

Engineering Human Tumor-specific Cytotoxic T Cells to Function in a Hypoxic Environment

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Hypoxia occurs in many tumors and reduces the effectiveness of radio- and chemotherapy. Hypoxia also impedes immune responses to tumors, reducing T lymphocyte production of cytokines such as interleukin-2 (IL-2) and interferon gamma, as well as the survival and proliferation of these cells. We constructed a lentiviral vector encoding a bidirectional hypoxia-inducible responsive element (HRE) derived from human vascular endothelial growth factor, which drives the *hIL-2* gene and a marker gene. We used a model of human B cell lymphoma to show that tumor-specific T cells modified with this vector upregulate *hIL-2* expression when oxygen tension is low *in vitro* and *in vivo*. The consequence of this effect is to increase T-cell survival and proliferation whilst sustaining effector function, even in O₂ concentrations as low as 1%. The phenotype of the transduced cells is unchanged, as is their ability to migrate to tumor. HRE-IL-2-modified cytotoxic T lymphocytes (CTLs) produce faster and more complete tumor regression than parental CTLs and increase overall survival. Hypoxia-resistant T cells may thus be of value in the treatment of human tumors in which areas of hypoxia may otherwise account for resistance to this therapeutic strategy.

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INTRODUCTION

Many human tumors contain areas of hypoxia.^{1–8} This phenomenon is usually due to inadequate neo-vasculature in a rapidly growing malignancy, increased vascular permeability (producing hemoconcentration and high interstitial pressure), and/or anemia⁹ (from the disease or its treatment). Hypoxic areas of tumors are often resistant to chemo-radiotherapy, because locally deficient drug biodistribution and altered cellular metabolism may reduce the effectiveness of these agents.^{10,11} Additionally, residual-surviving hypoxic cells may evolve into a more aggressive phenotype.¹²

Hypoxia also has the potential to adversely affect the cellular immunotherapy of tumors.¹³ While the results of adoptive cellular immunotherapy have been promising, for example in the treatment of melanoma and Epstein-Barr virus (EBV)-related lymphoma and nasopharyngeal cancer,^{14,15} it is clear that T-cell growth and survival is profoundly impaired at low oxygen tension levels. Hypoxia blocks many of the consequences of T cell receptor activation by antigen^{16–20} and inhibits the generation of cytotoxic T lymphocytes (CTLs) from precursor cells. Once CTL have been generated, hypoxia can cause a shift from a Th1 to a Th2 phenotype, with an increase in the secretion of interleukin-8 (IL-8), IL-1, and IL-6 and a decrease in IL-2 (refs. 18,21), IL-4, and interferon- γ .¹⁸

Although hypoxia is inhibitory to T-cell growth overall, low oxygen tension also increases expression of a number of different gene families, including vascular endothelial growth factor, erythropoietin,^{22,23} and a cluster of glycolytic enzymes that compensate for altered metabolic needs. These genes are regulated by the hypoxia-inducible factor (HIF)²⁴ a transcription factor which is upregulated during hypoxia, and binds to hypoxia responsive elements (HREs).²⁵ We reasoned that an HRE-based hypoxia-responsive expression vector could be used to stably introduce protective transgenes into T cells that would be expressed on exposure to low oxygen tension, and that this would prevent impairment of their growth, survival, and function. Since hypoxia profoundly reduces IL-2 accumulation, which in turn affects the growth of cytotoxic T cells, we chose to transfer the human IL-2 gene as a proof-of-principle experiment and placed it under the regulation of a hypoxia-inducible promoter containing five copies of the vascular endothelial growth factor-HRE.²⁶ To assess the potential of our approach, we have used T lymphocytes that target EBV antigen expressing solid tumors, since the latter exhibit hypoxic areas and are only partially amenable to immunotherapy.^{27,28} We grew these tumors in a severe combined immunodeficient mice xenograft model, and allowed them to develop hypoxic areas. We show that the HRE-IL-2 transgene is indeed expressed when pO₂ is reduced, and that in contrast to control T cells, such modified T cells can sustain their growth and function, even in severely hypoxic conditions.

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RESULTS

T-cell proliferation and survival is impaired by hypoxia

To evaluate the effect of low oxygen tension on T-cell proliferation, non-transduced (NT) Jurkat T cells were cultured in 20 or 1% oxygen for 20 days. Cell proliferation was determined by counting the cells each day with a hemocytometer, using trypan blue to distinguish live and dead cells. The oxygen tension of the chamber was readjusted daily. **Figure 1a** shows that the NT Jurkat (T-cell line) had a lower rate of proliferation when cultured under hypoxic, compared to normoxic conditions. An apoptosis assay on these Jurkat cells showed that after 3 days, 70% of T cells cultured under normoxic conditions were viable compared to only 34% of cells cultured under hypoxic conditions (**Figure 1b**), confirming a drastic decrease of recovery and viability under low oxygen tension. We have also tested the effects of hypoxia on primary CTLs, using an experimental EBV-lymphoblastoid cell (LCL) tumor system.^{29,30} Peripheral blood mononuclear cells were stimulated with EBV-LCL (see Materials and Methods) to generate EBV-specific CTL which

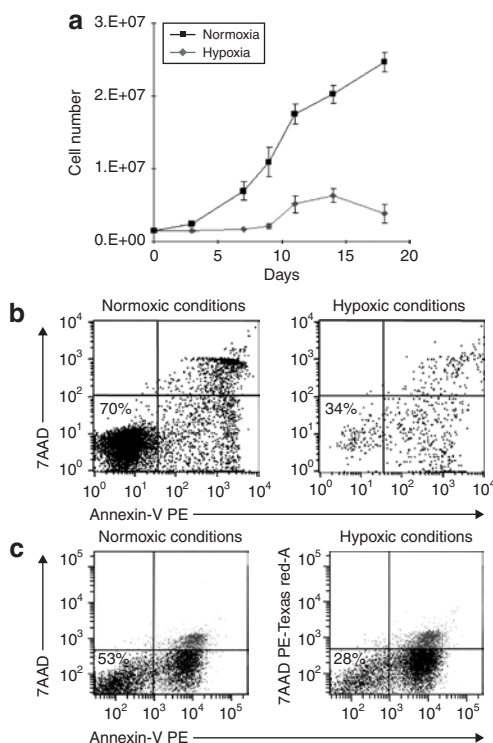


Figure 1 T-cell proliferation and apoptosis under hypoxic conditions. **(a)** Jurkat cells were cultured under hypoxic (diamonds) and normoxic (squares) conditions. Cell proliferation was determined by trypan blue staining and counting in a hemocytometer. **(b)** Apoptosis was detected by staining the cells with Annexin-V FITC, 7-amino-actinomycin D (7AAD), and propidium iodide (PI) followed by flow cytometric analysis after 72 hours. The results are expressed as the percentage of viable cells (Annexin-V FITC and/or PI negative) for each condition. **(c)** The hypoxia-induced apoptosis of cytotoxic T cells (CTL) was also investigated. CTLs were cultured in normoxia and hypoxia and then stained with Annexin-V FITC, 7AAD and PI followed by flow cytometric analysis. The results showed an increased CTL apoptosis in hypoxia. CTL, cytotoxic T lymphocyte; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

were cultured in normoxia and hypoxia and analyzed using an Annexin-V apoptosis assay. The results showed that up to 53% of the CTL were viable in normoxia but only 28% of the CTL were viable in hypoxia (**Figure 1c**), confirming that primary T cells are also adversely affected by low oxygen tension.

T-cell transduction with pHRE-IL-2 leads to hypoxia-dependent expression of the transgene

To rescue T cells from the negative effects of hypoxia on their function, we generated bi-directional hypoxia-inducible expression vectors encoding renilla luciferase and IL-2 genes. The genes were placed under the control of five tandem repeats of HREs flanked by two minimal cytomegalovirus (CMV) promoters (**Figure 2**). This expression construct was cloned in a self-inactivating lentiviral vector to generate Luc-HRE-IL-2 and was used to infect 293 cells. The level of renilla luciferase expression was measured in the cell extract. High levels of renilla were detected only in transduced cells cultured in hypoxic conditions (526 relative units in normoxia and 1.73×10^6 relative units in hypoxia) (**Table 1**). No signal was detected in NT cells (data not shown). Enzyme-linked immunosorbent assay was used to evaluate IL-2 production, and results revealed that a significant secretion of the cytokine occurred only when the cells were hypoxic (9,500 pg/ml versus non-detectable in normoxia **Table 1**).

Hypoxia-induced IL-2 transgene expression preserves T-cell proliferation and protects against apoptosis under hypoxic conditions

To verify the regulation of Luc-HRE-IL-2 by hypoxia, and the rescuing role of the induced IL-2, EBV-specific CTL were modified with our constructs. Luc-HRE-IL-2-modified and parental CTL were cultured in normoxia or in hypoxia ($n = 4$ wells per culture condition). For each condition, protein lysates were used to measure the renilla luciferase production (**Figure 3a**). Enzyme-linked immunosorbent assay was used to test the supernatant of the cultures for IL-2 production (**Figure 3b**). We found that renilla luciferase and IL-2 transgenes were both only expressed under

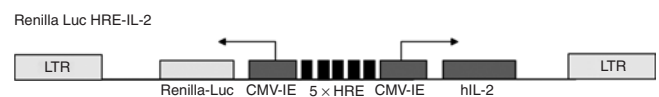


Figure 2 Structure of hypoxia responsive element (HRE) construct. Scheme of the self-inactivating-lentiviral vector encoding hypoxia-inducible interleukin-2 (IL-2) and renilla luciferase genes. We generated vectors encoding bi-directional repeats of five HREs linked to minimal cytomegalovirus (CMV) promoters and driving the renilla luciferase gene, and the *hIL-2* gene. LTR, long-terminal repeat.

Table 1 Self-inactivating (SIN)-lentivector tightly regulates interleukin-2 (IL-2) and renilla luciferase synthesis

	Normoxia	Hypoxia
Luc (RU)	526 (± 32)	1.73×10^6 ($\pm 10E4$)
Interleukin-2 (pg/ml)	0	9,500 (± 7)

Abbreviation: HRE, hypoxia responsive element.

293T cells were transduced with SIN-lentivirus Luc-HRE-IL-2. Transduced cells were exposed to normoxia or hypoxia for 24 hours and IL-2 was measured in the supernatant of the cultures using an enzyme-linked immunosorbent assay. The cells were lysed to evaluate the renilla luciferase production in both conditions using a luciferase assay kit.

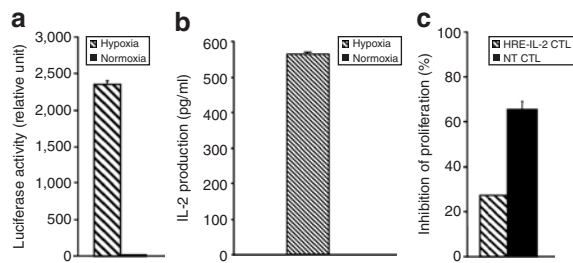


Figure 3 Hypoxia induces transgene expression and proliferation of Luc-HRE-IL-2 cytotoxic T lymphocyte (CTL). The renilla Luc-HRE-IL-2-vectors were pseudotyped with the vesicular stomatitis virus envelope glycoprotein and transfected with accessory plasmids (pCMV Δ R8.2, pCMV Δ R8.9). Filtered and concentrated vector preparations were used to spinfect CTL in the presence of polybrene. **(a)** Hypoxia induces renilla luciferase expression in HRE-IL-2 CTL. To test the hypoxic-mediated regulation of the HRE vector, the same numbers of CTL transduced with R-Luc-HRE-IL-2 vector were cultured under normoxic or hypoxic conditions for 3 days. The cells were then lysed, the renilla luciferase activity was measured (bars denote the SD for $n = 4$), and results showed robust transgene induction under hypoxic conditions. **(b)** Exogenous IL-2 production under hypoxia. The supernatants of HRE-IL-2 CTL cultured under normoxia and hypoxia ($n = 4$) were harvested 24 hours after activation and tested by enzyme-linked immunosorbent assay; results revealed that IL-2 is produced only in low oxygen tension. **(c)** HRE-IL-2 CTL proliferate under hypoxic conditions. To investigate the activity of transgenic IL-2, the same number of parental non-transduced CTL (NT CTL) and HRE-IL-2 CTL was cultured under normoxic and hypoxic conditions for 96 hours; the proliferation in each condition and each cell type was evaluated by counting the cells. The inhibition of proliferation due to hypoxia was calculated based on cell proliferation in normoxia (100% proliferation). Results are expressed as the percentage (mean + SD, $n = 4$) inhibition of proliferation obtained in 1% oxygen. CMV, cytomegalovirus; HRE, hypoxia responsive element; IL, interleukin.

hypoxia. The proliferation of the NT CTL and Luc HRE-IL-2 CTL (HRE-IL-2 CTL) was assessed by culturing these cells under normoxia and hypoxia ($n = 4$). The inhibition of proliferation due to hypoxia was calculated based on CTL proliferation during normoxic culture. The IL-2 produced during hypoxia appeared to rescue HRE-IL-2 CTL proliferation since the proliferation of HRE-IL-2-transduced CTL was less inhibited by hypoxia than NT CTL at 96 hours (Figure 3c).

Expressing HRE-IL-2 does not alter the phenotype and chemotactic responses of EBV-CTL

We next determined the effects of expressing Luc-HRE-IL-2 on the phenotype of tumor-specific CTL. The percentages of CD8⁺ cells were similar in transduced and NT populations (Figure 4a), although the intensity of the CD8 signal was slightly increased in the HRE-IL-2 CTL population. The expression of adhesion molecules and chemokine receptors (CXCR4, CCR5, CCR7, CD62, and CD54) typically associated with T-cell activation by antigen were only slightly altered by stable gene transduction (Figure 4b-1, $n = 3$). Dot plots are shown for CD62L and CCR5 staining (Figure 4b-2).

In vitro T-cell chemotaxis assay

To measure the chemotaxis and *in vitro* extravasation of the transduced cells, we measured their ability to migrate through a layer of cultured human umbilical vein endothelial cells in response to a chemotactic signal [stromal cell-derived factor-1 (SDF-1)]. Human umbilical vein endothelial cells were seeded in the upper

chamber of a transwell and when they reached confluence, transduced and NT CTL were seeded on top. The lower chambers contained LCL tumor cell supernatant, media supplemented with SDF-1 or plain media. Under normoxic conditions, both NT and transduced CTL migrated after 5 hours (60% versus 77% in the presence of tumor supernatant media and 83% versus 87% in the presence of SDF-1; Figure 4c) into wells containing either tumor cell supernatant or recombinant SDF-1 ($n = 3$). In both cases migration was blocked by CXCR4 neutralizing antibody but not by control antibody. Hence, both transduced and NT CTL migrate in an SDF-1/CXCR4-dependent manner.

Cytotoxic activity of CTL is increased after transduction with HRE-IL-2

Gene-modified T cells retained their cytotoxic activity against specific target cells in chromium (⁵¹Cr)-release assays. Modified and non-modified CTL were co-cultured at different ratios with autologous ⁵¹Cr-loaded LCL target cells under hypoxic or normoxic conditions ($n = 3$). The ⁵¹Cr-assays show that overall, HRE-IL-2 CTL kept their killing activity and displayed greater killing activity than non-modified CTL under both hypoxic and normoxic conditions and at all effector target ratios tested (Figure 4d).

Effects of transduction on T-cell accumulation in hypoxic lymphoma

We implanted EBV-LCL tumor cells in Matrigel subcutaneously into the flanks of irradiated severe combined immunodeficient mice and allowed the tumors to grow. We first injected the CMV-firefly luciferase-modified autologous tumor-specific CTL by the tail vein. The constitutive expression of the firefly luciferase allowed us to track circulating CTL and CTL homing to the tumor (into both normoxic and hypoxic areas). Mice were monitored by non-invasive bioluminescence imaging (IVIS imaging system) daily for 4–7 days after injection. A strong luciferase signal appeared in the tumors on day 7, suggesting that the CTL specifically migrated from the bloodstream to the tumor (Figure 5a). Mice were then injected with the hypoxia marker pimonidazole, killed and the tumors harvested for immunohistochemistry. Figure 5b shows that the tumor cells were positive for human CD20 expression (Figure 5b, panel A) and that injected CTL efficiently infiltrated the normoxic areas of the tumor (Figure 5b, panels B and H). In the probe-defined hypoxic areas (C), tumor cells were dividing/proliferating as judged by the markers PCNA, Ki-67, and MCM7 (Figure 5b, panels D–F). However, scanty CD3 positive CTL (Figure 5b, panels G and H) were present in these hypoxic regions compared to the normoxic regions. Figure 5b, panel I shows a macroscopic view of a tumor. Panels J and K show the primary antibody controls. We repeated these experiments using CTL expressing hypoxia-induced IL-2 and renilla luciferase (HRE-IL-2 CTL). Primary tumors were located in the inner flank region of the mice; because we monitored the mice for a longer period, we could also observe metastatic axillary tumors (Figure 5c). Seven days after CTL injection, the luciferase signal was detected at both sites indicating the presence of both CTL and hypoxia in the tumors. Seven to ten days after CTL injection, pimonidazole was administered and tumors were removed, sectioned, and stained with antibodies specific for hIL-2 and hCD3. Microscopic

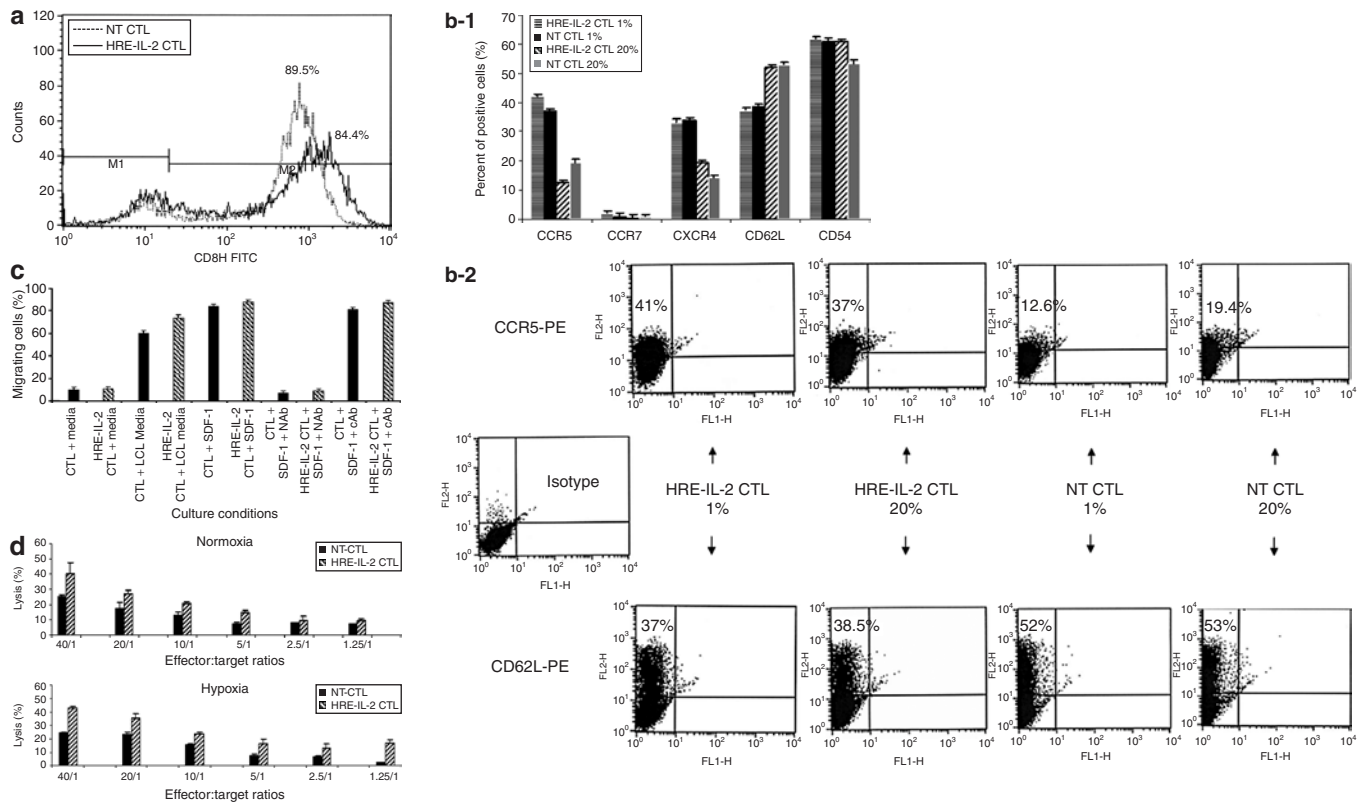


Figure 4 *In vitro* analysis of cytotoxic T lymphocyte (CTL) phenotype under hypoxia. Tumor-specific CTL were transduced with Luc-HRE-IL-2 lentivector and tested. **(a)** HRE-IL-2 CTL (solid line, HRE-IL-2) and non-transduced (dotted line, NT) CTL were stained with CD8 antibodies and analyzed by fluorescence-activated cell sorting (FACS). Results are expressed as the percentage of positive CD8 cells. **(b)** Engineered and parental CTL were stained with monoclonal antibodies specific for CCR5 [chemokine (CC motif) receptor 5], CCR7, CXC chemokine receptor 4 (CXCR4), CD54 (ICAM-1), CD62L (μ -selectin) and isotype controls. The stained CTL were analyzed by FACS. Results representative of five experiments are expressed as the percent of positive cells under normoxia or hypoxia (**b-1**) and representative dot plots are shown for CCR5 and CD62L staining (**b-2**). **(c)** Migration assay with Luc-HRE-IL-2-engineered (striped bars, HRE-IL-2 CTL) and parental (solid bars, CTL) tumor-specific CTL was performed using HUVEC cells cultured in the upper chamber of a transwell. Recombinant stem cell-derived factor (SDF-1), tumor media [lymphoblastoid cell (LCL) media], and plain media (media) were added to the lower chamber. Neutralizing antibodies (NAb) against CXCR4 were used to block the effect of SDF-1 on T cell CXCR4 (for each condition, $n = 3$). Both modified (HRE-IL-2) and NT CTL were placed in the upper chambers. The culture systems were then incubated in normoxia. Cell migration was evaluated by counting the cells in the media of the lower chamber. Results are expressed as percentage of cells migrating. **(d)** Increased cytotoxic activity of transgenic-CTL. Tumor-specific CTL modified with the hypoxia inducible IL-2 (HRE-IL-2 CTL, solid bars) and non-modified CTL (NT-CTL, striped bars) were incubated at different ratios with target cells ($n = 3$) under hypoxia and normoxia for 24 hours and their cytotoxicity tested under the same conditions, using a 4-hour ^{51}Cr release assay. Autologous Epstein-Barr virus-transformed LCLs were used as target cells. Results are expressed as the percentage of specific ^{51}Cr release lysis at multiple effector-to-target ratios. HRE, hypoxia responsive element. PE, phycoerythrin.

observation showed that, unlike NT CTL, the HRE-IL-2 CTLs were present in the hypoxic region of the tumors (positive for the hypoxia probe staining), where they produced IL-2 (**Figure 5d**).

Modified CTL eradicate tumor and enhance survival

To evaluate the *in vivo* effects of HRE-IL-2 CTL, non-obese diabetic severe combined immunodeficient mice at 6–8 weeks of age were irradiated (250 rad) and injected subcutaneously in their right flanks with $10\text{--}15 \times 10^6$ aggressive tumor cells (LCLp) resuspended in Matrigel (BD Bioscience). Two weeks later, we injected 40 tumor-bearing mice intravenously with either (i) HRE-IL-2-modified CTL, (ii) NT parental EBV-specific CTLs (NT CTL) with no rIL-2, (iii) NT parental EBV-specific CTLs plus rIL-2 (1,000 IU every second day), or (iv) phosphate-buffered saline alone (injected for control mice). We used 10 mice in each group. Mice were observed every 2–3 days for local tumors and for development of signs of systemic disease (loss of weight and activity, ruffled

fur, ascites, etc.). All 10 HRE-IL-2 CTL-infused mice had a rapid reduction in tumor size, followed by complete tumor eradication (**Figure 6a**, square symbol). In the NT CTL group, tumor growth was delayed compared to the control phosphate-buffered saline group (**Figure 6a**, cross symbol), but all 10 mice had tumor progression. The injection of rIL-2 every second day delayed growth and then produced regression, but only in 8 of 10 mice. However, this regression was delayed compared to HRE-IL-2 CTL-treated mice, occurring 8–14 days later than the same effects in the HRE-IL-2 CTL-treated group. All mice in the control group injected with phosphate-buffered saline had progressive tumor growth and died or were killed (**Figure 6a**, diamond symbol). All HRE-IL-2 CTL-treated mice survived for the long term (**Figure 6b**), while in the parental-CTL-treated group, only mice that received repeated rIL-2 injections survived (**Figure 6c**). In the HRE-IL-2 CTL and parental CTL + rIL-2 groups, all mice were followed to the very end of the experiment and survived and were censored.

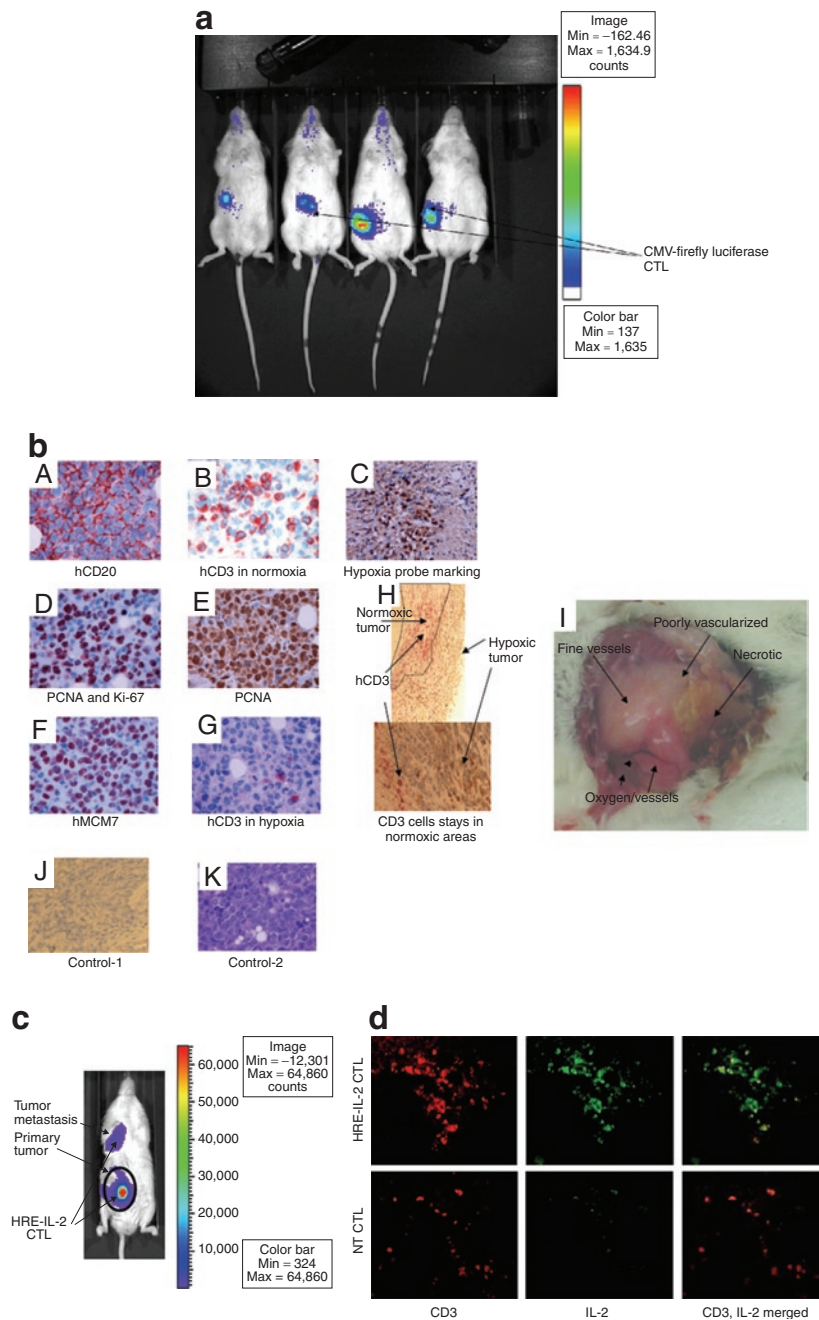


Figure 5 Cytotoxic T lymphocytes (CTLs) are present in lower numbers in hypoxic areas of tumor. **(a)** At 21 days after tumor cell implantation, severe combined immunodeficient (SCID) mice bearing large tumors ($n = 6$) were injected in the tail vein with 5×10^6 autologous tumor-specific CTL, modified with a cytomegalovirus (CMV)-firefly luciferase retrovirus (this vector functions independently of hypoxia and allows us to track all the injected CTL using the firefly signal). Seven days later, the presence of CTL was determined by imaging live mice with an IVIS imaging system (bioluminescence imaging detection). Firefly-luciferase substrate was injected intraperitoneally. Ten minutes after injection, images were taken. **(b)** The mice were injected with hypoxia probes, and tumors harvested for immunohistochemistry. The tumor sections were stained with antibodies specific for CD20 (A), CD3 (B, G and H), proliferation markers Ki-67 (D) and PCNA (D and E), hMCM7 (F) and hypoxia probes: pimonidazole (C and H, detection of hypoxia region). CTL were detected primarily in normoxic areas (B), with few CTL infiltrating or surviving in hypoxic areas (G, H). A macroscopic view of the tumor showed poorly and well-vascularized areas (I). J and K are primary antibodies control slides. **(c)** Functional HRE-IL-2 CTLs efficiently infiltrate the tumor. Lymphoblastoid cell tumor cells were injected subcutaneously in SCID mice ($n = 6$ per group). Twenty-one days later, the same number of hypoxia-engineered (HRE-IL-2) or non-transduced (NT) CTLs (5×10^6 cells) were injected intravenously. Seven days after CTL infusion, homing of the CTL to the hypoxic tumor was visible by IVIS imaging system, which detected the HRE-driven renilla luciferase, indicating that modified CTL had entered hypoxic areas. **(d)** Six days after detecting renilla luciferase producing CTL in the tumor, the mice were killed and tumor masses were collected and frozen in optimum cutting temperature formulation. Hypoxic areas of the tumor were sectioned ($6 \mu\text{m}$) and human CTLs were detected by immunofluorescence with anti-CD3 (Texas red) and with anti-IL-2 (FITC). Fluorescence signals were sequentially acquired from each section and are shown individually. Although CTL are present in both tumors, only HRE-IL-2 CTL produced IL-2. FITC, fluorescein isothiocyanate; HRE, hypoxia responsive element.

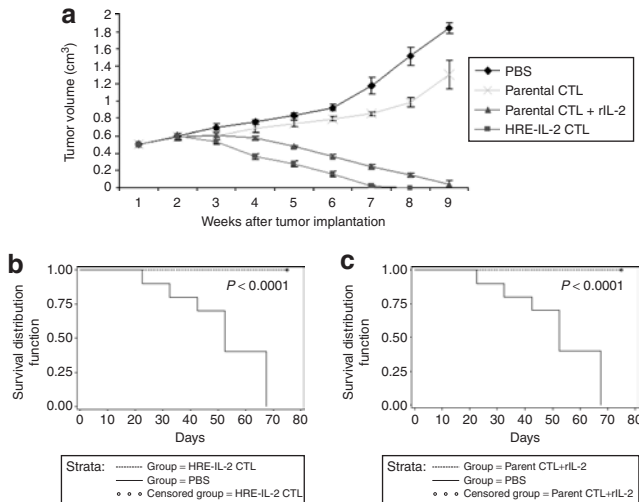


Figure 6 HRE-IL-2 cytotoxic T lymphocytes (CTLs) promote complete and rapid tumor eradication and prolong survival. **(a)** Tumor growth. Tumor-bearing mice were injected with CTL: Luc-HRE-IL-2 CTL (squares), parental CTL (crosses), parental CTL and recombinant interleukin-2 (rIL-2) (1,000 IU) every second day (triangles) or phosphate-buffered saline (PBS) alone (diamonds). $n = 10$ mice for each group. Tumor size was measured every other day with a caliper. Results are expressed as tumor volume (cm³) and show that HRE-IL-2 CTL rapidly shrink and completely eradicate the tumors, while parental CTLs only reduced tumor size if exogenous rIL-2 is provided every other day, and this effect is delayed compared to HRE-IL-2 CTL efficacy. Moreover, the absence of rIL-2 in the parental CTLs group leads to resurgence of tumor growth (cross symbol). **(b)** and **(c)** Murine survival. Tumor-bearing mice were intravenously injected with HRE-IL-2 CTL (broken line, **b**) or non-transduced parental CTLs plus rIL-2 (2,000 IU) every second day (broken line, **c**). Control mice were injected with PBS (solid line, **b** and **c**). $n = 10$ for each group. All mice treated with PBS died early (not censored), while all mice treated with HRE-IL-2 CTL survived (censored). Mice treated with parental CTL + rIL-2 also survived. HRE, hypoxia responsive element.

DISCUSSION

Hypoxia is a characteristic of solid tumor growth and is a challenge for cellular immunotherapy, as the condition impedes T-cell proliferation, activation, survival, and homing. In this study, we have confirmed that hypoxia affects T-cell proliferation and survival, and that immunosuppressive effects can be overcome by genetically modifying tumor-specific T cells so that they express a hypoxia-inducible transgenic *IL-2* gene. This modification restores T-cell expansion under hypoxic conditions *in vitro* and augments tumor infiltration even in areas of hypoxia *in vivo*.¹⁻⁸ In the great majority of studies, T cells utilized for cancer immunotherapy are grown and stimulated *in vitro* to generate CTL with optimal phenotype and function at 20% oxygen. These conditions, however, are not representative of those that are present at many sites of human tumors, in which oxygen tension is variable and is often significantly lower. In the circulation, T cells are exposed to 40–100 mmHg (13% oxygen tension), but even in normal or inflamed tissues, the oxygen tension can decrease to <5% (refs. 18,31,32), and in some primary tumor sites and their metastases it can drop to 0.1–1% pO₂ (refs. 33,34). Hence, there may be a misperception of the actual T-cell functionality at tumor sites *in vivo*. Our modification to T lymphocytes was intended to become functional only when these cells were exposed to low oxygen tension, and we found limited changes to the phenotype or function of CTL under normoxic conditions. We found

a 5% decrease in CD8 positive cells in the HRE-IL-2 CTL population. In normoxia, modified CTL have increased CXCR4 and CD54 expression and as well as improved migration across an endothelial cell layer. They also show improved killing activity both in normoxia and in hypoxia. Strikingly, however, under hypoxic conditions, many of the functions known to be impaired in parental T cells were sustained, including production of IL-2, survival, and proliferation.

As previously described by Makino *et al.*,³² we found that activated tumor-specific CTL were less sensitive to hypoxia once they had engaged their tumor antigen-specific receptors. T cell-receptor activation induces high levels of HIF-1 α proteins that boost glucose catabolic pathways.³⁵ Even in these activated CTL, however, the protective effects of HRE-IL-2 modification could be observed. Of note, lytic activity was unaffected by low oxygen tension in contrast to proliferation and survival.³⁶ Both unmodified and HRE-IL-2 CTLs revealed higher lytic activity against autologous target cells, under hypoxic rather than normoxic oxygen tension level. The superior lytic activity of HRE-IL-2 CTL can be attributed to activation by engagement of antigens on the tumor targets during the cytotoxic assay itself. Once activated by antigen-receptor engagement, T cells stabilize HIF-1 protein.³⁷ In the parental population the stabilization of HIF-1 has no direct effect on endogenous IL-2 production. However, in HRE-IL-2 CTL, stabilization of HIF-1 leads to the production of transgenic IL-2 even under normoxia, leading to enhanced activation and killing by modified CTL. *In vivo*, HRE-IL-2 CTLs were present in larger numbers than parental CTL, the latter being present at the tumor site only if the mice received repeat doses of exogenous rIL-2. As anticipated from these data, tumor regression studies confirmed that HRE-IL-2 CTL were more efficient (achieving 100% tumor eradication) than parental CTL in the absence of exogenous IL-2 (achieving no tumor eradication). Nonetheless, even in the presence of exogenous rIL-2, treatment with parental CTL remained less efficient at eradicating tumors or at preventing recurrence than treatment with transduced cells without exogenous rIL-2 (80% efficacy versus 100% for HRE-IL-2).

The overall consequence of all the above was that renilla-HRE-IL-2-engineered CTL were more readily detected in hypoxic areas of a tumor *in vivo* than unmodified T cells, a result consistent with previously reported observations of significantly lower levels of CD3⁺ cells in hypoxic compared to non-hypoxic tumors.

How does modification of CTLs with HRE-IL-2 produce these benefits? When T cells infiltrate hypoxic areas of a tumor, the level of transcription of the HRE-IL-2 transgene is increased due to hypoxia-mediated stabilization of the HIF transcription factor. While only the Luc-HRE-IL-2-transduced CTL actually produce IL-2, the surrounding CTL may also benefit from this secreted cytokine. IL-2 is essential for T-cell proliferation, survival, and activation³⁸ and therefore loss of IL-2 expression in the CTL infiltrating hypoxic areas of tumors is strongly immunosuppressive. The virtual absence of unmodified CTLs in hypoxic regions in our tumor system suggests that they either do not infiltrate the hypoxic areas or die by apoptosis once they become hypoxic thereby losing IL-2 expression. We have also demonstrated, by this study, that arming the CTL with an HRE-IL-2 transgene neutralizes this effect.

In this proof-of-principle study, we have demonstrated that tumor-specific cytotoxic T cells can be engineered to circumvent hypoxia-induced immunosuppression caused by the CTL's reduced ability to produce IL-2, a proliferative and antiapoptotic soluble factor, when exposed to hypoxic conditions at tumor sites. Although we have demonstrated these effects only in an EBV-associated lymphoma, the same approach may be applicable to T cell therapies for other malignancies³⁹ and to the production of other hypoxia-protective factors. Hence, the approach we have described may be of value in enhancing CTL functionality for the treatment of solid tumors using adoptive immunotherapy.^{40–42}

MATERIALS AND METHODS

Cells. 293T (human embryonic cells¹³) were cultured in Dulbecco's modified Eagle's medium (BioWhittaker, Walkersville, MD). Jurkat (T leukemia), LCL (EBV-LCL) were cultured in Roswell Park Memorial Institute-1640 medium. CTL were cultured in Roswell Park Memorial Institute/Click's media (vol:vol, 1:1) (Life Technologies, Gaithersburg, MD), CTL media were supplemented with hrIL-2 (proleukin; Chiron, Emeryville, CA) (30–50 U/ml). All media were supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 100 U/ml penicillin, and 100 µg/ml streptomycin (Life Technologies, Gaithersburg, MD) and cells were maintained at 37°C, 5% CO₂ in a humidified incubator.

Generation of EBV-specific CTLs. EBV-specific CTLs were prepared by stimulating peripheral blood mononuclear cells with the autologous EBV-transformed LCLs. Peripheral blood mononuclear cells (2×10^6) were co-cultured with 5×10^4 gamma-irradiated (40 Gy) autologous LCLs per well in a 24-well plate. On day 10, the responder cells were restimulated with irradiated (40 Gy) LCLs at a responder-to-stimulator ratio of 4:1. From day 14, recombinant human IL-2 (50 IU/ml) was added two times a week.³⁰

Vector construction. A vector was generated by cloning the *hIL-2* gene downstream of an expression vector (pBI-V5R) containing a hypoxia-inducible promoter with five copies of the vascular endothelial growth factor gene HREs.²⁶ The renilla luciferase gene was cloned on the other side of the bidirectional 5 × HRE, and then introduced into a self-inactivating lentivirus.²⁶ Although we did not quantitate the CTL with this marker gene, we were able to use it to ensure that the CTL reached the hypoxic areas.

Vector production and transduction. Retroviruses encoding CMV-firefly luciferase pseudotyped with RD114 and lentiviral vectors (self-inactivating) encoding Renilla-5(HRE)-hIL-2 pseudotyped with vesicular stomatitis virus envelope glycoprotein were prepared using 293T cells. The cells were transfected with the lentiviral constructs and the envelope and accessory plasmids (pCMVΔR8.2, pCMVΔR8.9). The culture supernatants were filtered and concentrated by ultracentrifugation and added to the T cells, which were then spinfected at 1,000 rpm for 90 minutes in the presence of the vector and Polybrene (8 µg/ml; Sigma, St Louis, MO). The CMV-firefly luciferase vector was used to trace CTL in the total tumor while the lentivirus–renilla luciferase–HRE–IL-2 was used to trace CTL entering hypoxic areas; firefly and renilla luciferase use different substrates.

Hypoxia/normoxia culture conditions. For all experiments, cells were grown for 48 or 72 hours at either ambient oxygen (normoxia, 20% oxygen) with 5% CO₂, or 1% oxygen (hypoxia) concentration with 5% CO₂. Hypoxia was established using a Modular Incubator Chamber (MIC-101, Billups-Rothenberg), which was gassed at a rate of 20 l/minute to completely purge the chamber with 1% O₂, 5% CO₂, 94% N₂ and placed in a standard humidified incubator at 37°C.

CTL proliferation. pHRE-IL-2–transduced and pHRE-IL-2–NT CTL were seeded at 0.5×10^6 cells per well, stimulated or not with EBV-LCLs, and

incubated under hypoxic conditions; control cells were incubated under normoxic conditions. Proliferation was determined by trypan blue exclusion on alternate days.

Phenotyping. Cell-surface phenotype was investigated using the following monoclonal antibodies: CD3, CD4, CD8, CXCR4, CD54, CD62L, CCR5, CCR7 (Becton Dickinson). Appropriately matched isotype controls (Becton Dickinson) were used in each experiment. Cells were analyzed with a FACScan flow cytometer (Becton Dickinson).

IL-2 production. Transduced cells were cultured under normoxia or hypoxia. The concentration of IL-2 in the culture supernatants was measured by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN).

Apoptosis assay. T cells were cultured under normoxic or hypoxic conditions and analyzed for apoptosis using a BD Bioscience kit (Becton Dickinson Bioscience, Mountain View, CA). The cultured cells were stained with 7 amino-actinomycin D and Annexin-V, and analyzed by flow cytometry using a FACScalibur and CellQuest software (Becton Dickinson).

Luciferase detection. Cells were cultured under normoxic or hypoxic conditions, lysed, and protein extracts analyzed using the bicinchoninic acid protein assay kit. Luciferase was quantified for a given amount of protein using a renilla-luciferase assay kit (Promega, WI) and the luminosity measured with a luminometer (Monolight 3010; Pharmingen).

Cytotoxicity assay. The cytotoxic activity of the CTL was evaluated in a ⁵¹Cr release assay, as previously described.³⁰ Autologous LCL target cells incubated in complete medium or 1% Triton X-100 (Sigma, St Louis, MO) were used to determine spontaneous and maximum ⁵¹Cr release. The mean percentage of specific lysis was calculated as $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximal release} - \text{spontaneous release})$.

CTL in vitro chemotaxis and extravasation assay. We measured the ability of T cells to extravasate through a layer of endothelial cells in a modified Boyden chamber assay.⁴³ Human umbilical vein endothelial cells seeded in the transwells (pore: 0.5 µm) of 12-well plates. 1×10^5 HRE-IL-2 CTL, or control CTL, was added to the upper chambers and 800 µl of Roswell Park Memorial Institute medium with 120 ng/ml of SDF-1 (Chemokines, R&D Systems) in serum-free media was added to the lower chambers. Control wells consisted of: (i) LCL culture media or plain Roswell Park Memorial Institute medium in the lower chambers, and (ii) pre-incubation of the CTL with neutralizing anti-CXCR4 (fusin antibody; R&D Systems) or isotope control antibody. After 5 hours of incubation in normoxic conditions at 37°C, migration was evaluated by counting CTL that migrated to the lower chambers.

In vivo experiments. Non-obese diabetic severe combined immunodeficient mice were kept in accordance to institutional guidelines. We have selected aggressive tumors (LCLp) that developed hypoxia *in vivo*. LCL cells were implanted in mice. The largest tumors were then expanded and re-implanted in mice. Selected fast-growing tumors cells (LCLp) allowed us to obtain aggressive hypoxic tumors *in vivo*. Mice at 6–8 weeks of age were irradiated (250 rad) and injected subcutaneously in their right flanks with $10–15 \times 10^6$ LCL resuspended in Matrigel (BD Bioscience). Mice with palpable identical tumor masses were injected intraperitoneally with recombinant human IL-2 (2,000 U, Proleukin; Chiron) then with CTLs (5×10^6) via their tail veins. CTL were tracked using the IVIS imaging system. To visualize CTL independent of the oxygen level, we used firefly-luciferase substrate. On day 10, a hypoxia probe was injected intraperitoneally (pimonidazole hydrochloride, Chemicon International, Temecula, CA) and tumors were harvested. Tumors from mice that received only NT CTLs (5×10^6) or phosphate-buffered saline were used as controls. We monitored the mice every 2–3 days for tumor regression (using a caliper) and survival studies.

Immunohistochemistry. We used mouse monoclonal anti-CD3 or CD20 antibodies (Dako, CA) for immunohistochemical analysis of formalin-fixed tumor sections. Antibodies specific for hypoxia probes, and PCNA Ki-67, hMCM7, HIF-1 α (Chemicon, CA), were also used to analyze the tumor sections. Prepared sections were counterstained with hematoxylin, and mounted in aqueous medium (Dako Faramount; Dako, Carpinteria, CA). Tumor cryostat sections (6 μ m) were blocked and incubated with mouse anti-human CD20 and CD3 antibodies (Dako, Carpinteria, CA), or mouse anti-human IL-2 antibody (GeneTex, San Antonio, TX), followed by the fluorescein isothiocyanate-conjugated horse anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA). Sections were mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Staining was visualized with a fluorescence microscope.

Statistical analysis. The student *t*-test was used to analyze each experiment. For each test, *P*-values of <0.05 were considered statistically significant. Kaplan–Meier estimates were used for the curves representing mice survival.

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