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Antitumor Activity of EBV-specific T Lymphocytes Transduced With a Dominant Negative TGF- β Receptor

Aaron E. Foster^{*,†}, Gianpietro Dotti^{*,‡}, An Lu^{*}, Mariam Khalil^{*}, Malcolm K. Brenner^{*,†,‡}, Helen E. Heslop^{*,†,‡}, Cliona M. Rooney^{*,†,§,||}, and Catherine M. Bollard^{*,†,‡,§}

^{*}Center for Cell and Gene Therapy, The Methodist Hospital and Texas Children's Hospital

[†]Department of Pediatrics, Baylor College of Medicine, Houston, TX

[‡]Department of Medicine, Baylor College of Medicine, Houston, TX

[§]Department of Immunology, Baylor College of Medicine, Houston, TX

^{||}Department of Virology, Baylor College of Medicine, Houston, TX

Summary

Transforming growth factor (TGF)- β is produced in most human tumors and markedly inhibits tumor antigen-specific cellular immunity, representing a major obstacle to the success of tumor immunotherapy. TGF- β is produced in Epstein-Barr virus (EBV)-positive Hodgkin disease and non-Hodgkin lymphoma both by the tumor cells and by infiltrating T-regulatory cells and may contribute the escape of these tumors from infused EBV-specific T cells. To determine whether tumor antigen-specific cytotoxic T lymphocytes (CTLs) can be shielded from the inhibitory effects of tumor-derived TGF- β , we previously used a hemagglutinin-tagged dominant negative TGF- β RII expressed from a retrovirus vector to provide CTLs with resistance to the inhibitory effects of TGF- β in vitro. We now show that human tumor antigen-specific CTLs can be engineered to resist the inhibitory effects of tumor-derived TGF- β both in vitro and in vivo using a clinical grade retrovirus vector in which the dominant negative TGF- β type II receptor (DNRII) was modified to remove the immunogenic hemagglutinin tag. TGF- β -resistant CTL had a functional advantage over unmodified CTL in the presence of TGF- β -secreting EBV-positive lymphoma, and had enhanced antitumor activity, supporting the potential value of this countermeasure.

Keywords

non-Hodgkin lymphoma; Hodgkin disease; TGF- β ; cytotoxic T lymphocyte

Encouraging results have been obtained for T-cell immunotherapy of tumors such as melanoma and Epstein-Barr virus (EBV)-associated malignancies.^{1–4} Success is, however, often incomplete, and an explanation for treatment failure is that potentially immune-sensitive tumors have acquired immune-escape mechanisms.⁵ One of the commonest of these is the production of transforming growth factor (TGF)- β both by tumor cells and by infiltrating T-regulatory cells. TGF- β has anti-inflammatory, immune inhibitory, and proangiogenic effects, all of which may favor tumor survival and growth.^{6,7} The action of TGF- β on antigen-specific T lymphocytes is pleiotropic, but primarily inhibitory.⁸ For example, whereas EBV-positive Hodgkin lymphoma (HL) cells express and present EBV antigens in the presence of

Reprints: Catherine M. Bollard, Department of Pediatrics, Baylor College of Medicine, Houston, TX (cmbollard@txccc.org).

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costimulatory molecules, these tumors produce sufficient TGF- β to actively inhibit T-cell effector function,⁹ a strategy that obstructs current efforts to enhance antitumor killing through adoptive T-cell therapy.

Although tumor cells, including EBV-HL, have a range of strategies by which they evade the immune system, we have suggested that an effective countermeasure even against a single major mechanism would allow a sufficient increase in overall immune activity to produce significant clinical benefit.¹⁰ Because of the potency of TGF- β , we have studied the effects of blocking T-cell responses to this molecule. We have previously shown in vitro that antigen-specific T cells expressing a transgenic dominant negative TGF- β receptor type II (DNRII) will respond to tumor antigens by proliferation, cytokine secretion, and tumor antigen-specific killing, in the presence of TGF- β concentrations that were inhibitory to unmodified T cells.⁸ We have now generated a DNRII vector for clinical use in EBV-positive Hodgkin disease (HD) and non-Hodgkin lymphoma (NHL) and determined whether the potential advantages we demonstrated in vitro also apply to T-cell functionality in the tumor bearing host in vivo.

Methods

Tumor Cell Lines

EBV-negative HL-derived cell lines HDLM-2 and L1236, were obtained from the German Collection of Cell Cultures (DMSZ, Braunschweig, Germany). EBV-infected B-lymphoblastoid cell lines (LCLs) were generated as previously described.^{8,11}

Measurement of TGF- β Production by Enzyme-linked Immunosorbent Assay

Duplicate samples of tumor cell lines (5×10^4 /well) were cultured in 96-well round bottom plates (Costar). After 48 hours, the supernatants were harvested and 200 μ L harvested from each well and analyzed for human TGF- β 1 using 96-well plates coated with human TGF- β 1 monoclonal antibody (R&D Systems) by enzyme-linked immunosorbent assay, according to the manufacturer's instructions. Supernatant was also collected in this manner for use in the proliferation assay as described below.

³H-Thymidine Uptake Assay

The effects of TGF- β on the proliferation of nontransduced cytotoxic T lymphocytes (CTL) and SFG:DNRII-transduced CTLs were evaluated using [³H]-thymidine uptake assays.⁸ Briefly, CTL were seeded at 5×10^5 cells per well and maintained in the absence or presence of either 100 μ L supernatant obtained from the HL cell line HDLM-2 (as described above) or human TGF- β (R&D Systems Inc, Minneapolis, MN) (2.5 ng/ 5×10^5 cells as determined by previously published titration experiments⁸) for 72 hours at 37°C. Eight hours after pulsing the wells with 5 μ C [³H]-thymidine (Amersham Pharmacia Biotech, Piscataway, NJ), we harvested them onto filtered glass filter strips and measured the incorporated [³H]-thymidine using a TriCarb 2500 TR β -counter (Packard Bioscience, Downers Grove, IL).

Cytotoxicity Assays

To compare the cytotoxic specificity of transduced and nontransduced CTL in the presence of TGF- β 1, standard ⁵¹Cr release assays were performed. At 72 to 96 hours before performing the cytotoxicity assay, TGF- β 1 (R&D Systems Inc, Minneapolis, MN) was added to 5×10^6 transduced and nontransduced CTL at a concentration of 5 ng/mL, which we have shown yields similar TGF- β concentrations in cultured supernatants as TGF- β -secreting lymphoma cell lines.⁸ Dilutions of CTL were cocultured in triplicate for 4 hours with 5000 ⁵¹Cr-labeled target cells (Amersham Pharmacia Biotech), in a total volume of 200 μ L in a V-bottom 96-well plate (Costar) as previously described.¹² The targets tested were autologous LCL, human

leukocyte antigen class I and II mismatched LCL, and HSB-2. At the end of a 4-hour incubation period at 37°C and 5% CO₂, supernatants were harvested, and ⁵¹Cr release was measured on a gamma counter (Tri-CARB 4640, Packard BioScience Company, Downers Grove, IL) as previously described.⁸

Generation of Plasmids

A human type II TGF-β receptor cDNA was generated as previously described¹³ with truncation at nt597 leaving only 7 amino acids in the intracellular signaling domain.¹³ The DNRII was then ligated into the retroviral vector SFG.⁸ To reduce immunogenicity for the future clinical trial, the hemagglutinin sequence initially inserted was removed before the generation of the vector used in this in vivo study.

Production of Recombinant Retrovirus

DNRII Vector—To make a producer line for the DNRII retrovirus vector, PG13 cells were transduced with transient retroviral supernatant from transfected Phoenix-Eco cells. The transduced PG13 cells were single-cell cloned, and those with the highest biological titer were expanded in T-75 flasks (Falcon) to 80% confluence. Media¹¹ was then replaced and cells incubated at 37°C for 24 hours. Retrovirus supernatant was then harvested, filtered, and stored at – 80°C.

Luciferase Vector—A retrovirus vector SFG.EGFPluciferase, (EGFPluc) encoding a fusion protein formed between enhanced green fluorescence protein (EGFP) and firefly luciferase (Promega) was prepared as previously described.⁸ EBV-LCLs were transduced on Retronectin (Takara Bio, Otsu, Japan) coated plates and subsequently purified for EGFP expression (> 95%) using a MoFlo flow cytometric cell sorter (Dako, Glostrup, Denmark). To monitor in vivo T-cell proliferation and survival, we transduced EBV-CTL with EGFPluc (transduction efficiency of 30% to 60%, data not shown) and then split into 2 groups, 1 transduced with DNRII (EGFPluc⁺/DNRII⁺) and the other mock transduced (EGFPluc⁺/DNRII[–]). Bioluminescence was measured in a luminometer (Monolight; BD Biosciences Pharmingen, San Diego, CA).

Generation and Transduction of EBV-specific CTL Lines

We prepared EBV-specific polyclonal CTLs from healthy donors under our Institutional Review Board-approved protocol, as previously described.⁸ After 3 stimulations with autologous EBV-infected LCLs, we transduced our CTLs with retroviral supernatant for 72 hours and expanded them by weekly restimulation with LCLs in the presence of recombinant human interleukin (rhIL)-2.¹¹

In Vivo Study in a Xenogeneic Severe Combined Immunodeficient Mouse Model

To examine the antitumor effect of the DNRII-transduced and nontransduced EBV-specific CTL against TGF-β expressing tumors in vivo, we imaged both tumor cells and infused T cells in a severe combined immunodeficient (SCID) mouse xenograft model.¹¹ Briefly, EBV⁺ lymphoma cells (LCLs) or EBV-CTLs were transduced with a retrovirus coding EGFPluc. SCID mice (8 to 10 wk old) were sublethally irradiated (250 rad) and injected intraperitoneally (IP) with 5 × 10⁶ tumor cells suspended in Matrigel (Becton Dickinson). Seven days later, 1 × 10⁷ CTL were injected IP. rhIL-2 (1000 U) was administered IP every other day. For in vivo imaging of tumor cells or CTL expressing EGFPluc, mice were injected IP with D-luciferin (150 mg/kg). To examine migration of EBV-CTL to LCL, 1 × 10⁷ LCLs were resuspended in matrigel and injected subcutaneously in the right flank. After 7 days, 1 × 10⁷ EBV-CTL transduced with EGFPluc were injected via tail vein. Mice were imaged on days 1, 3, and 7

using the Xenogen-IVIS Imaging System (Caliper Life Sciences, Hopkinton, MA) to determine T-cell biodistribution.¹¹

Flow Cytometry Analysis

To assess expression of the dominant negative DNR II, transduced CTLs (1×10^6) were incubated with 10 μ L of TGF- β RII-specific antibody (R&D Systems Catalog no. FAB241P), which we have shown to have high specificity for the DNR II. For each sample, we analyzed 10,000 cells using the FACSCalibur with the Cell Quest Software (Becton Dickinson).

Statistical Analysis

For the bioluminescence experiments, intensity signals were log-transformed and summarized using mean $\pm$ 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 paired *t* tests or the Wilcoxon signed rank test.

Results

DNR II-transduced CTLs are Resistant to the Antiproliferative Effects of Exogenous TGF- β 1 and Supernatant From TGF- β -secreting HL Cell Line

A median of 31.7% (range, 26.71% to 37.42%) of CD3+ T cells transduced with the clinical grade vector expressed the mutant receptor construct (DNR II) (Fig. 1A). To confirm that this new vector expressed sufficient DNR II to overcome the antiproliferative effects of TGF- β , we added recombinant TGF- β 1 to DNR II-transduced and nontransduced EBV-CTLs and compared their thymidine uptake 72 hours later (Fig. 1B). We also determined if the level of expression was sufficient for the transduced cells to resist the TGF- β present in the supernatant of the TGF- β secreting HL cell line HDLM-2 (Fig. 1C, D). Both recombinant TGF- β 1 and HDLM-2 supernatant had a consistent antiproliferative effect on nontransduced EBV-CTL, inhibiting thymidine uptake by 80% and 53%, respectively. By contrast, the inhibition of thymidine uptake by DNR II-transduced EBV-CTLs was 17% with recombinant TGF- β and 12% using HDLM-2 supernatant. This resistance to the antiproliferative effects of TGF- β in DNR II-transduced CTL was statistically significant when compared with nontransduced CTL ($P=0.03$).

DNR II-transduced CTLs are Resistant to Functional Inhibition by Exogenous TGF- β 1

CTL transduced with retrovirus DNR II maintained their cytolytic activity in the presence of TGF- β . The cytotoxic activity of DNR-transduced and nontransduced CTLs with and without TGF- β 1 were compared in standard 4-hour ⁵¹Cr release assays. At an effector:target ratio of 20:1, a mean of 63% (range, 40% to 83%) of autologous LCL were lysed by nontransduced CTL compared with a mean of 37% (range, 18% to 55%) after 96-hour incubation with 5 ng/mL TGF- β 1 ($P=0.01$) (Fig. 2A). By comparison, at the same effector:target ratio, 61% (range, 42% to 80%) of autologous LCL were lysed by DNR-transduced CTL in the absence of TGF- β 1 and a mean of 73% (range 65% to 81%) were lysed in the presence of TGF- β 1 ($P=0.5$). (Fig. 2B) The CTL lines did not have significant (< 20%) reactivity with allogeneic LCL or HSB-2 (data not shown).

DNR II-expressing EBV-CTLs Have Greater In Vivo Antitumor Activity Than Control EBV-CTL

To determine the in vivo activity of DNR II-transduced CTL, we used an IP SCID xenograft model, in which sublethally irradiated SCID mice ($n=27$) were implanted IP with EGFP-labeled EBV+ TGF- β -secreting tumor cells (LCL) (Fig. 1B). Light emission was monitored as an indication of tumor growth. Once a progressive increase of bioluminescence occurred (7 d after tumor injection), mice received control EBV-CTLs ($n=9$), or DNR II EBV-CTLs ($n=$

9), or phosphate-buffered solution only (n = 9) all by IP injection, followed by IP rhIL-2 1000 U on alternate days. Over 2 weeks, we observed a reduction of light emission in mice treated with DNR II EBV-CTLs (Fig. 3A) indicating control of tumor growth albeit not complete eradication in all mice. In contrast, photon emissions, and thus tumor size, initially was stable and then increased in mice receiving control EBV-CTLs (Fig. 3A). By 28 days, the mice infused with DNR II EBV-CTLs continued to have no tumor growth in contrast to the control mice in which tumors continued to expand, consistent with high cytotoxicity by DNR II CTLs (Fig. 2) and maintenance of perforin expression⁸ even in the presence of TGF- β 1. (Fig. 3B) When the CTLs were labeled with luciferase, we detected their continued persistence in both the control and the DNR II groups (Fig. 3C), consistent with our in vitro observations, which showed superior cytotoxicity by DNR II CTLs (Fig. 2) and maintenance of perforin expression⁸ even in the presence of TGF- β 1.

Although these findings support translation to clinical study, the potential concern remains that the genetically modified cells may fail to traffic to tumor. Figure 3D, however, shows that intravenously administered EBV-specific CTLs traffic well to EBV-positive tumors in our murine model, an effect matching our observations in clinical studies.¹⁴

Discussion

TGF- β is likely to be a major obstacle to the expansion and maintenance of a tumor antigen-specific cytotoxic T-cell response in vivo, thereby reducing the clinical value of an immunotherapeutic approach. Efforts have been made, in vitro and in vivo, to enhance the antitumor response by overcoming the effect of tumor-derived or induced TGF- β secretion.^{15,16} Neutralization efforts using monoclonal antibodies targeting TGF- β and TGF- β RII¹⁵ have been reported, but systemic inhibition of a multifactorial molecule and potent immune response modifier such as TGF- β will likely have unpredictable adverse consequences.¹⁷ The alternative strategy of targeting the inhibition of TGF- β to desired sites and cell types should be better tolerated. We have previously shown that CTLs transduced with a retroviral vector expressing a DNR II can resist the antiproliferative effects of TGF- β in vitro.⁸ EBV-positive HD and NHL are known to secrete TGF β and are surrounded by TGF- β -secreting regulatory T cells.¹⁸ In view of our experience using adoptive immunotherapy to treat these lymphoma patients,^{4,14} we wanted to evaluate the efficacy of the CTL transduced with the clinical grade DNR II vector against TGF- β -secreting lymphomas. We have therefore now extended the in vitro data to show the resistance of the DNR II-transduced CTL in the presence of lymphoma tumor supernatants containing TGF- β . We have also now shown the benefits for antilymphoma activity in vivo, because CTL transduced with DNR II maintain their tumor antigen-specific killing even in the presence of the TGF- β -secreting lymphoma.

The potent cell-inhibitory activities of physiologically produced TGF- β raise the concern that T lymphocytes lacking a response to the cytokine will be insensitive to an important homeostatic regulatory mechanism and will undergo abnormal lymphoproliferation. Reassuringly, however, studies in an immunocompetent (nontumor) murine model have shown that DNR II-specific CTL do not spontaneously proliferate in the absence of antigenic stimulation.¹⁹

A countermeasure, such as the one we have described, which is active against just one of many tumor immune evasion strategies, may be insufficient for clinical benefit. Indeed, the presence of other immune evasion mechanisms such as tumor-derived IL-10²⁰ may be one reason why tumor eradication in our study was incomplete. We suggest, however, that tumor cells possess multiple evasion strategies because the limitations of each individually mean that several are needed for effective tumor protection. Hence, disruption of even one of them may greatly increase tumor vulnerability to immune destruction. Failure to completely eradicate tumors in

this xenograft model may therefore relate more to the deficiencies of the murine environment for human T-cell growth than to the effectiveness of residual tumor evasion strategies. Certainly, the potential antitumor efficacy of TGF- β -resistant T cells has been demonstrated in a murine model of prostate cancer, in which the functional limits of xenografted T cells are avoided.¹⁵ Ultimately, the value of this countermeasure will have to be assessed clinically in individual tumor types.

Our report demonstrating the efficacy of TGF- β -resistant T cells in a lymphoma model uses a strategy, which will be directly translatable to the clinic, and it will be of interest to learn whether the adoptive transfer of EBV-CTLs grafted with a DNRII further improves the outcome of T-cell immunotherapy of patients with EBV⁺ HD and NHL.

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FIGURE 1.

A to D, DNR-transduced CTLs are resistant to TGF- β -mediated inhibition of proliferation. A, EBV-specific CTL transduced with SFG:DNRII (truncated TGF- β RII) and nontransduced CTLs were stained with anti-CD3 and the TGF- β RII monoclonal antibody and analyzed by flow cytometry. B, Supernatants obtained from Hodgkin lymphoma cell lines L1236 and HDLM-2 and a lymphoblastoid cell line (LCL) were analyzed for concentrations of TGF- β 1 by ELISA assay. C, Nontransduced CTL (black), and DNR-transduced CTL (gray) were incubated with IL-2 $\pm$ 5 ng/mL TGF- β 1. Proliferative responses were measured after 72 hours incubation by measurement of 3 [H] thymidine uptake. The results are shown as proliferation of each CTL line incubated in the presence and absence of TGF- β . D, Nontransduced CTL (black), and DNR-transduced CTL (gray) were then incubated with irradiated LCL $\pm$ HDLM-2 supernatant. Proliferative responses were measured after 72 hours incubation by measurement of 3 [H] thymidine uptake. The results are shown as proliferation of each CTL line incubated in the presence and absence of tumor supernatant. The graphs show 1 representative experiment out of 3. CTL indicates cytotoxic T lymphocytes; DNR, dominant negative receptor; DNRII, dominant negative TGF- β type II receptor; EBV, Epstein-Barr virus; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; TGF, transforming growth factor.

FIGURE 2.

A and B, DNR-transduced CTL are resistant to TGF- β -mediated inhibition of cytolytic activity. Percent specific ^{51}Cr release was determined 4 hours after coincubation with DNR II-transduced CTL and nontransduced CTL with autologous LCL. TGF- β was added to CTL cultures 96 hours before cytotoxicity assay. The graphs show the percent-specific lysis at effector to target ratios of 20:1, 10:1, and 5:1 in a representative (A) nontransduced CTL line and (B) DNR-transduced CTL line. CTL indicates cytotoxic T lymphocytes; DNR, dominant negative receptor; DNR II, dominant negative TGF- β type II receptor; LCL, lymphoblastoid cell line; TGF, transforming growth factor

FIGURE 3.

A to D, DNRII-expressing EBV-CTLs have enhanced antitumor activity in vivo against EBV⁺ lymphomas compared with control CTL. A, Analysis of the antitumor effect of DNRII-transduced EBV-specific CTL was compared with that of nontransduced CTL and PBS controls. SCID mice were injected intraperitoneally with EGFPluc-transduced autologous EBV-LCL followed by injection of PBS, CTL-NT, or CTL-DNRII 7 days later. In vivo bioluminescence imaging was performed on days 0, 7, 14, and 20. One representative animal out of a total of 9 mice for each group is shown. B, Region-of-interest (ROI) bioluminescence emission measurements were calculated for each group, PBS (open squares), CTL-NT (open triangles), and CTL-DNRII (black circles) and plotted over 3 weeks. * indicates a statistically

significant difference of CTL-NT and CTL-DNR^{II} against the PBS control, whereas ** indicates a statistical difference between CTL-DNR^{II} against both CTL-NT and PBS ($P<0.05$). To evaluate the proliferation and migration of EBV-CTL in vivo (C) EBV-CTL transduced with EGFP^{luc} and either DNR^{II} or NT were examined for in vivo proliferation against autologous EBV-LCL using bioluminescence after intraperitoneal injection. D, EBV-CTL transduced with EGFP^{luc} exhibits in vivo migration to subcutaneous EBV-LCL tumors after intravenous injection (tail vein). EBV-CTL can be observed in the lungs and tumor after 1 day postinjection, and preferentially migrate to the tumor site by day 7. CTL indicates cytotoxic T lymphocytes; DNR^{II}, dominant negative TGF- β type II receptor; EBV, Epstein-Barr virus; LCL, lymphoblastoid cell line; PBS, phosphate-buffered saline; SCID, severe combined immunodeficiency.