 different EBV-CTL lines were tested in vivo. Briefly, SCID mice were sublethally irradiated (250 rad) and injected subcutaneously with 10^7 LCLs suspended in Matrigel (Becton Dickinson). Between 15 and 20 days later when the tumor was palpable (0.5-0.8 cm in diameter), 10^7 autologous CTLs were injected intravenously. No exogenous cytokines were administered. For the in vivo imaging of EBV-CTLs expressing eGFP-FFLuc, mice were injected intraperitoneally with D-luciferin (150 mg/kg) and analyzed using the Xenogen-IVIS Imaging System, as previously described. Briefly, a constant region-of-interest (ROI) was drawn over the tumor region and the intensity of the signal measured as total photon/sec/cm^2/sr (p/s/cm^2/sr). Mice were euthanized if their tumor was >1.2 cm in maximum diameter. Mice with smaller tumors or complete tumor regression were followed until day 50 post CTL infusion and then euthanized to analyze the tumors after autopsy. A group of control mice engrafted with LCLs received unmanipulated EBV-CTLs and rhIL-2 i.p. 1000 U/mL every two days.

In vivo validation of the iCasp-9 suicide gene. To evaluate the functionality of the suicide gene, mice bearing LCLs and receiving EBV-CTLs transduced either with iC.ΔCD34/IL-2v or iC.ΔCD34/IL-15v vector and labeled with the eGFP-FFLuc gene, were treated with CID (50 μg) i.p. 2-3 doses every other day. CID treatment was initiated when the bioluminescent signal was exponentially increasing, indicating active expansion of the transgenic cells. Mice were then imaged as described above.

Statistical analysis. All in vitro data are presented as mean ± 1 SD. Student’s t test was used to determine the statistical significance of differences between samples, and P < .05 was accepted as indicating a significant difference. For the bioluminescent experiments, intensity signals were log-transformed and summarized using mean ± SD at baseline and multiple subsequent time points for each group of mice. Changes in intensity of signal from baseline at each time point were calculated and compared using the Wilcoxon signed-ranks test. Tumor free survival was analyzed by Kaplan-Meier analysis (using SPSS software), and the statistical significance of observed differences was assessed by log-rank and Breslow testing.
Results

Transgenic expression of IL-2 and IL-15 sustains the expansion of EBV-CTLs. Fig. 1A shows the construction schema for the vectors SFG.iCasp-9.2A.ΔCD34.2A.IL-2 (iC.ΔCD34/IL-2v), SFG.iCasp-9.2A.ΔCD34.2A.IL-15 (iC.ΔCD34/IL-15v) and SFG.ΔCD34 (ΔCD34v) used to transduce EBV-CTL lines. To validate expression from these vectors, we performed a Western blot analysis using COS-7 transduced cell lines. Fig. 1B illustrates that CD34 and Caspase-9 proteins were expressed as single proteins in cells transduced either with iC.ΔCD34/IL-2v or iC.ΔCD34/IL-15v vectors, indicating efficient cleavage mediated by the 2A-like sequence. We were not able to determine the presence of the cytokines IL-2 and IL-15 by Western blot likely because these cytokines were rapidly secreted. However, these cytokines were detectable in the culture supernatants of transduced but not control COS-7 cells (data not shown). We obtained EBV-CTLs after three stimulations of T cells with autologous LCLs from five healthy EBV-seropositive donors, and transduced them either with iC.ΔCD34/IL-2v or iC.ΔCD34/IL-15v or ΔCD34v vectors. We determined the transduction efficiency of EBV-CTLs by surface detection of the CD34 molecule, and obtained values of 33% ± 15%, 37% ± 16% and 56% ± 20% for the iC.ΔCD34/IL-2v, iC.ΔCD34/IL-15v and ΔCD34v vector, respectively (Fig. 1C). To confirm the functionality of the constructs, we used specific ELISAs to measure the amount of IL-15 and IL-2 cytokines released in the supernatants of EBV-CTL lines stimulated with autologous EBV-LCLs. IL-15 was undetectable in control EBV-CTL supernatants, but was 41 pg/mL/10^6 cells (range 21 to 72) in supernatants from EBV-CTLs transduced with iC.ΔCD34/IL-15v, 48-72 hours after the first stimulation with autologous LCLs. The production of IL-15 by transgenic CTLs remained detectable, although at lower levels, after antigen stimulation in supernatants of transgenic CTLs maintained in culture for more than 4 weeks (data not shown). The maximum concentration of IL-2 was detected 24 hours after the first stimulation with LCLs. It was 40 pg/mL/10^6 cells (range, 0 to 101) in control EBV-CTLs and 108 pg/mL/10^6 cells (range, 24 to 264) (p=0.08) in EBV-CTLs transduced with the iC.ΔCD34/IL-2v. Although the increased production of IL-2 by iC.DCD34/IL-2v transduced CTLs did not reach statistical significance compared to control CTLs after the first LCL stimulation, IL-2 remained detectable (7-10 pg/mL) in the supernatant for up to 5 rounds of re-stimulation with antigen. In contrast, IL-2 was always undetectable in control CTLs immediately after the first stimulation with EBV-LCLs and these cells did
not significantly expand. To confirm that IL-2 produced by IL-2 transgenic CTLs was responsible for the growth of CTL lines transduced with iC.ΔCD34/IL-2v vector, we used blocking antibodies to the IL-2 cytokine or to its high affinity receptor (CD25) (R&D systems). In both cases, we observed significant inhibition of the proliferation of IL-2 transgenic CTLs (Supplemental Fig. 1).

We also evaluated the kinetics of IL-2 and IL-15 cytokine production by CTLs transduced either with iC.ΔCD34/IL-2v or iC.ΔCD34/IL-15v or ΔCD34v vectors. Fig. 1D shows that cytokine production was antigen dependent and that both IL-2 and IL-15 secretion from CTLs became undetectable 6-7 days after antigen stimulation.

We next evaluated whether the quantity of cytokine production was sufficient to sustain the expansion of transgenic EBV-CTLs in response to specific antigenic stimulation. After transduction, EBV-CTLs were maintained in culture and stimulated once a week with autologous LCLs (E:T ratio of 1:1) without addition of exogenous cytokines. In parallel, control EBV-CTLs transduced with ΔCD34v vector alone were maintained in culture with exogenous rhIL-2 (50 U/mL) or rhIL-15 (10 ng/mL). As shown in Fig. 2A, a significant expansion of IL-2 transgenic CTLs (1838 fold expansion, range 289-5044) and IL-15 transgenic CTLs (1156 fold expansion, range 511-3069) was observed after 35 days of culture. As expected, control EBV-CTLs transduced with ΔCD34v vector also expanded when stimulated with LCLs, but only in the presence of exogenous rhIL-2 (3576 fold expansion, range 915-5000) or rhIL-15 (1389 fold expansion, range 335-2838). In the absence of such exogenous cytokines, LCL stimulated ΔCD34v CTLs did not significantly expand (<2 fold). Similar results were observed using CD4+ EBV-CTL lines (Supplemental Fig. 2). We found that the continued proliferation of transgenic EBV-CTLs remained strictly antigen dependent, as a progressive reduction in the proportion of stimulator cells (CTL:LCL ratio from 4:1 to 10:1) resulted in a corresponding reduction in the CTL’s expansion rate (data not shown). Moreover, both IL-2 and IL-15 transgenic EBV-CTLs maintained in culture without antigen stimulation did not significantly expand and died within 2-3 weeks (Fig. 2B).

**IL-2 and IL-15 transgenic-CTLs retain their antigen specificity.** To confirm that the genetic manipulation of EBV-CTLs did not modify their antigen specificity, we monitored their phenotype, polyclonality and antigen repertoire over 5-6 weeks of culture. The phenotypic profile of CTLs was not altered by cytokine transduction, as the great majority of EBV-CTLs remained CD3+/CD8+ (96 % ± 1%, 97% ± 2% and 98% ± 2% for
ΔCD34v, iCD34ΔCD34/IL-2v and iCD34ΔCD34/IL-15v vectors), while less than 5% were CD3+/CD4+. In all cases, fewer than 15% of cells were CD3+/CD56+ (Fig. 3A). EBV-CTL lines growing in exogenous rhIL-2, are mainly effector cells with a proportion of effector memory cells (CD45RA-, CD45RO+, CD62L+) ranging from 2% to 15%. This pattern was similarly maintained in CTLs expanded with exogenous rhIL-15 or in CTLs transgenic for IL-2 or IL-15. In addition, neither IL-2 nor IL-15 transgenes modified the antigen specificity of the EBV-CTLs. Hence, cytotoxic activity, measured by 51Cr release assay, remained specific for autologous LCLs and the percentage of autologous LCLs lysed by the control EBV-CTLs was 56% ± 20% at E:T ratio 20:1, versus 54% ± 12% for IL-2 and 46% ± 9% for IL-15 transgenic cells (Fig. 2B). Killing of allogeneic LCLs was significantly lower in all cases, confirming retained MHC-restriction (Fig. 3B). No significant reactivity (<10%) was observed against the K562 cell lines (Fig. 3B and Supplemental Fig. 3).

To further demonstrate that EBV-CTLs maintained the pattern of antigen response after transduction, we evaluated the frequency with which each EBV-CTL population recognized HLA class I restricted EBV peptides, using specific peptide-HLA tetramers and flow cytometric analysis to measure binding, and IFNγ ELIspots to measure responsiveness. As shown in Fig. 3C and 3D and Table 1, the frequency of EBV-specific CTLs in control and transgenic CTLs was retained. Although the distribution of antigen specificity and Vβ T-cell receptor (αβTCR) drifted over time, there was continued polyclonality of the transgenic EBV-CTLs, with no evidence for progressive clonal outgrowth compared to control CTLs growing with exogenous rhIL-2 (Supplemental Table 1).

Expression of transgenic cytokines improves in vivo expansion and anti-tumor activity of EBV-specific CTLs. To assess the in vivo functionality of transgenic CTLs, we used a SCID mouse tumor model, in which the animals were engrafted subcutaneously with EBV-LCLs and then infused i.v. with autologous EBV-CTLs. Transduced EBV-CTLs obtained from 4 healthy donors were selected with anti-CD34 microbeads and then transduced with the vector encoding eGFP-FFLuc. After the second transduction, the percentage of GFP+ EBV-CTLs ranged from 43% to 64% for ΔCD34v, from 34% to 46% for iCD34ΔCD34/IL-2v and from 16% to 46% for iCD34ΔCD34/IL-15v. SCID mice were engrafted with autologous LCLs (10^7 cells), and when the tumor was palpable (0.5-0.8 cm in diameter), 10^7 EBV-CTLs were injected i.v. No exogenous
cytokines were administered. Localization and expansion of the CTLs in the tumor area was measured using the Xenogen-IVIS Imaging System. As illustrated in Fig. 4A and 4B that summarize results from 20 mice per group, the EBV-CTLs homed to the tumor and progressively expanded. Maximum bioluminescence photon emission occurred 2-4 weeks after CTL infusion and the median increase was 2 fold (range, 0.5 – 45) for control \( \Delta CD34v \), 13 fold (range, 1.2 – 587) for iC.\( \Delta CD34/IL-2v \) (\( p=0.0015 \)) and 33 fold (range, 1.5 – 97) for iC.\( \Delta CD34/IL-15v \) (\( p=0.0008 \)).

The increase of bioluminescence from IL-2 and IL-15 transgenic CTLs remained antigen dependent and MHC-restricted in vivo: when the mice were engrafted with allogeneic EBV-LCLs, transgenic CTLs homed to the tumor but, unlike with autologous EBV-LCLs, did not expand significantly (Fig. 4B). The increase in the bioluminescence signal for the cytokine-transgenic CTLs corresponded to an actual increase in number of CTLs accumulated within the tumor and not just to an increase in bioluminescence due to enhanced expression by the same number of cells. Fig. 4C shows the increased CTL numbers in tumors excised from animals in the high (cytokine-transduced) versus the low (control) bioluminescence groups.

The increased expansion in vivo of cytokine transgenic CTLs was associated with an enhanced anti-tumor effect, as in both the IL-2 and the IL-15 transgenic CTL groups, 47% to 53% of the mice were tumor free 4-6 weeks after adoptive T-cell transfer, compared to none of the mice receiving control CTLs (\( p=0.001 \)) for iC.\( \Delta CD34/IL-2v \) CTLs and \( p=0.0009 \) for iC.\( \Delta CD34/IL-15v \) CTLs (Table 2 and Supplemental Fig. 4). Tumor regression was observed in mice treated with transgenic IL-2 and IL-15 from 3 of the 4 EBV-CTL lines used (25% for donor #1, 75% for donor #2, 64% for donor #3 and 0% for donor #4). These results also compared favorably to mice receiving unmanipulated CTLs and exogenous rhIL-2 in which we observed a 17% rate of complete tumor regression\(^3\) (\( p=0.045 \)) for iC.\( \Delta CD34/IL-2v \) CTLs and \( p=0.019 \) for iC.\( \Delta CD34/IL-15v \) CTLs, respectively (Table 2).

**Activation of the iCasp-9 suicide gene eliminates transgenic EBV-CTLs ex vivo and in vivo.** For clinical applications, constitutive expression of transgenic cytokines would likely raise concerns about autonomous and uncontrolled growth of CTLs\(^{16,17}\). We therefore incorporated the iCasp-9 suicide gene in our cytokine encoding retroviral vectors\(^{16}\). This pro-apoptotic gene product is activated after exposure to a small chemical inducer of dimerization (CID) (AP20187), which is an analog of FK506.
Addition of CID (50nM) to cultures of EBV-CTLs transduced either with iC.ΔCD34/IL-2v or iC.ΔCD34/IL-15v vectors led to a significant reduction in the percentage of ΔCD34+ cells (from 42% ± 11% to 5% ± 1% p=0.002 for iC.ΔCD34/IL-2v and from 63% ± 15% to 8% ± 3% p<0.001 for iC.ΔCD34/IL-15v) within 24 - 48hrs (Fig. 5A and 5B). In contrast, the percentage of ΔCD34+ cells did not change when the drug was added to EBV-CTLs transduced with the ΔCD34v control vector, which lacks the iCasp-9 gene. Elimination of the transgenic cells in vitro was dose dependent (Fig. 5C and 5D) and was mediated by induction of apoptosis as assessed by Annexin-V/7-AAD staining. Although up to 10% of ΔCD34dim cells persisted 24-48 hrs after incubation with CID, the near complete elimination of ΔCD34bright cells meant that the (linked) production of IL-2 and IL-15 cytokines fell to unmeasurable levels (Fig. 6A). To further demonstrate elimination of the transgenic cells, we evaluated whether exposure to CID terminated the growth of IL-2 or IL-15 transgenic CTLs in long-term culture experiments. Transgenic CTLs were selected based on the expression of ΔCD34 (Supplemental Fig. 5) and incubated with CID (50nM) for 24-48 hours. CTLs were then collected, washed and restimulated once a week with autologous LCLs for a total of 3 stimulations without any addition of CID. As shown in Fig. 6B, one single dose of drug was sufficient to abrogate the long-term growth of IL-2 or IL-15 transgenic CTLs and the cells progressively died. To evaluate whether the fraction of apoptotic cells (Annexin-V+/7-AAD-) detected 24-48 hrs after CID exposure could recover and become a viable cell population (Annexin-V-/7-AAD-) after drug withdrawal, we sorted Annexin-V+ cells using magnetic beads and maintained them in culture with or without stimulation with autologous LCLs. As shown in Fig. 6C, Annexin-V+ cells were not restored to an Annexin negative population even after antigenic stimulation and instead they invariably progressed to late apoptosis/necrosis (Annexin-V+/7-AAD+). We also evaluated whether the CID could induce apoptosis in resting transgenic CTLs. IL-2 and IL-15-transgenic CTLs sorted for CD34 expression and maintained in culture for 8-9 days after the last stimulation were incubated with CID (50nM). As shown in figure 6D, the drug continued to significantly deplete the transduced cells within 24-48 hours.

The iCasp-9 system was also functional in vivo iC.ΔCD34/IL-2v or iC.ΔCD34/IL-15v transduced EBV-CTLs were selected for ΔCD34 expression and then transduced with the eGFP-FFLuc gene, as described above. Ten million EBV-CTLs were injected i.v. in tumor bearing SCID mice. Once CTL expansion was detectable at the tumor site
(assessed by progressively rising photon emission), the animals were injected with CID i.p. As illustrated in Fig. 7A and 7B, photon emission at the time of CTL localization at the tumor site ranged from $5 \times 10^4$ to $1 \times 10^5$ p/s/cm$^2$/sr, increasing to $1.8 \times 10^6$ - $1.5 \times 10^7$ p/s/cm$^2$/sr ($p<0.01$) by day 10-15. Mice were then treated with CID (50 μg) and the bioluminescence fell by more than 1 log ($6.3 \times 10^4$ to $5.2 \times 10^5$ p/s/cm$^2$/sr) within 24-72 hours returning to the pre-expansion level ($p=0.1$), compared to the bioluminescence at the time of CTL localization. In contrast, the bioluminescence increased over time in mice infused with IL-2 or IL-15 transgenic CTLs without the CID (Fig. 7A and 7B). As shown in Fig. 7C, the reduction of the bioluminescence after CID administration corresponded to a significant decrease in CTLs infiltrating the tumor, assessed by FACS analysis of biopsied tumors.

**Discussion**

We have used retroviral mediated transgenic expression of IL-2 or IL-15 in tumor specific CTLs to improve their expansion and efficacy. We also determined whether the inclusion of a suicide gene could remove the transgenic cells, to further increase safety should unwanted T-cell proliferation or autonomy occur. Using EBV as our model system, we found that IL-2 or IL-15 transgenic EBV-CTLs have enhanced expansion *ex vivo* and *in vivo*, and retained their antigen specificity and effector function. Their improved survival and expansion was associated with increased anti-tumor activity *in vivo*. Importantly, we found that the growth of cytokine transgenic CTLs remained dependent on antigen stimulation and that the pharmacologic activation of a suicide gene iCasp-9, efficiently eliminated cytokine producing CTLs.

Preclinical and clinical trials of adoptive transfer with anti-tumor CTLs have shown that the limited *in vivo* expansion of transferred CTLs is one of the barriers that needs to be overcome to improve the clinical outcome of this therapeutic approach.$^{7,34}$ Transgenic expression of growth promoting cytokines by the adoptively transferred CTLs represents a strategy to support their own expansion/persistence.$^{13,14}$ In addition, the localized production of cytokines by transgenic cells might avoid the toxicity of systemic administration of recombinant cytokines, and reduce the expansion of other cells such as regulatory T cells that impair the anti-tumor immune response.$^{7,9,11,12}$ However, before translating this genetic modification in a clinical application, several issues need to be
addressed such as: confirming that progeny of genetically modified cells maintain antigen specificity and dependence; providing evidence that genetically modified CTLs possess enhanced anti-tumor effects; and ensuring that these cells remain safe.

Our model of EBV-tumor and EBV-CTLs demonstrates that retroviral gene transfer of IL-2 or IL-15 produces sufficient cytokines to sustain CTL expansion ex vivo and in vivo for more than 5 weeks providing an improved anti-tumor effect. Importantly, the antigen specificity and dependence, and the MHC restriction of the CTL progeny were maintained. Although extensive ex vivo T cell culture can alter the antigenic and TCR repertoire of CTL lines, we found that IL-2 and IL-15 transgenic CTL lines retained broad reactivity to EBV-epitopes and showed no progressive restriction of their antigen specificity or TCR repertoire compared to control CTL lines.

In a previously described immunocompetent mouse model, the presence of IL-15 resulted in the polarization to central memory phenotype of adoptively transferred CTLs and improved anti-tumor effects compared to IL-235. In our model, we did not find differences in anti-tumor effects between IL-2 or IL-15 transgeneic EBV-CTLs or a polarization to central memory phenotype in IL-15 transgenic CTLs. Since the gene transfer was performed in CTL lines obtained after 2-3 stimulation ex vivo, in cells exposed to IL-2 for 10-15 days, it is possible that at the time of transduction cells are already polarized to an effector memory phenotype and that the transgenic production of IL-15 cannot then affect this phenotype.

The use of cytokine-expressing CTLs in clinical adoptive transfer raises potential concerns, since leukemic transformation or immortalization of T cells has been reported in a mouse model engrafted with murine T-cell lines expressing IL-236, in IL-15 transgenic mice37 and in a human T cell clone14. By contrast, our study of cytokine expressing EBV-CTLs failed to show any evidence for progressive clonality or for the development of antigen independent proliferation. In the absence of antigen, the transgenic CTLs did not expand and died within 2-3 weeks. Moreover, we found that the bioluminescence signal from transgenic CTLs progressively declined in vivo after elimination of the tumor. This is likely related to the fact that transgenic cytokine production depends on the activation of the retroviral LTR, which in turn is strictly dependent on the activation status of the T cells, and is optimal only in the presence of adequate αβTCR stimulation13,38. Moreover, the cells we transduce are mature effector T
cells which are likely less vulnerable to malignant transformation than the less committed T progenitor cells or cell lines used in the earlier studies.36,37

Although our observations support the safety of transgenic expression of cytokines in antigen specific CTLs, we cannot exclude rare oncogenic events associated with retroviral integration near promoters or genes involved with critical elements of growth and survival14,16,17. We therefore incorporated a suicide gene based on the inducible caspase-9 molecule within our constructs. To generate these retroviral vectors encoding for three genes, we co-expressed iCasp-9, ΔCD34 and cytokine genes using 2A-like peptides22,23, which use a ribosomal skip mechanism to allow translation of multiple genes encoded by one single mRNA39. We found this approach for expressing multiple genes preferable to an internal ribosome entry site (IRES) or additional promoters, since these approaches were associated with suboptimal or unequal expression of one of the transgenes23. Consistent co-expression of multiple transgenes is particularly important for our application, as the activation of the suicide gene needs to have its highest probability of success against those cells that produce the highest quantity of growth-promoting cytokines. While our data show that a fraction of transgenic CTLs (<10%) did not undergo apoptosis/necrosis shortly after exposure to the dimerizer drug, these cells were all characterized by low expression of the ΔCD34 selectable marker, and undetectable cytokine secretion. Hence, cytokine production falls to undetectable levels after suicide gene activation, and the increased proliferation following antigen stimulation is no longer observed.

The inclusion of the selectable marker within the retroviral cassette might be useful for the enrichment of transgenic CTLs for a clinical trial. We used a truncated form of human CD34 since CD34+ selection can be performed using clinical grade reagents. However, ΔCD34 can be easily substituted by other selectable markers, such as CD19 (Tey SK et al. Bio Bone Marrow Transpl. 2007 in press) in case it is found that CD34 expression impairs the trafficking in vivo of antigen specific CTLs.

In conclusion, the data we report specifically support the use of transgenic cytokines to improve the expansion and anti-tumor effects of antigen specific CTLs. The incorporation of an effective suicide gene should further increase the safety of the approach and increase its potential clinical applicability.
Authorship
C.Q. and J.V. contributed equally to the work
C.Q., J.V., B.S., G.G. and G.D. designed and performed the experiments
J.V., M.P. and G.D. designed and constructed the vectors
G.D., B.S., J.V., C.Q. and M.K.B. designed the research and analyzed the data
A.F. contributed to perform the in vivo experiments
H.E.H. and C.M.R. provided expertise in T-cell generation, analyzed the data and reviewed the manuscript
G.D., B.S., J.V., C.Q. and M.K.B. wrote the manuscript.

The authors declare no competing financial interests

Acknowledgements. This work was supported in part from Leukemia and Lymphoma Society Specialized Center of Research (SCOR; grant no. 7018) and MDACC SPORE in Head and Neck. B.S. is supported by NIH 5R21AI65549. G.D. is supported by the Doris Duke Charitable Foundation/Clinical Scientist development award and by a Leukemia and Lymphoma Society Translational Research grant. J.V. and H.E.H. are supported by a Doris Duke Distinguished Clinical Scientist Award (H.E.H.). We express gratitude to Fernando Jimenez for FACS analysis, Jessie Wu for statistical analysis and David Spencer for providing expertise with the suicide gene.
Figure legend

**Fig. 1. Construction and functionality of the retroviral vectors.** Panel A is the schema of the retroviral vectors used to transduce EBV-CTLs. Panel B is a Western blot analysis showing the expression of ΔCD34 (upper panel) and caspase-9 (middle panel) in COS-7 cells transduced either with iC.ΔCD34/IL-2v or iC.ΔCD34/IL-15v or ΔCD34v vectors. The lower gel shows the membrane re-probed with anti-GAPDH antibody. Panel C shows the transduction efficiency of EBV-CTLs measured as expression of a truncated form of CD34 (ΔCD34) on the cell surface by FACS analysis. Plots from a representative experiment are shown. Panel D illustrates the kinetics of cytokine release by EBV-CTLs transduced either with iC.ΔCD34/IL-2v or iC.ΔCD34/IL-15v or ΔCD34v vectors and stimulated with EBV-LCLs. Cytokines were detected in the culture supernatant at the indicated time post EBV-LCL stimulation and measured by specific ELISAs.

**Fig. 2. IL-2 and IL-15 transgenic CTLs expand in response to antigen stimulation.** EBV-CTLs transduced either with iC.ΔCD34/IL-2v or iC.ΔCD34/IL-15v or ΔCD34v vectors were maintained in culture and stimulated once a week with autologous LCLs (E:T ratio 1:1) without addition of exogenous cytokines. Control EBV-CTLs transduced with the ΔCD34v vector were maintained in culture by adding rhIL-2 (50 U/mL). Data represent the mean ± SD of cell expansion of 5 donors (Panel A). IL-2 or IL-15 transgenic EBV-CTLs did not significantly expand when they were maintained in culture without specific antigen stimulation. Data represent the mean ± SD of cell expansion of 3 donors (Panel B).

**Fig. 3. IL-2 and IL-15 transgenic CTLs maintain their antigen specificity.** EBV-CTLs transduced either with ΔCD34v or iC.ΔCD34/IL-2v or iC.ΔCD34/IL-15v vectors were analyzed for their phenotype and antigen specificity. Panel A illustrates the phenotypic analysis of transduced EBV-CTLs. Data represent the mean ± SD of 5 donors. Panel B illustrates the result of a standard \(^{51}\)Cr release assay in which killing by CTLs of autologous LCLs, allogeneic LCLs, HSB-2 and K562 cell lines was tested at an E:T ratio of 20:1. Data represent the mean ± SD of 5 donors. Panel C illustrates a representative staining of EBV-CTLs using tetramers targeting the HLA-B8 BZLF1 peptide RAKFKQLL and the HLA-A2 LMP-2 peptide CLGGLLTV. Panel D illustrates the IFN-γ ELISpot assay of EBV-CTLs tested against the EBV-peptides HLA-A2 LMP-2 CLGGLLTMV.
HLA-B8 EBNA3A FLRGRAYGL and HLA-B8 BZLF1 QAKWRLQTL. This is representative of 3 donors.

Fig. 4. In vivo expansion of IL-2 and IL-15 transgenic CTLs. SCID mice engrafted with LCLs were injected either with EBV-CTLs control (ΔCD34v) or EBV-CTLs transgenic for IL-2 (iC.ΔCD34/IL-2v) or IL-15 (iC.ΔCD34/IL-15v) (10^7 cells). To track their homing and in vivo expansion, CTLs were transduced with the vector encoding eGFP-FFLuc. CTL localization and expansion was monitored using an in vivo imaging system (Xenogen-IVIS Imaging System). Mice did not receive exogenous cytokines after CTL transfer. Panel A shows images of representative mice. The signal intensity measured as photon/sec/cm²/sr (p/s/cm²/sr) was increased in mice receiving CTLs transgenic for IL-2 or IL-15 compared to control cells (ΔCD34v). Panel B illustrates the maximum increase in bioluminescence obtained in 20 mice per group. The expansion of iC.ΔCD34/IL-15v CTLs and iC.ΔCD34/IL-2v CTLs was statistically significant compared to control ΔCD34v (p=0.0008 and p=0.0015, respectively). IL-2 and IL-15 transgenic CTLs did not significantly expand in response to allogeneic EBV-LCLs. Panel C. To evaluate whether the increase in bioluminescence signal corresponded to an increased number of CTLs infiltrating the tumor, mice were euthanized and T-cell infiltration in the biopsy samples was measured using anti human CD3 staining and FACS analysis (ΔCD34v = 3.8x10^5 p/s/cm²/sr; iC.ΔCD34/IL-2v = 3x10^6 p/s/cm²/sr; iC.ΔCD34/IL-15v = 3.7x10^6 p/s/cm²/sr).

Fig. 5. Activation of the iCasp-9 suicide gene significantly eliminates IL-2 and IL-15 transgenic CTLs. EBV-CTLs transduced either with ΔCD34v or iC.ΔCD34/IL-2v or iC.ΔCD34/IL-15v vectors were plated at 10^6 cells/well and incubated with or without CID AP20187 at 50nM. Twenty-four hours later cells were collected, stained with CD34-PE antibody and the percentage of residual transgenic CTLs was evaluated by FACS analysis. Significant reduction of CD34+ cells after incubation with CID was obtained only for EBV-CTLs transduced either with iC.ΔCD34/IL-2v or iC.ΔCD34/IL-15 vectors incorporating the iCasp-9 gene. Panel A illustrates ΔCD34 expression in a representative experiment. Panel B summarizes the effects of the CID on 4 different EBV-CTL lines transduced either with iC.ΔCD34/IL-2v, iC.ΔCD34/IL-15v or ΔCD34v vectors. The y axis represents the mean ± SD for CD34+ CTLs in the CTL lines before or after incubation with CID. The percentage of CD34+ cells remained unchanged in control CTLs transduced with the ΔCD34v vector lacking the suicide gene. In contrast, a
significant reduction in the percentage of CD34+ CTLs was observed for CTLs transduced either with iC.ΔCD34/IL-2v or iC.ΔCD34/IL-15v vectors.

Panels C show that the elimination of transgenic CTLs was mediated by induction of apoptosis as assessed by Annexin-V/7-AAD staining. Panel D shows that the induction of apoptosis/necrosis by CID was dose dependent. CTLs transgenic for IL-2 or IL-15 and selected using CD34 magnetic beads were incubated with different doses of CID and then 24 hours later the induction of apoptosis/necrosis was evaluated by staining with Annexin-V/7-ADD and FACS analysis.

**Fig. 6. Activation of the iCasp-9 suicide gene abrogates cytokine production and long term expansion of IL-2 and IL-15 transgenic CTLs.** EBV-CTLs transduced either with iC.ΔCD34/IL-2v or iC.ΔCD34/IL-15v vectors were plated at 10⁶ cells/well and incubated with or without the CID AP20187 (50nM). Panel A illustrates the production of transgenic cytokines IL-2 or IL-15 by iC.ΔCD34/IL-2v and iC.ΔCD34/IL-15v EBV-CTLs before and after exposure to the CID. Neither IL-2 nor IL-15 cytokines could be detected in the supernatants from CTLs treated with the CID. Data represent the mean ± SD of 4 experiments. Panel B illustrates the long-term expansion of EBV-CTLs transduced either with iC.ΔCD34/IL-2v or iC.ΔCD34/IL-15v vectors and stimulated once a week with autologous LCLs. Viable cells were counted by trypan blue exclusion once a week before EBV-LCL restimulation. Exposure to a single dose of CID (50nM) ablated CTL expansion, while non-exposed CTLs continued to expand. Data represent mean ± SD of 4 experiments. Panel C illustrates a representative experiment in which IL-2 and IL-15 transgenic CTLs obtained 24 hours post CID exposure were sorted based on the expression of Annexin-V. These CTLs were then cultured without any further addition of CID and stimulated with EBV-LCLs. Annexin-V/7ADD staining showed that these cells progressed to a late stage of apoptosis/necrosis (Annexin-V+/7ADD+) by day 7-9. Panel D shows that CID induced elimination of CD34+ cells for IL-2 or IL-15 transgenic CTLs even in their resting phase, 7 days after the last antigen stimulation.

**Fig. 7. Activation of the iCasp-9 suicide gene eliminates the IL-2 and IL-15 transgenic CTLs in vivo.** SCID mice engrafted s.c. with LCLs were injected i.v. with EBV-CTLs transduced either with iC.ΔCD34/IL-2v or iC.ΔCD34/IL-15v vector, sorted for ΔCD34 expression and transduced with eGFP-FFLuc vector. When the CTLs were expanding, mice were treated with 2-3 doses of the CID AP20187 (50 μg) i.p. two days apart. The persistence of the transgenic cells was monitored in vivo using the
bioluminescence system. Panels A illustrate in a representative experiment the reduction of the bioluminescence after CID administration. Bioluminescence was significantly reduced in mice receiving IL-2 or IL-15 transgenic CTLs after treatment with CID. In contrast, the signal was not diminished in mice receiving control ΔCD34+ CTLs lacking the expression of the suicide gene. The bioluminescence continued to increase in mice receiving IL-15 transgenic CTLs non treated with CID. Panel B shows the kinetics of bioluminescence in 7 mice (closed symbols) before and after treatment with CID. In mice receiving EBV-CTLs expressing either iC..ΔCD34/IL-2v or iC..ΔCD34/IL-15v followed by the CID >1 log reduction in bioluminescence was observed. In contrast, bioluminescence continued to increase in mice non treated with CID (4 representative mice, open symbols). Panels C. Mice showing > 1x10^6 photons were euthanized to evaluate the infiltrate of CTLs within the tumor by FACS analysis after staining with anti human CD3 antibody (upper left panel). Mice with similar level of bioluminescence signal were treated with CID and 24-72 hours later euthanized to evaluate the effective reduction of CTL infiltration by FACS analysis after staining with anti human CD3 antibody (upper right panel). CID did not reduce the number of CD3+ cells in mice receiving control CTLs transduced with ΔCD34v vector (lower left and right).
Table 1. Tetramer analysis of EBV-CTL lines

<table>
<thead>
<tr>
<th>EBV-CTLs lines</th>
<th>HLA type</th>
<th>Transgene expression</th>
<th>B8 RAK (%)</th>
<th>B8 QAK (%)</th>
<th>A2 CLG (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>HLA-A2,24;B8,65</td>
<td>ΔCD34</td>
<td>8.9%</td>
<td>4.3%</td>
<td>NT</td>
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<tr>
<td></td>
<td></td>
<td>iC.ΔCD34/IL-2</td>
<td>9.6%</td>
<td>4.7%</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>iC.ΔCD34/IL-15</td>
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<td>1.8%</td>
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<td>9.3%</td>
<td>NT</td>
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<td></td>
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<td>3.6%</td>
<td>0.4%</td>
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ΔCD34 = CTLs transduced with the ΔCD34 control vector; iC.ΔCD34/IL-2v = CTLs transduced with the iC.ΔCD34/IL-2v; iC.ΔCD34/IL-15v = CTLs transduced with the iC.ΔCD34/IL-15v. The following HLA class I restricted tetramers were used: HLA-A2 LMP2 epitope CLGGLLTML (CLG), HLA-B8 BZLF1 epitope RAKFQLL (RAK), HLA-B8 EBNA3A QAKWRLQTL (QAK); NT = not tested.
Table 2. Antitumor effect of EBV-CTL adoptive transfer.

<table>
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<tr>
<th>Mice</th>
<th>EBV-CTLs $\Delta$CD34v</th>
<th>EBV-CTLs + rhIL-2</th>
<th>EBV-CTLs iC.$\Delta$CD34/IL-2v</th>
<th>EBV-CTLs iC.$\Delta$CD34/IL-15v</th>
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<tbody>
<tr>
<td>Inoculated with EBV-LCLs</td>
<td>17</td>
<td>24</td>
<td>19</td>
<td>17</td>
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<tr>
<td>Tumor free 4-6 weeks after CTL transfer</td>
<td>0</td>
<td>4(17%)</td>
<td>9 (47%)*</td>
<td>9 (53%)**</td>
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$\Delta$CD34v = CTLs transduced with the $\Delta$CD34 control vector; iC.$\Delta$CD34/IL-2v = CTLs transduced with the vector encoding for IL-2; iC.$\Delta$CD34/IL-15v CTLs transduced with the vector encoding for IL-15v. Mice injected with these CTLs did not receive exogenous cytokines. EBV-CTLs = CTLs non transduced. Mice infused with control EBV-CTLs received exogenous rhIL-2 (1000 U/mL) i.p. every 2 days. (*p=0.001; **p=0.0009 when compared to control EBV-CTLs $\Delta$CD34v by Fisher exact test).
Reference List


(12) Malek TR. The main function of IL-2 is to promote the development of T regulatory cells. J Leukoc Biol. 2003;74:961-965.

(13) Liu K, Rosenberg SA. Transduction of an IL-2 gene into human melanoma-reactive lymphocytes results in their continued growth in the absence of


Fig. 1

A

\[ \Delta \text{CD34} \quad \text{LTR} \quad \Psi \quad \Delta \text{CD34} \quad \text{LTR} \]

\[ \text{iC.} \Delta \text{CD34/IL-2v} \quad \text{LTR} \quad \Psi \quad \text{iCasp-9} \quad 2A \quad \Delta \text{CD34} \quad 2A \quad \text{IL-2} \quad \text{LTR} \]

\[ \text{iC.} \Delta \text{CD34/IL-15v} \quad \text{LTR} \quad \Psi \quad \text{iCasp-9} \quad 2A \quad \Delta \text{CD34} \quad 2A \quad \text{IL-15} \quad \text{LTR} \]

B

Control NT

\[ \Delta \text{CD34} \quad \text{LTR} \quad \Psi \quad \text{LTR} \]

\[ \Delta \text{CD34v} \quad \text{LTR} \quad \Psi \quad \text{LTR} \]

\[ \text{iC.} \Delta \text{CD34/IL-2v} \quad \text{LTR} \quad \Psi \quad \text{iCasp-9} \quad 2A \quad \Delta \text{CD34} \quad 2A \quad \text{IL-2} \quad \text{LTR} \]

\[ \text{iC.} \Delta \text{CD34/IL-15v} \quad \text{LTR} \quad \Psi \quad \text{LTR} \]

C

\[ \begin{array}{c}
\text{CD34 PE} \\
\text{Counts} \\
\text{IL-2 pg/mL/10^6 cells} \\
\text{IL-15 pg/mL/10^6 cells}
\end{array} \]

\[ \begin{array}{c}
\text{ΔCD34v} \\
\text{ΔCD34/IL-2v} \\
\text{ΔCD34/IL-15v}
\end{array} \]

\[ \begin{array}{c}
\text{Counts} \\
\text{ΔCD34v} \\
\text{ΔCD34/IL-2v} \\
\text{ΔCD34/IL-15v}
\end{array} \]

\[ \begin{array}{c}
\text{ΔCD34v} \\
\text{ΔCD34/IL-2v} \\
\text{ΔCD34/IL-15v}
\end{array} \]

\[ \begin{array}{c}
\text{ΔCD34v} \\
\text{ΔCD34/IL-2v} \\
\text{ΔCD34/IL-15v}
\end{array} \]
Fig. 2

A

- ΔCD34v no cytokines
- ΔCD34v + rhIL-2
- iC.ΔCD34/IL-2v
- iC.ΔCD34/IL-15v

B

- iC.ΔCD34/IL-2v + LCL
- iC.ΔCD34/IL-2v no LCL
- iC.ΔCD34/IL-15v + LCL
- iC.ΔCD34/IL-15v no LCL

Cell counts x10^6

Days of culture
Fig. 3

**A**

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**B**

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**C**

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**D**

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</table>
Fig. 4

A

Day 7 15 19 27

ΔCD34v eGFP-FFLuc

dC.ΔCD34/IL-2v eGFP-FFLuc

dC.ΔCD34/IL-15v eGFP-FFLuc

B

Bioluminescence fold expansion

ΔCD34v  iC.ΔCD34v  iC.ΔCD34/IL-2v  iC.ΔCD34/IL-15v  + Allo LCL

C

CD3-PerCP
0.4% 1%
59% 6%
78% 12%

CD19-PE
0.6%
Fig. 5

A

ΔCD34

iC.ΔCD34/IL-2v

iC.ΔCD34/IL-15v

Counts

CD34-PE

No CID + 24h

81%

48%

64%

ΔCD34+ cells

Plus CID (50nM) + 24h

77%

7%

10%

B

% of ΔCD34+ cells

ΔCD34

iC.ΔCD34/IL-2v

iC.ΔCD34/IL-15v

Media

CID 50nM

C

No CID

10pM CID

1000pM CID

7-AAD

Annexin V-PE

7%

17%

35%

26%

48%

60%

D

% vital cells

CID concentration pM

0

0.1

1

10

10^2

10^3

10^4

10^5
**Fig. 6**

**A**

- IL-2 pgr/mL/10^6 cells
- IL-15 pgr/mL/10^6 cells
- iC.ΔCD34/IL-2v
- iC.ΔCD34/IL-15v

**B**

- Cell counts x10^6
- Days of culture
- CID

**C**

- Cells Annexin-V+ sorted after CID exposure
- 7-AAD
- 44%

**D**

- Resting CTLs
- Resting CTLs after CID
- iC.ΔCD34/IL-2v
- iC.ΔCD34/IL-15v
- 55% 2%
- 76% 5%
Fig. 7

A

<table>
<thead>
<tr>
<th>Day 4</th>
<th>9</th>
<th>13</th>
<th>15</th>
<th>21</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔCD34 eGFP-FFLuc</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>iC.ΔCD34/IL-2v eGFP-FFluc</td>
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<tr>
<td>iC.ΔCD34/IL-15v eGFP-FFluc</td>
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</tr>
<tr>
<td>iC.ΔCD34/IL-15v eGFP-FFluc not treated with CID</td>
<td></td>
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</tr>
</tbody>
</table>

B

CID

Day after CTL infusion

Bioluminescence p/s/cm²/sr x 10⁶

4 9 13 15 21

C

no CID post CID

c.ΔCD34/IL-15v

ΔCD34

CD3-PerCP

CD19-PE

0.2% 1%
1.6% 1.5%
20% 27%
1.1% 0.9%
Co-expression of cytokine and suicide genes to enhance the activity and safety of tumor specific cytotoxic T lymphocytes

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