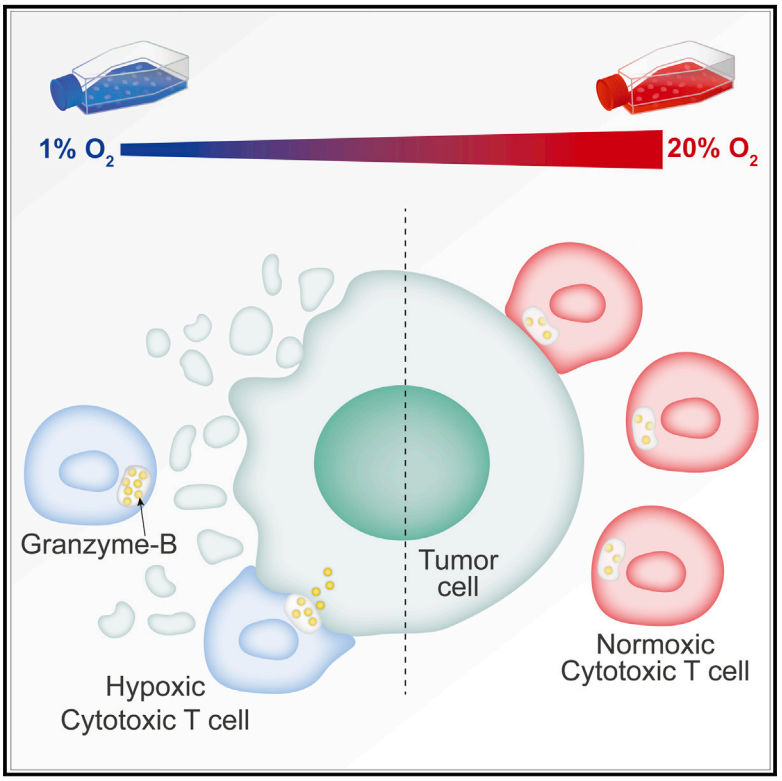
Report

Cell Reports

Culturing CTLs under Hypoxic Conditions Enhances Their Cytolysis and Improves Their Anti-tumor Function

Graphical Abstract



Highlights

- CD8 T cells activated under hypoxic conditions survive and mature well
- Hypoxic CTLs package more granzyme-B into each granule they produce
- Hypoxic CTLs do not penetrate more deeply into avascular tumor regions
- Mice treated with hypoxic CTLs exhibit better tumor rejection and survival

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In Brief

Gropper et al. show that antigen-specific CD8 T cells activated under hypoxic, rather than normoxic, conditions disperse normally in cognate tumors and do not migrate farther away from blood vessels but package more granzyme-B into their granules, show enhanced cytotoxicity, and reject tumors more efficiently.

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Cell Reports
Report

Culturing CTLs under Hypoxic Conditions Enhances Their Cytolysis and Improves Their Anti-tumor Function

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SUMMARY

Cytotoxic T lymphocytes (CTLs) used in immunotherapy are typically cultured under atmospheric O_2 pressure but encounter hypoxic conditions inside tumors. Activating CTLs under hypoxic conditions has been shown to improve their cytotoxicity in vitro, but the mechanism employed and the implications for immunotherapy remain unknown. We activated and cultured OT-I CD8 T cells at either 1% or 20% O_2. Hypoxic CTLs survived, as well as normoxic ones, in vitro but killed OVA-expressing B16 melanoma cells more efficiently. Hypoxic CTLs contained similar numbers of cytolytic granules and released them as efficiently but packaged more granzyme-B in each granule without producing more perforin. We imaged CTL distribution and motility inside B16-OVA tumors using confocal and intravital 2-photon microscopy and observed no obvious differences. However, mice treated with hypoxic CTLs exhibited better tumor regression and survived longer. Thus, hypoxic CTLs may perform better in tumor immunotherapy because of higher intrinsic cytotoxicity rather than improved migration inside tumors.

INTRODUCTION

In the past few years, immunotherapy has come to the forefront in the fight against cancer. Adoptive cell therapies (Fesnak et al., 2016; Rosenberg and Restifo, 2015), such as tumor-infiltrating lymphocytes (TILs), T cell receptor (TCR)-transgenic T cells, and chimeric antigen (Ag) receptors (CAR)-T cells, are in advanced clinical trials, and the latter have shown positive results in treating hematologic malignancies. On the other hand, when treating solid tumors, transferred cells often fail to fully reject them. Some of the obstacles T cells face are restricted access to distant sites of tumor growth (Angell and Galon, 2013) and an immunosuppressive tumor environment (Shalapour and Karin, 2015).

To effectively reject solid tumors, T cells, in particular cytotoxic T lymphocytes (CTLs), must disseminate widely inside the tumors and reach all target cells; unfortunately, they often fail (Erdag et al., 2012). It is still unclear which specific factors underlie restricted dissemination, but the hypoxic microenvironment in tumors seems to play a role.

Hypoxia is a major feature of the tumor microenvironment. Stemming from suboptimal density and architecture of blood vessels, it characterizes the majority of solid tumors (Vaupel and Mayer, 2014). Hypoxia can lead to accumulation of adenosine in avascular tumor areas, which, in turn, suppresses the function of T cells (Hatfield et al., 2014), and perhaps their motility. Indeed, CD8 T cells have been shown to avoid hypoxic areas inside tumors (Hatfield et al., 2015), but this effect may be secondary to changes in the secretory repertoire of tumor cells (Hasmim et al., 2013) or in the adhesive profile of endothelial cells, (Bellone and Calcinotto, 2013) both of which are triggered by hypoxic conditions inside tumors.

Better studied is how hypoxia interferes with the function of T cells within tumors (Chouaib et al., 2017). Hypoxic areas in the tumor attract M2-like tumor-associated macrophages, (Casazza et al., 2013; Movahedi et al., 2010; Van Overmeire et al., 2014) as well as regulatory T cells (Facciabene et al., 2011), which may contribute to T cell dysfunction. In addition, anaerobic glycolysis produces lactate resulting in acidification, which, together, may suppress T cell activation, proliferation, and cytotoxicity (Fischer et al., 2007; Nakagawa et al., 2015).

Paradoxically, examining the direct effect of hypoxia on CD8 T cells, most studies point to enhanced effector function. Caldwell et al. (2001) were the first to report that, when cultured in low O_2 tension, CTLs acquire more lytic activity. Two other studies (Nakagawa et al., 2015; Vuillefroy de Silly et al., 2015) found that reactivation of CD8 T cells under hypoxic conditions resulted in better killing in vitro. The mechanism through which CTLs became more effective was also studied: interferon-gamma (IFNγ) yields inconsistent results (Caldwell et al., 2001; Doedens et al., 2013; Nakagawa et al., 2015; Vuillefroy de Silly et al., 2015), whereas granzyme-B expression tends to be higher (Doedens et al., 2013; Vuillefroy de Silly et al., 2015). Surprisingly,

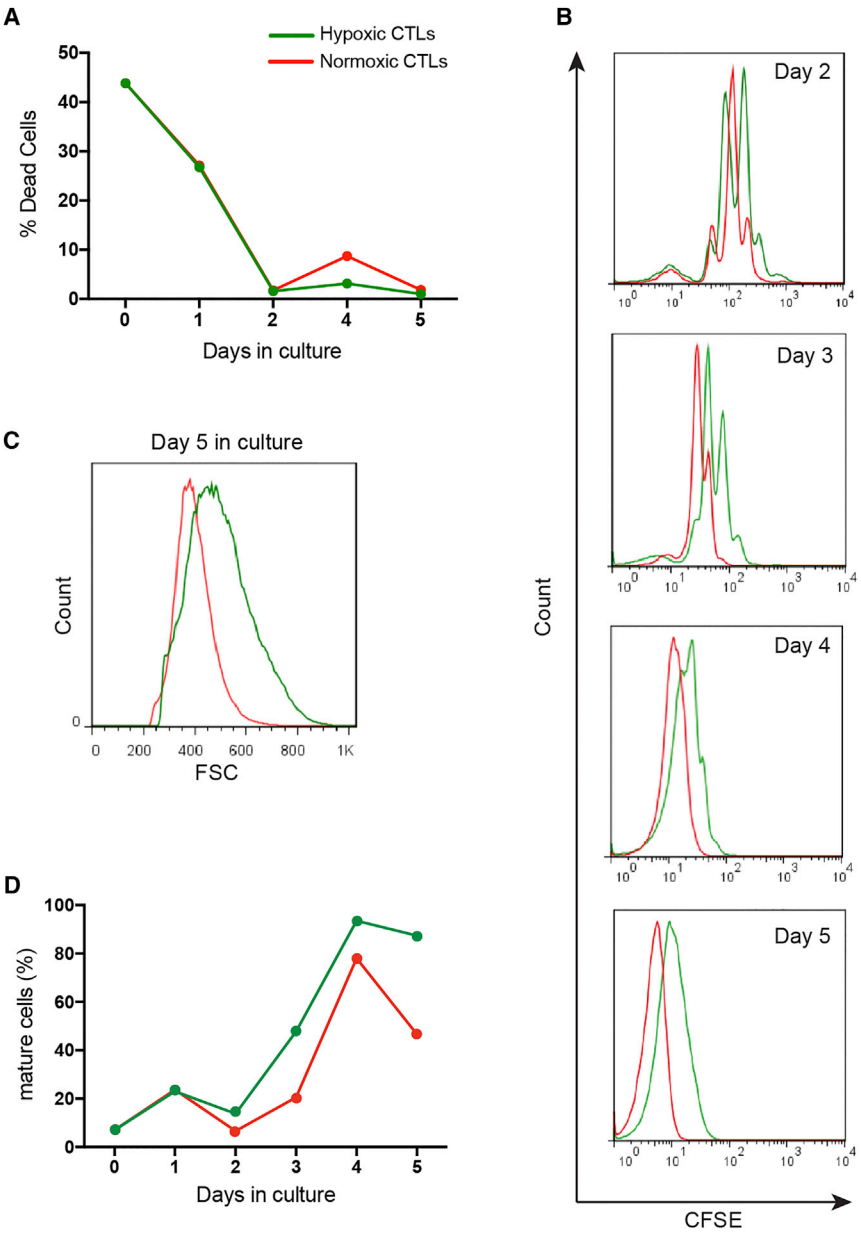

Figure 1. CD8 T Cells Survive and Mature Well under Hypoxic Conditions

(A–D) Flow cytometry was used to analyze the fate of CD8 T cells cultured under hypoxic (1% O_2) or normoxic (20% O_2) conditions.

(A) CD8 T cell death in culture was analyzed based on DAPI staining. Hypoxic cells survived at least as well as normoxic ones.

(B) CD8 T cell proliferation in culture was analyzed based on CFSE dilution. Normoxic cells started to proliferate earlier and, by day 2, underwent an extra proliferation cycle.

(C) Analysis of cell size after 5 days in culture revealed that hypoxic CTLs are bigger than the normoxic ones.

(D) The percentage of mature CTLs ($CD44^{hi}$ $CD62L^{lo}$) increased in the first 4 days of culture, but a higher proportion of hypoxic CTLs exhibited a mature phenotype. Data are representative of two experiments.

See also Figures S1 and S2 and Table S1.

no study reported the utility of CTLs cultured under such conditions in adoptive cell therapy of tumors.

In this study, we explore whether adaptation to hypoxia improves anti-tumor CD8 T cell function and how. We culture and activate CD8 T cells under 1% O_2 tension (hypoxic CTLs) or 20% O_2 tension (normoxic CTLs) and compare their activity and behavior as effector cells.

Thus, we characterize the cells' survival, proliferation, and activation profile during culture, examine their effector function in vitro and in vivo, and quantify the granzyme-B and perforin content inside their granules. To gain insight into the behavior of the differentially cultured CTLs inside tumors, we intravitally image their migration and record their distribution. Hypoxic CTLs do not benefit from higher velocity or wider distribution inside tumors but show better cytolytic activity and package more granzyme-B into each granule. Correspondingly, hypoxic CTLs reject Ag-specific tumors more effectively, leading to better mouse survival.

RESULTS

CD8 T Cells Survive Hypoxic Conditions and Mature Well but Proliferate More Slowly than under Normoxic Conditions

Naive CD8 T cells were harvested from the spleen of OT-I mice, activated, and cultured for five days under atmospheric pressure (20% O_2) to produce "normoxic CTLs" or under hypoxic conditions (1% O_2) to produce "hypoxic CTLs." Transcriptomic analysis verified that the hypoxic CTLs upregulated genes associated with hypoxia, glucose transport, and glycolysis (Table S1). In parallel, they downregulated genes needed for oxidative phosphorylation without losing mitochondrial mass, which was actually higher in these cells (Figure S1).

To better understand how hypoxia influences the activation of CTLs in vitro, we followed the cells' survival and proliferation in culture. Hypoxic CTLs as well as normoxic ones survived in culture and did not exhibit excess cell death during maturation in vitro (Figure 1A). However, hypoxia inhibited the proliferation of CD8 T cells in culture, slowing their division rate by one cell cycle over 5 days (Figure 1B) and leading to lower cell counts (data not shown). A difference in cell size was observed by day 3 of culture, where the hypoxic CTLs became bigger and remained so until day 5 of culture (Figure 1C). During their activation, both CD8 T cell populations gradually acquired a mature

cellular phenotype ($CD44^{hi}CD62L^{lo}$) consistent with effector memory CTLs, but by the third day of culture hypoxic CTLs contained a higher percentage of such cells (Figures 1D and S2A).

We also assessed whether culturing CTLs under normoxic or hypoxic conditions leads to senescence or exhaustion. Both populations contained a negligible proportion of cells showing senescence-associated beta-galactosidase (SA-β-gal) activity (Figure S2B) and exhibited almost complete nuclear localization of high mobility group box 1 (HMGB1) protein (Figure S2C). Both also contained a large proportion of replicating cells based on Ki67 staining (Figure S2D). Together these indices show that neither population is approaching senescence. The exhaustion markers T cell immunoglobulin and mucin-domain containing-3 (TIM3) and lymphocyte-activation gene 3 (LAG3) showed higher expression in the hypoxic cells, but cytotoxic T-lymphocyte-associated protein 4 (CTLA4) and programmed cell death protein 1 (PD1) expression was essentially the same in both cell types (Figure S2E).

Higher Production of Granzyme-B in Hypoxic CTLs

To assess the cytolytic potential of CTLs cultured in hypoxic conditions, we examined their granzyme-B and perforin content. Hypoxic CTLs contained more granzyme-B than did normoxic ones, as shown by flow cytometry (Figure 2A) and by imaging flow cytometry (IFC) (mean fluorescence intensity [MFI] 4,131 ± 18.11 versus 2,580 ± 7.13) (Figures 2B and 2C). This difference persisted even when cell sizes were corrected for (MFI 33.45 ± 0.12 versus 22.06 ± 0.04).

Given that granzyme-B levels were higher in hypoxic CTLs, we next checked whether the cells also produced more cytotoxic granules to package this protein. We stained both cell populations with LysoTracker and counted their granules using imaging flow cytometry (Figure 2D). The average number of granules per cell was very similar in both cell populations (9.7 ± 6 in hypoxic CTLs versus 8.2 ± 5 in normoxic ones). Correspondingly, the mean intensity of anti-granzyme-B in granules in hypoxic cells was significantly higher (Figure 2E). Hypoxic CTLs thus produce the same number of granules but package more granzyme-B into each one. In contrast to granzyme-B, perforin expression was similar in the hypoxic and normoxic CTLs, as shown by mass cytometry (CyTOF) analysis (Figure 2F).

To analyze their ability to degranulate cytotoxic granules containing granzyme-B and perforin, we challenged OT-I CTLs with B16-OVA target cells at the presence of antibody against LAMP-1, a membranal granule marker (Figure 2G). The accumulation of LAMP-1 on the cell membrane was similar in the CTL populations, even when they were robustly stimulated with PMA and ionomycin.

Several researchers (Roman et al., 2010; Vuillefroy de Silly et al., 2015) have reported higher secretion of the pro-inflammatory effector molecule IFNγ in hypoxic CTLs, we recapitulated these findings (Figure S3A). Conversely, secretion of the anti-inflammatory cytokine interleukin-10 (IL-10) had also been reported to be elevated following restimulation of CD8 effector cells under hypoxic conditions (Vuillefroy de Silly et al., 2015). We observed the same phenomenon in our unchallenged hypoxic CTLs, although absolute IL-10 levels were very low (Figure S3B).

Hypoxic CTLs Kill Target Cells More Efficiently than Normoxic CTLs

To test the cytotoxic potential of hypoxic CTLs, we used an effector-tumor target model of OT-I CTLs and OVA^+ $dTomato^+$ B16 cells (B16-OVA) and performed a florescence-based killing assay under normoxic or hypoxic conditions (Figure 2H). Hypoxic CTLs, however, consistently exhibited better performance under either O_2 tensions.

Similar Migration Speed and Distribution Patterns of Hypoxic and Normoxic CTLs inside Tumors

To investigate the ability of CTL populations to infiltrate hypoxic tumor regions located away from blood vessels, we intravenously (i.v.) co-injected hypoxic and normoxic OT-I CTLs to mice bearing B16-OVA tumors. Two days later, we examined CTL movement inside the tumors using intravital 2-photon microscopy (Figure 3A). Both cell types moved at similar velocities inside the tumor and slowed down to the same extent as they moved away from blood vessels (Figures 3B and 3C; Movie S1).

Two-photon microscopy allowed us to watch only a restricted area of the tumor and observe it to a limited depth. We therefore also examined the parallel distribution of both CTL populations in the entire tumor using fluorescent immunohistology of tumor sections (Figure 3D). CTLs populated the tumor by 2 days after injection and accumulated in higher numbers by 5 days, especially in the tumor periphery. There were no differences, however, in the distribution of the different cell types inside the tumors. In addition, the distances of the CTLs from blood vessels were similar at either 2 or 5 days (Figures 3E and 3F).

Hypoxic CTLs Reject Tumors in Mice More Efficiently than Normoxic CTLs

To check if the enhanced in vitro cytotoxicity would improve the therapeutic potential of hypoxic cells, we used an implanted tumor rejection assay with OT-I CTLs and B16-OVA tumors. Seven days after implanting 2×10^6 tumor cells subcutaneously (s.c.), we treated the mice i.v. with 5×10^6 hypoxic CTLs, normoxic CTLs, or not at all. Mice treated with hypoxic CTLs showed significantly better tumor regression (Figure 4A) and, as a result, survived significantly longer (Figure 4B). This result was consistent and was repeated in three independent experiments with 56 mice in total.

DISCUSSION

Investigating the influence of hypoxia on the activation and function of CD8 T cells in vitro, we demonstrated its enhancing effects. Our results are supported by several previous studies that have reported such effects of hypoxia but disagree about the mechanisms involved (Caldwell et al., 2001; Doedens et al., 2013; Nakagawa et al., 2015; Vuillefroy de Silly et al., 2015). Our study expands on these results by investigating the in vivo implications for cell motility and tumor rejection, and pinpointing granzyme-B as a potential target for hypoxia.

Although CD8 T cells were activated under severe hypoxia (1% O_2), they survived in this environment just as well as under normoxic conditions (20% O_2) (Figure 1A). This finding is consistent with some (Makino et al., 2003), but not all (Gaber et al.,

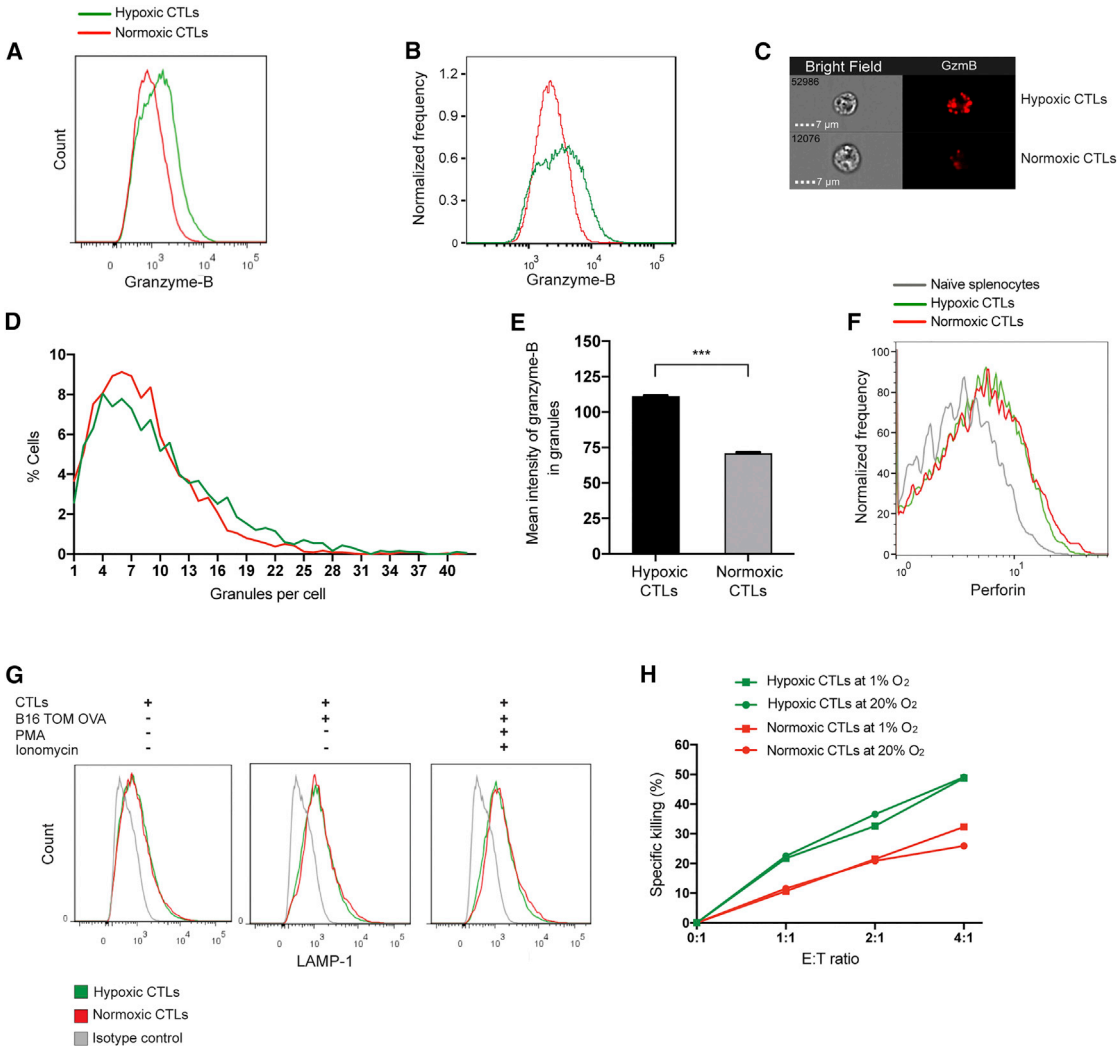

Figure 2. Hypoxic CTLs Package More Granzyme-B in Lytic Granules and Are More Cytotoxic

(A–E) Flow cytometry analysis (A) and imaging flow cytometry analysis (B–E) of intracellular granzyme-B content in hypoxic and normoxic CTLs that had been cultured for 5 days. (B) The bright detail intensity (intensity of bright spots) of granzyme-B. (C) Sample images of granzyme-B staining in normoxic and hypoxic CTLs. (D) Percentage of cells that contain different numbers of LysoTracker^{+} granules in hypoxic and normoxic CTLs. Both CTL populations contain similar numbers of granules per cell (two-way ANOVA, $p = 0.08$). (E) The mean intensity of granzyme-B per granule in both cell types (t test, $p < 0.0001$). Bars denote ± SEM. (A–E) Hypoxic cells have granules richer in granzyme-B.

(F) Mass cytometry (CyTOF) analysis of intracellular perforin content in the hypoxic and normoxic CTLs showing similar expression.

(G) Flow cytometry analysis of anti-LAMP-1 in CTLs that were cultured for 2.5 hr with or without B16-OVA target cells, in the presence or absence of phorbol 12-myristate 13-acetate (PMA) and ionomycin. The CTL secretory capacity was similar.

(H) Following culture, both CTL populations were placed with B16-OVA target cells for 2.5 hr at different O_2 tensions. Hypoxic CTLs killed target cells in vitro better than did the normoxic ones. The results shown are representative of three independent experiments.

See also Figure S3.

2013; Larbi et al., 2010), previous studies. High survival is perhaps not surprising since the O_2 tension in lymph nodes ranges from 0.5% to 6% (Huang et al., 2007), 4- to 10-fold lower than the atmospheric conditions used in standard protocols. That the degree of CD8 T cell death did not increase also implies that during their activation CD8 T cells adapted to the hypoxic conditions rather than undergoing a selection process.

In contrast to their survival, the proliferation of hypoxic CD8 T cells was diminished (Figure 1B), and so they exhibited lower cell yields, in accordance with previous reports (Atkuri et al., 2005; Cretenet et al., 2016; Nakagawa et al., 2015). A related observation was that hypoxic CTLs were bigger (Figure 1C). The unchanged number of granules in these cells (Figure 2D), and the high percentage of ki67 expressing cells (Figure S2D) implies that they include more lymphoblasts awaiting division rather than cells overloaded with cytolytic granules. Even though they underwent fewer division cycles, the hypoxic cells reached full maturation faster, expressing lower levels of CD62L by day 3

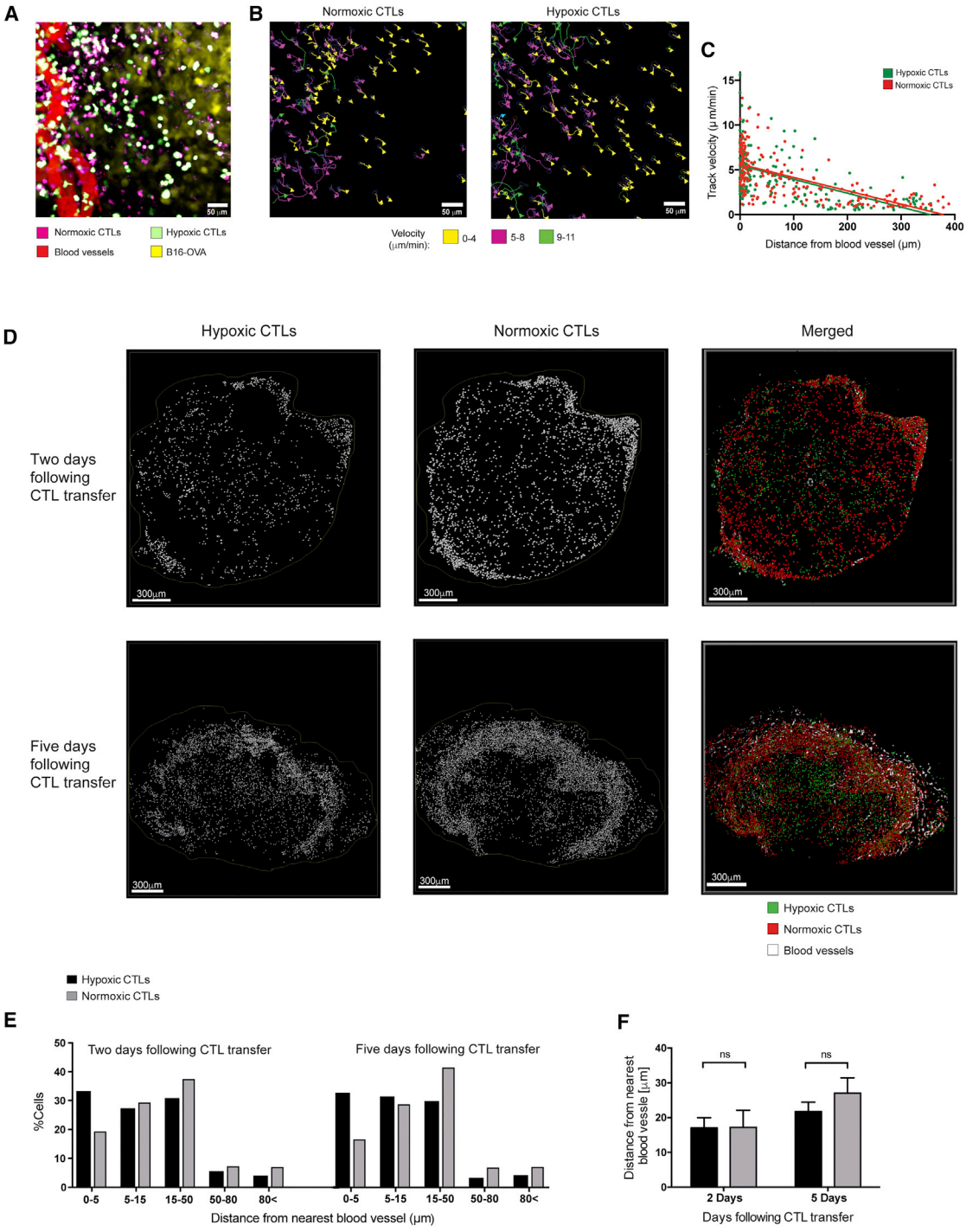

Figure 3. Hypoxic and Normoxic CTLs Slow Down as They Move Away from Blood Vessels and Similarly Distribute Inside Tumors

(A–C) Mice were inoculated with a B16-OVA-tdTomato tumor, co-treated 7 days later with CFP-tagged normoxic and GFP-tagged hypoxic OT-I CTLs, and imaged intravitally 2 days later. (A) A frame from intravital 2-photon movie (Movie S1) shows the position of the three cell populations in relation to blood vessels. (B) Intratumoral motility was examined in relation to blood vessels. Arrows represent cell trajectories of hypoxic and normoxic CTLs, color-coded by track velocity. (C) The track velocity of CTLs was inversely related to their mean distance from the nearest blood vessel ($r = 0.60$, $p < 0.0001$), but ANCOVA indicated that there was no difference in the overall velocities ($p = 0.38$) or dependency on distance ($p = 0.74$) between the two CTL populations.

(D–F) Mice were inoculated with a B16-OVA tumor and co-treated 7 days later with CD45.1$^+$ normoxic (red) and GFP-tagged hypoxic (green) OT-I CTLs. Tumors were harvested and sectioned two or 5 days later, CTL positions were analyzed in relation to CD31$^+$ endothelium (white). (D) Immuno-histochemical sections. CTLs populated the tumor by 2 days and further accumulated by 5 days (t test, $p < 0.001$, $n = 6$). Although more cells accumulated in the periphery of the tumors on

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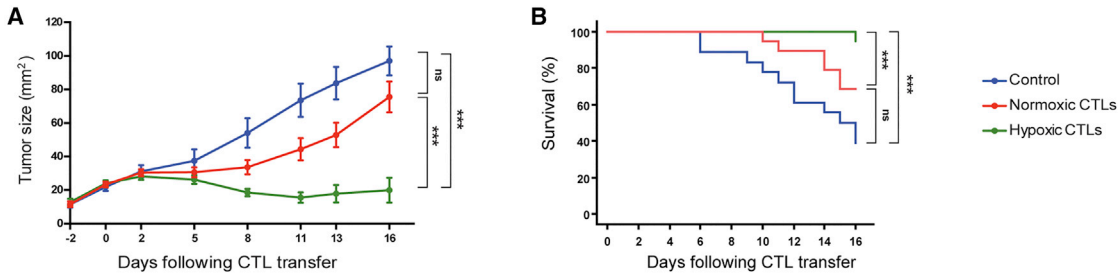

Figure 4. Hypoxic CTLs More Efficiently Reject Tumors and Prolong Mice Survival

(A) Mice were implanted with B16-OVA tumors and treated 7 days later with no CTLs, normoxic CTLs, or hypoxic CTLs. Two-way repeated-measures ANOVA, $p < 0.0001$, $n = 56$.

(B) Survival curves of the three treatment groups shown in (A) (log rank, $p < 0.001$). Bars denote ± SEM. The results indicate that hypoxic CTLs reject the tumors more efficiently than do normoxic ones and prolong the survival of mice.

of culture (Figures 1D and S2A). This result is supported by previous findings (Vuillefroy de Silly et al., 2015). The reduced proliferation could pose a problem if such conditions are used in adoptive cell therapies, but this could be offset by the higher proportion of mature effector cells.

The mechanism through which hypoxia shapes CD8 T cell fate is still unclear. It has been shown that activated CD8 T cells that highly express the glucose transporter Glut-1 (Cretenet et al., 2016) or exhibit higher glucose uptake (Sukumar et al., 2013) show poor proliferation, bigger cell size, and more terminally differentiated cell markers (regardless of the O_2 tension they were cultured in). By blocking glycolysis and observing arrested maturation, Sukumar et al. (2013) showed that glycolysis drives CD8 T cell maturation toward effector fate. Our transcriptomic analysis showed that hypoxic cells express higher levels of Glut-1 as well as glycolysis-related genes (Table S1). The accumulating evidence on the similarity between the effect of glycolysis and the effect of hypoxia on CD8 T cells suggests that hypoxia indirectly affects the cells by modulating the metabolic pathways in the cells.

In an attempt to explain the high cytotoxicity of hypoxic CTLs, we assessed their perforin and granzyme-B content. It has been shown that VHL-deficient CTLs, which in many respects mimic hypoxic cells, express slightly higher perforin levels (Doedens et al., 2013). Although the mRNA levels of perforin in our hypoxic CTLs were higher (Table S1), the protein levels were the same in both cell populations (Figure 2F). In contrast, hypoxic CTLs showed higher granzyme-B protein levels (Figures 2A–2C). This finding is consistent with some (Doedens et al., 2013; Vuillefroy de Silly et al., 2015), but not all (Xu et al., 2016), previous studies. Because granzyme-B mRNA levels were lower in the hypoxic cells (Table S1), elevated translation or reduced degradation of the protein could explain the higher protein content. An alternative explanation could be reduced secretion of granules from the hypoxic cells. Unlike Xu et al. (2016), we failed to detect higher surface concentration of LAMP-1 in hypoxic cells (Figure 2G) and the number of granules in each cell was similar in both populations (Figure 2D), ruling this option out. Instead, our data suggest that hypoxic cells package more granzyme-B in each granule (Figure 2E). This feature may be a key reason for the enhanced cytolytic activity of the hypoxic cells (Figure 2H) and their improved efficacy in tumor rejection (Figure 4A).

Since normoxic cells went through more proliferation cycles and exhibited reduced cytotoxic activity, it could be suggested that they became senescent or exhausted. This however was not supported by our findings. Indicators of senescence were similarly absent in both populations (Figures S2B–S2D), and mRNA levels of the senesence marker KLRG1 were almost undetectable in both cell types (Table S1). In addition, the exhaustion markers TIM3 and LAG3 were even higher in hypoxic cells (Figure S2E). This seemingly paradoxical finding, of highly efficient effector cells that nonetheless express more inhibitory molecules has been reported before in VHL-deficient CTLs (Doedens et al., 2013). We propose that the cells employ inhibitory molecules to counteract their high activation, but these do not block their cytolysis fully.

Previous findings from our lab (unpublished data) show that T cells slow down as they move away from blood vessels, and immediately arrest when blood flow is cut off. These observations point to rapid regulation of motility which is independent of transcription or energetic deficits. Such regulation may help CTLs save energy when they enter hypoxic regions. The end result is accumulation of CTLs near blood vessels and out of hypoxic areas (unpublished data), an observation also made by Hatfield et al. (2015). Based on these findings, we hypothesized that hypoxic CTLs may infiltrate tumors better. However, although both populations slowed down as they move away from blood vessels (Figure 3B) and accumulated in perivascular areas (Figures 3D–3F), they decelerated to the same extent (Figure 3C) and exhibited similar mean distances from blood vessels (Figures 3D–3F). Overall, it seems that the hypoxic cells do not benefit from better penetration into avascular tumor areas.

day 5 (ratio of cells in the core versus the periphery 1.14 ± 0.13 on day 2 and 0.30 ± 0.04 on day 5, $p < 0.001$) normoxic and hypoxic CTLs distributed similarly in the tumors (0.79 ± 0.26 versus 0.64 ± 0.12; paired t test, $p = 0.36$). (E) The percentage of cells at different distances from the nearest blood vessel in the corresponding images in (D) (Two-way ANOVA, $p > 0.9$). (F) The average distance of CTLs from nearest blood vessel (two-way ANOVA, $p = 0.57$, $n = 6$). Bars denote ± SEM. CTL populations distributed similarly in relation to the blood vessels.

See also Movie S1.

To date, no one has reported the in vivo anti-tumor activity of CTLs cultured under hypoxic conditions. We showed here that mice treated with hypoxic CTLs benefit from significantly better tumor rejection (Figure 4A), and as a result survive significantly longer (Figure 4B). The magnitude and robustness of the result indicate a valuable biological process that may be applied clinically.

Corresponding findings were obtained using effector T cells genetically or pharmacologically manipulated to mimic hypoxic cells. Thus, VHL-deficient OT-I CD8 T cells better rejected B16-OVA tumors (Doedens et al., 2013). Likewise, CD4 cells treated with an inhibitor of the O_2-sensing PHD enzymes showed better rejection of a B16 tumor (Clever et al., 2016). This effect seems to employ a distinct mechanism, as treated CD4 cells exhibited a Th1-like phenotype and contained fewer regulatory cells.

In contrast, inhibiting glycolysis in CD8 T cells promoted memory cell formation at the expense of effector cells (Sukumar et al., 2013). Surprisingly, treating tumor-bearing mice with such cells proved more effective, adding complexity to the putative pathways engaged in increased cytotoxicity under hypoxia.

Although hypoxia seems to benefit T cell effector function in mice, it is not clear if this also applies in humans, as hypoxia sometimes affects human T cells differently (Bollinger et al., 2014). While some findings recapitulated ours in mice, showing that hypoxia reduced proliferation (Larbi et al., 2010) and enhanced cytotoxicity (Xu et al., 2016) in human T cells, others showed that hypoxia reduced cell survival (Larbi et al., 2010; Gaber et al., 2013) and the expression of perforin and granzyme B (Xu et al., 2016). It is still unclear whether inter-species differences underlie these discrepancies. To obtain more relevant results, future pre-clinical studies should study TILs produced from cancer patients and reactivated under hypoxic conditions. These should be tested against primary human melanoma cells in vitro and against xenografts in mice.

We believe that using hypoxia to enhance CD8 T cell activity has clinical potential: making small adjustments to present protocols may improve adoptive cell immunotherapies. Still, better understanding of the effects of hypoxia is needed to sensibly manipulate CD8 T cell metabolism, survival, and antitumor effector function.

EXPERIMENTAL PROCEDURES

Details can be found in the Supplemental Experimental Procedures.

Mouse Strains

Mice were maintained in a specific pathogen-free facility under conditions approved by the institutional animal care and use committee (protocol number 15250115-2) at the Weizmann Institute of Science. C57BL/6 mice were purchased from Harlan laboratories Ein Kerem Breeding Farm Jerusalem. Several transgenic mouse strains were purchased from the Jackson Laboratories and bred locally, including mice expressing the ECFP under the β-actin promoter B6.129(ICR)-Tg(ACTB-ECFP)CK6Nagy/J, mice expressing the EGFP under the human ubiquitin C promoter (Jackson laboratory C57BL/6-Tg(UBC-EGFP)30Scha/J), and TCR-transgenic OT-I mice whose TCR recognized the peptide SIINFEKL from the OVA protein presented by on H2Kb MHC-I molecules C57BL/6-Tg(TcraTcrb)1100Mjb/J. Male mice at the age of 7–13 weeks were used in the experiments.

T Cell Culture

Splenocytes from OVA-specific OT-I mice were activated using SIINFEKL peptide (50 μg/ml), supplemented by IL-2 (100 U/ml) 3 days later. At the fifth day of culture, the culture contained >95% OT-I CTLs. Hypoxic CTLs were activated and cultured in 1% O_2 balanced with N_2 in a tri-gas incubator, whereas normoxic CTLs were kept at 20% O_2 conditions. During culture, cell death and proliferation were assessed using DAPI and (5-(and 6) carboxyfluorescein diacetate succinimidyl ester) (CFSE), respectively.

LAMP-1 Lysosomal Fusion Assay

After 5 days of culture, CTLs were placed with B16-OVA target cells for 2.5 hr at a ratio of 2.5:1 in the presence of anti-LAMP-1 for analysis using flow cytometry. T cell activation with PMA and ionomycin was used as a positive control.

Fluorescence-Based In Vitro Killing Assay

In-vitro-generated OT-I CTLs were co-incubated with B16-OVA-tdTomato target cells or with control tdTomato cells at different E:T ratios for 2.5 hr at 1% or 20% O_2. Loss of the fluorescent content indicated target cell death.

Tumor Imaging

B16-OVA-tdTomato (2×10^6) tumor cells were injected intradermally into the flank skin of C57BL/6 mice. After 7 days, tumor-bearing mice were co-injected i.v. with differentially tagged hypoxic and normoxic OT-I CTLs at 1×10^7 each. Mice were used at the indicated days for the two following procedures.

Intravital Two-Photon Imaging of Tumors

Two days after CTL injection, mice were anesthetized, and a skin flap was separated from the abdominal muscle to expose the intradermal tumor, which was then covered with a glass-bottom imaging chamber. The mice were kept at a core temperature of 37°C and were ventilated throughout the imaging process. CPF, GFP, tdTomato, and Qtracer-655 Qdots were imaged together using a pulsed InfraRed (IR) laser tuned to 890 nm.

Immunohistochemistry of Tumor Sections

Two or five days after CTL injection, mice were sacrificed and tumors were excised, sectioned, stained for CD31, GFP, CD45.1, and Hoechst and imaged using a confocal microscope.

Tumor Rejection Assay

B16-OVA-tdTomato (2×10^6) tumor cells were injected intradermally into the flank skin of C57BL/6 mice. Seven days after tumor inoculation, CTLs (5×10^6 cells) that were cultured in vitro for 5 days in hypoxia or normoxia were i.v. injected to three recipient mouse groups: controls (no CTLs treatment), mice treated with hypoxic CTLs, and mice treated with normoxic CTLs. The tumor area was measured every 2 days for 16 days using a caliper.

Statistical Analysis

Statistical tests were performed using GraphPad Prism or SAS StatView software. For simple comparisons, a two-tailed Student's t test was used. Multiple comparisons were performed by two-way ANOVA or two-way repeated-measures ANOVA followed by Tukey's multiple comparisons test. Analysis of differential cell velocity was performed using ANCOVA. Differential survival was assessed using the log rank test. Significance was set at $p < 0.05$. Data in figures are shown as mean ± SEM.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, one table, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.08.071.

AUTHOR CONTRIBUTIONS

Y.G. planned and executed the experiments and wrote the manuscript; T.F. planned and performed the tumor rejection and intravital two-photon experiments; T.S. performed the RNA sequencing data analysis; T.-M.S. performed

the CyTOF analysis; Z.P. performed the imaging flow cytometry analysis; and G.S. conceived and supervised the project and wrote the manuscript. All the authors reviewed the manuscript.

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