Glycolysis determines dichotomous regulation of T cell subsets in hypoxia

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Hypoxia occurs in many pathological conditions, including chronic inflammation and tumors, and is considered to be an inhibitor of T cell function. However, robust T cell responses occur at many hypoxic inflammatory sites, suggesting that functions of some subsets are stimulated under low oxygen conditions. Here, we investigated how hypoxic conditions influence human T cell functions and found that, in contrast to naive and central memory T cells (Tn and Tcm), hypoxia enhances the proliferation, viability, and cytotoxic action of effector memory T cells (Tem). Enhanced TEM expansion in hypoxia corresponded to high hypoxia-inducible factor 1α (HIF1α) expression and glycolytic activity compared with that observed in Tn and Tcm. We determined that the glycolytic enzyme GAPDH negatively regulates HIF1α expression by binding to adenylate-uridylate-rich elements in the 3′-UTR region of HIF1α mRNA in glycolytically inactive Tn and Tcm. Conversely, active glycolysis with decreased GAPDH availability in Tem resulted in elevated HIF1α expression. Furthermore, GAPDH overexpression reduced HIF1α expression and impaired proliferation and survival of T cells in hypoxia, indicating that high glycolytic metabolism drives increases in HIF1α to enhance Tem function during hypoxia. This work demonstrates that glycolytic metabolism regulates the translation of HIF1α to determine T cell responses to hypoxia and implicates GAPDH as a potential mechanism for controlling T cell function in peripheral tissue.

Introduction

T cells experience a broad range of O2 tension in vivo, varying from 13% in peripheral arterial blood (1) to 5% in normal tissues with increased distance from blood vessels (2), to less than 2% in chronically inflamed tissues (3) and solid tumor microenvironments (4). Local O2 tension is an environmental factor that affects T cell function (5, 6). In particular, low O2 tension (1% O2; hypoxia) impairs the proliferation and viability of human peripheral blood T cells in vitro and the activation of mouse splenic T cells in vivo (7, 8). However, the inhibitory effects of hypoxia is thought to have on T cells are inconsistent with the robust expansion of T cells in many hypoxic inflammatory sites (9–11). Recent studies demonstrate that hypoxia-related pathways can facilitate the differentiation of CD8+ cytotoxic T lymphocytes (CTLs) (12) and clearance of chronic viral infection and tumors (13). These new findings suggest that low O2 tension in tissues can be inhibitory for certain T cell subsets, but stimulatory for other T cell subsets that must be functional in hypoxic inflamed or neoplastic tissues. For instance, circulating T cells and those located in secondary lymphoid organs are mainly naive cells (Tn) and central memory T cells (Tcm), while T cells in peripheral tissues in pathological conditions such as inflammation or tumors are predominantly effector memory T cells (Tem) and effector T cells (Te) (14). Whether the low O2 tension has distinct effects on T cell memory subsets that are differentially located within tissues remains unknown.

Hypoxia-inducible factors (HIFs) are transcription factors that facilitate cellular responses to hypoxia. HIFs are heterodimeric proteins consisting of α (HIF1α, HIF2α, and HIF3α) and β (HIF1β) subunits. While the β subunit is constitutively expressed, the α subunits are dynamically regulated by various mechanisms (2). In normoxia, the α subunits undergo O2-dependent hydroxylation and proteosomal degradation via the E3 ligase von Hippel Lindau (VHL) complex (15). By contrast, α subunits are stabilized under conditions of low O2 tension (15) or genetic deletion of VHL (16). In T cells, HIF1α expression is also induced both transcriptionally and translationally by T cell receptor (TCR) stimulation (16, 17), which drives glycolytic metabolism by transcriptionally activating enzymes involved in glycolysis (12, 18). Increased glycolysis mediated by HIF1 resembles the “metabolic switch” occurring during T cell activation (17, 19): while resting T cells primarily use oxidative phosphorylation (OXPHOS) to generate ATP, activated T cells reprogram the metabolism to favor glycolysis to fulfill the bioenergetic and biosynthetic requirement for rapid proliferation, even when oxygen is available for OXPHOS (17). Because the HIF1 pathway is active during T cell stimulation (13, 17, 18), the hypoxia/HIF1-facilitated glycolysis may converge in activated T cells with endogenous glycolytic induction to synergistically support the proliferative and effector functions.

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Here, we show that TEM have immediate superior proliferation and effector function under hypoxic conditions, while TN and TCM are inhibited under these conditions. This distinct pattern of hypoxia response is attributed to the differential expression of HIF1α and related glycolytic activity in T cell memory subsets. Furthermore, the differential HIF1α expression is linked to a novel mechanism of translational regulation by the glycolytic enzyme GAPDH in T cells.

Results

Proliferation and survival of human TEM are enhanced in hypoxia.

Previous studies have suggested an inhibitory role of hypoxia in activated human T cells (20). We confirmed that freshly isolated peripheral blood T cells (PB-Ts) have impaired proliferation and viability upon activation with OKT3/α-CD28 Abs in hypoxia (1% O2) as compared with that observed in normoxia (20% O2) (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI85834DS1). However, unselected PB-Ts predominantly consist of T cells with a TNI (CD45RA-CCR7+) or TCM (CD45RA CCR7−) phenotype, with the TEM (CD45RA CCR7−) subset underrepresented in PB-Ts (Supplemental Figure 2, A and B). Additionally, because TNI and TCM show higher intrinsic proliferative potential upon antigen stimulation than do TEM (14), any specific effect of hypoxia in TEM could be underrepresented when analyzing unFractionated PB-Ts, given the predominance of TNI and TCM. Therefore, we aimed to specifically study TEM upon activation in hypoxia. To acquire sufficient numbers of TEM, we activated human peripheral blood mononuclear cells (PBMCs) with OKT3/α-CD28 Abs and cultured them with IL-2 for 12 days. These ex vivo–expanded T cells (TEXP) were enriched in CD45RA−CCR7− cells (62.8% ± 3.1% vs. 12.4% ± 2.5% in PB-Ts) and were phenotypically similar to the TEM detected in PB-Ts, based on various T cell differentiation markers (CD25, CD27, CD28, CD45RO, and CD62L) (Supplemental Figure 2).

We activated TEM with OKT3/α-CD28 Abs under hypoxic or normoxic conditions and measured proliferation and cell viability 72 hours after stimulation. In sharp contrast to unselected PB-Ts, cell counts of TEM were higher in hypoxia than in normoxia (Figure 1A) as a result of both enhanced cell division (Figure 1, B–D) and reduced cell apoptosis (Figure 1E). In hypoxia, TEM showed lower expression of the death receptor CD95/Fas (Figure 1, F and G) and reduced caspase activity (Figure 1H), which further suggests a reduced potential for induction of apoptosis. In addition, mRNA expression of the antiapoptotic genes BCL2 and BNIP3 was significantly higher in TEM activated in hypoxia, while the expression of FAS mRNA decreased (Supplemental Figure 3), correlating with the reduced apoptosis of TEM in hypoxia. TEM showed a comparable percentage of CD4+ and CD8+ T cells when activated in hypoxia or normoxia (Supplemental Figure 4A). Furthermore, both CD4+ and CD8+ TEM dis-
played enhanced proliferation and survival in hypoxia compared with that observed in normoxia (Supplemental Figure 4, B–D). These results suggest that the dichotomous influence of hypoxia on PB-Ts versus that on TEXP was not due to the disparities in CD4 and CD8 composition. Finally, hypoxia did not affect the production of IL-2, IFN-γ, or TNF-α nor did it affect the expression of the IL-2 receptor CD25 or the early T cell activation marker CD69 (Supplemental Figure 4, E and F). Overall, these data suggest that TEXP show a unique pattern of response upon activation in hypoxia.

Because TEXP are generated through ex vivo culture, which may alter the native characteristics of these cells, even if they are phenotypically similar to the TEM detected in the PB-Ts, we sorted TN, TCM, and TEM from PB-Ts on the basis of the expression of CD45RA and CCR7 (Supplemental Figure 5 and ref. 21). These cells were then stimulated with OKT3/a-CD28 Abs in hypoxia or normoxia for 72 hours after activation. (C) Quantitative analysis of the CFSE dilution in B. The division index was calculated using Flowjo software. n = 4. **P = 0.003 for TN and P = 0.008 for TCM and *P = 0.018, 2-way ANOVA with Bonferroni’s post-hoc analysis. (D) Expression of Ki67 in TN, TCM, and TEM 72 hours after activation. n = 3. (E) Percentage of live TN, TCM, and TEM 72 hours after activation. n = 6. ****P < 0.0001, 2-way ANOVA with Bonferroni’s post-hoc analysis. (F) Expression of CD95/Fas in TN, TCM, and TEM 48 hours after activation. n = 3. **P = 0.0011, 2-way ANOVA with Bonferroni’s post-hoc analysis. Error bars indicate SD. MFI, mean fluorescence intensity.

Cytotoxic function of tumor-specific TEXP is enhanced by hypoxia. Because hypoxia positively regulates the proliferation and survival of TEXP, we speculated that hypoxia might also modulate their cytotoxic function. To confer antigen specificity to TEXP, we transduced activated human T cells with a retroviral vector encoding a GD2-specific chimeric antigen receptor (CAR.GD2-T) (23) and cocultured CAR.GD2-T with GD2+ neuroblastoma tumor cells (LA-N-1) in normoxia or hypoxia for 48 hours. Fewer tumor cells survived when cocultured with CAR.GD2-T in hypoxia than occurred in normoxia. That this was a result of cytotoxicity rather than impaired tumor cell growth in hypoxic conditions was illustrated by coculture of tumor cells with nontransduced T cells (NTs), in which a similar percentage and number of surviving tumor cells were observed in both hypoxic and normoxic conditions at the end of the experiment (Figure 3, A and B). Although TEXP showed enhanced cell expansion in hypoxia at 72 hours upon OKT3/a-CD28 stimulation, after 48 hours of coculture, the number of CAR.GD2-T was similar in hypoxic and normoxic conditions (Figure 3C). To determine whether the observed enhanced antitumor activity in hypoxia is due to altered tumor cell susceptibility to the cytotoxic activity of CAR.GD2-T, we used LA-N-1 cells cultured in hypoxia or normoxia as target cells in cytotoxicity assays performed in normoxia. LA-N-1 cells cultured in hypoxia did not show increased susceptibility to lysis by CAR.GD2-T compared with that observed with LA-N-1 cells growing in normoxic conditions (Figure 3D). In addition, we observed a similar CD4/CD8 composition of CAR.

Figure 2. TEXP in PB-Ts show the same pattern of response to hypoxia as TEXP. FACS-sorted human TEXP, TCM, and TEM from blood samples were activated with OKT3/a-CD28 Abs in normoxia or hypoxia. (A) Cell counts 72 hours after activation. n = 6. ****P < 0.0001 and ***P = 0.0002, paired Student’s t test. (B) CFSE dilution of CFSE-labeled TEXP, TCM, and TEM 72 hours after activation. (C) Percentage of live TEXP, TCM, and TEM 72 hours after activation. n = 6. ****P < 0.0001, 2-way ANOVA with Bonferroni’s post-hoc analysis. Error bars indicate SD. MFI, mean fluorescence intensity.
Tumor-related hypoxia promotes the cycling of tumor-specific T_{exp} in vivo. Solid tumors are well recognized as hypoxic tissues, with O_2 tension ranging from 0% to 4%, depending on the distance from the blood vessel (4, 24, 25). Previous studies have indicated that tumor-infiltrating T cells are present in both O_2-rich and O_2-poor areas of tumor (26, 27). We thus used xenograft tumor models to determine whether low O_2 tension within the tumor affects the proliferation of TEM in vivo. We engrafted NOD/SCID/γc–/– (NSG) mice s.c. with neuroblastoma tumor cells. After engraftment, we intra-tumorally inoculated the mice with CAR.GD2-T or control TEXP expressing a nontumor-targeting CAR specific for the CD19 antigen (CAR.CD19-T) (Figure 4A). The hypoxia-labeling chemical pimonidazole (hydroxyprobe [HP1]) (28, 29) specifically labeled in vivo CAR.GD2-T located in either hypoxic (O_2 <1.3% HP1+) or normoxic (O_2 >1.3% HP1–) tumor areas (ref. 28 and Figure 4B). Strikingly, as assessed by expression of the mitotic marker Ki67, CAR.GD2-T were characterized by greater proliferation within O_2-poor tumor areas (HP1+) when compared with those in O_2-rich areas (Supplemental Figure 6A), while CAR.GD2-T displayed superior cytotoxicity against LA-N-1 cells in hypoxic conditions compared with that observed in normoxic conditions in a 51Cr release assay (Supplemental Figure 6B). Collectively, these data suggest an increase in the per-cell cytotoxicity of CAR.GD2-T cells in hypoxia.

Unlike mouse CD8+ CTLs, which express increased levels of lytic enzymes in hypoxia (12, 13), the expression of perforin and granzyme B in activated human CAR.GD2-T remained unchanged in hypoxia (Figure 3E). However, the superior antitumor activity of CAR.GD2-T in hypoxia correlated with enhanced degranulation of CD8+ CAR.GD2-T upon specific CAR.GD2 activation (Figure 3F). Stimulation of CAR.GD2-T in hypoxia with OKT3/α-CD28 Abs also induced more degranulation (Figure 3G), indicating that the enhanced degranulation in hypoxia is not due to an intrinsic property of the CAR.GD2 molecule but is recapitulated upon TCR stimulation. Collectively, our data indicate that the cytotoxic function of T_{exp} is enhanced under hypoxic conditions.

Figure 3. The cytotoxic function of CAR-redirected T_{exp} is superior in hypoxia. CAR.GD2-T were cocultured with LA-N-1 GFP+ neuroblastoma cells at a 1:4 ratio for 48 hours in normoxia or hypoxia. NTs were used as negative cytotoxic controls. (A–C) The percentage and number of GFP+CD3+ tumor cells and number of GFP CD3- CAR.GD2-T were determined by flow cytometry after 48 hours of coculture. The percentages in the upper left and bottom right quadrants in A indicate the percentage of T cells and tumor cells, respectively. n = 8 (A, left) and n = 4 (B and C). ****P < 0.0001 (A) and **P = 0.004 (B), 2-way ANOVA with Bonferroni’s post-hoc analysis. (D) LA-N-1 GFP+ cells were precultured in hypoxia or normoxia for 48 hours, labeled with 51Cr, and cocultured with CAR.GD2-T in normoxia at E/T ratios of 50:1, 25:1, 10:1, and 5:1. The cytotoxic activity of CAR.GD2-T was determined after 4 hours of coculture. n = 3. (E) Expression of perforin and granzyme B in CAR.GD2-T stimulated with the anti-CAR idiotype Ab (1A7) for 24 or 48 hours in normoxia or hypoxia. n = 3. (F and G) Surface expression of CD107a/b in CAR.GD2-T stimulated with either 1A7 Ab (F) or OKT3/α-CD28 (G) Abs for 6 hours. The percentages in the upper right and bottom right quadrants in F indicate the percentage of degranulated CD8 and CD4 T cells, respectively. n = 3. **P = 0.0059 and ****P < 0.0001, 2-way ANOVA with Bonferroni’s post-hoc analysis. Error bars indicate SD.
We found that HIF1α was required and sufficient for promoting the proliferation of TEXP (Supplemental Figure 7, A–F), which highlights the critical role of HIF1α in TEXP under hypoxic conditions. Previous studies have also hinted at a role of HIF2α in regulating TE functions (13). Though we found that EPAS1 (which encodes for HIF2α) mRNA expression was higher in TEXP cells than in PB-Ts, it was expressed at very low levels in both cell types (10–6-fold expression compared with ACTB mRNA) (Supplemental Figure 7G), and we were unable to detect HIF2α protein expression in either TEXP or PB-Ts by Western blotting (data not shown). Thus, we focused our study on HIF1α expression for subsequent analysis.

HIF1α accumulates in normal cells in hypoxia but is also constitutively overexpressed in some tumor cells in normoxia (25, 30), which facilitates aerobic glycolytic metabolism (the Warburg effect) (25). We postulated that HIF1α may also be overexpressed in TEXP and facilitate their metabolism and survival in hypoxia. Accordingly, we found that while HIF1α accumulated in PB-Ts after antigen stimulation in hypoxia, TEXP showed detectable gene transfer. We found that HIF1α was required and sufficient for promoting the proliferation of TEXP (Supplemental Figure 7, A–F), which highlights the critical role of HIF1α in TEXP under hypoxic conditions. Previous studies have also hinted at a role of HIF2α in regulating TEXP functions (13). Though we found that EPAS1 (which encodes for HIF2α) mRNA expression was higher in TEXP cells than in PB-Ts, it was expressed at very low levels in both cell types (10–6-fold expression compared with ACTB mRNA) (Supplemental Figure 7G), and we were unable to detect HIF2α protein expression in either TEXP or PB-Ts by Western blotting (data not shown). Thus, we focused our study on HIF1α expression for subsequent analysis.
HIF1α expression in hypoxia without TCR/CD28 stimulation, and expression increased after stimulation in either hypoxia or normoxia (Figure 5A). Since we could not acquire a sufficient number of freshly isolated TEM for immunoblot analysis via FACS sorting (Supplemental Figure 5), we compared HIF1α expression in FACS-sorted TN, TCM, and TEM by quantitating intracellular HIF1α staining and flow cytometry. Using this approach, HIF1α protein expression dynamics mirrored the responses to the hypoxia and antigen receptor stimulation observed in TEXP (Supplemental Figure 8, A and B). Notably, HIF1α only accumulated after activation in hypoxia in TTEM and TCM, while TTEM showed elevated HIF1α expression in hypoxia without stimulation, which was further increased after activation, as observed in TEXP (Supplemental Figure 8, C and D). These data suggest that the differential HIF1α expression in TEXP and PB-Ts represents a bona fide difference between TN/TCM and TEM subsets.

Consistent with HIF1α expression, the HIF1-targeted glycolytic genes GLUT1 and LDHA were expressed at higher levels in TEXP compared with levels detected in PB-Ts (Figure 5, B and C). The higher expression of these genes in TEXP was associated with more glucose uptake (Figure 5D) and lactate secretion (Figure 5E). Notably, the glycolytic activity in unstimulated TEXP was almost comparable to that of activated PB-Ts, suggesting that TEM are imprinted to preferentially...
The high glycolytic activity of TEXP is critical for their functionality in hypoxia, given that partial inhibition of glycolysis in TEXP using low doses of the competitive glycolytic inhibitor 2-deoxyglucose (2-DG) was sufficient to abolish the enhanced viability and proliferation of TEXP in hypoxia (Figure 5, F–H, and Supplemental Figure 9, A–C). The mitochondrial inhibitor oligomycin, however, did not affect the proliferation or survival of TEXP in either normoxic or hypoxic conditions, which suggests that, like tumor cells (25), TEXP may have intrinsically lower or dispensable mitochondrial functions that allow them to overcome the severe impairment in hypoxia of ATP synthesis from OXPHOS. In contrast, oligomycin suppressed the expansion and viability of PB-Ts in normoxia but did not contribute synergistically to the inhibitory effects of hypoxia (Supplemental Figure 9, D–F). These latter observations suggest that the reduction of PB-T proliferation and survival in hypoxia may be attributed to a mechanism related to the inhibition of mitochondrial function. In summary, our data demonstrate that TEXP express high levels of HIF1α and display a glycolysis-centric metabolism, which is critical for their functionality in hypoxia.

Differential expression of HIF1α in T cell memory subsets is translationally regulated by GAPDH. To mechanistically explain the distinct hypoxia response patterns in T cell memory subsets, we investigated the molecular programs that regulate HIF1α expression in both TEXP and PB-Ts. The steady-state expression of the mRNA transcript encoding HIF1α was comparable between unstimulated TEXP and PB-Ts and was similarly induced after T cell activation (Supplemental Figure 10A). These results excluded a primarily transcriptional regulatory mechanism underlying the differential relative expression of HIF1α protein in PB-Ts and TEXP. Together, these data suggest the possibility that translation of the HIF1A mRNA transcript might be inhibited in PB-Ts. Because the translation of HIF1A in T cells is facilitated by IL-2 signaling and the PI3K/mTOR pathway (12, 33), we evaluated the phosphorylation status of mTOR and its effector S6 kinase in both normoxia and hypoxia in TEXP and PB-Ts, but did not find a significant difference (Supplemental Figure 10C).

Messenger RNAs containing cis-acting adenyate-uridylate-rich elements (AREs) in their 3’-UTRs are often targets of RNA-binding proteins that regulate their stability (34, 35). In situ analysis revealed
7 “AUUUA” pentamer AREs and 3 “UUAUUUAUU” nonamer AREs within the HIF1A 3′-UTR (Supplemental Figure 1A). Many of these AREs are highly evolutionarily conserved among different species (3′-UTR analyzed using the AREsite database; http://nibiru.tbi.univie.ac.at/AREsite2/welcome) (36), suggesting that these elements may be pivotal in HIF1A mRNA regulation. We thus performed a 3′-UTR-based reporter assay to determine whether the HIF1A 3′-UTR alone is adequate to confer translational regulation in T cells. The HIF1A 3′-UTR specifically reduced the reporter expression in PB-Ts but not in TEXP (Figure 6A). Notably, the reduction in reporter expression by HIF1A 3′-UTR in PB-Ts was partially reversed when the AREs were mutated. These data suggest a specific translational regulation of HIF1A mRNA mediated by the AREs in its 3′-UTR in PB-Ts, but not in TEXP. Importantly, we found that the insensitivity to translational suppression by the HIF1A 3′-UTR of TEXP may be attributed to their high glycolytic activity. TEXP with dampened glycolysis by 2-DG showed reduced reporter expression with the HIF1A 3′-UTR compared with control 3′-UTR or the HIF1A 3′-UTR ARE mutant (Supplemental Figure 11B). These data suggest a link between the regulation of HIF1α expression via its 3′-UTR and glycolytic status in T cells.

The glycolytic enzyme GAPDH can function as an RNA-binding protein (37, 38) and can negatively regulate the translation of IL-2 and IFN-γ in activated T cells via specific binding to AREs within the mRNAs encoding these gene products (38, 39). Notably, this translational regulation only occurs in T cells with low glycolytic activity when GAPDH is not engaged in its main function as a glycolytic enzyme (39). Thus, we tested whether GAPDH may also act as a suppressor for HIF1A mRNA translation, preferentially in PB-Ts that have less active glycolysis as compared with TEXP that have high glycolytic activity. We performed RNA IP assays using GAPDH Ab and found a greater than 5-fold enrichment of HIF1A mRNA in the α-GAPDH Ab immunoprecipitate compared with the IgG control immunoprecipitate in PB-Ts (Figure 6B). In contrast, no enrichment of HIF1A mRNA was observed in TEXP. Furthermore, we did not find enrichment of β-actin (ACTB) mRNA in either TEXP or PB-Ts, suggesting that the binding of GAPDH to HIF1A mRNA is specific.

To further demonstrate the critical role of GAPDH in suppressing HIF1α protein expression, we overexpressed GAPDH in TEXP (Supplemental Figure 12, A and B) and found that HIF1α protein, but not mRNA, expression was reduced (Figure 6C, Supplemental Figure 12C). Reduction of HIF1α in TEXP by GAPDH overexpression did not impair the differentiation of TEXP (Supplemental Figure 12D), but caused a reduction of the glycolytic activity, cell expansion, survival, and proliferation of TEXP, in hypoxia (Figure 6D and Supplemental Figure 12, E and F). Notably, using the in vivo model we developed (shown in Figure 4), we found that CAR.GD2-T in which we overexpressed GAPDH showed significantly reduced proliferation in hypoxic tumor areas compared with that seen in mock CAR.GD2-T (Supplemental Figure 12G). GAPDH is thus a novel regulator for HIF1A mRNA translation and links HIF1α protein expression to the glycolytic activity in T cell memory subsets.

**Discussion**

T cell activation relies not only on TCR and costimulatory signals but also on environmental inputs including O2 availability. Here, we demonstrate dichotomous roles of hypoxia on human T cell subsets. Specifically, while Tα and TCM are suppressed in hypoxia, TEXP show elevated proliferation, survival, and cytotoxic activity. Therefore, hypoxia may serve as both a tolerance mechanism for Tα and TCM in secondary lymphoid organs by preventing excessive proliferation and a license mechanism for TEXP in inflamed or malignant tissues by facilitating proliferation and effector functions. Tα, TCM, and TEXP have highly specified functional distinctions, and thus it is intuitive that low O2 supply may be perceived differently by these subsets. In particular, the assumption that hypoxia impairs T-cell–mediated immune responses (40, 41) does not take into account the fact that hypoxia is present in almost all pathological conditions in which immune cells must execute essential protective functions. We have found that TEXP, but not Tα or TCM, react to low O2 supply by proliferating. Of note, although TEXP have a proliferative potential inferior to that of Tα and TCM upon stimulation in normoxia (14), this deficiency is corrected in hypoxia, in which TEXP divisions are almost comparable to those of Tα and TCM in normoxia. These findings suggest that TEXP are not intrinsically restricted in their proliferative capacity compared with Tα or TCM but require additional environmental input to fully exploit it. Alternatively, the O2-rich condition may pose an inhibitory effect on TEXP possibly through the generation of ROS, which is relieved in hypoxia (25, 42). Further studies are needed to elucidate these mechanisms.

The enhanced functions of TEXP in hypoxia correspond to a “hypoxia adaptation signature” with higher expression of the master hypoxia transcriptional regulator HIF1α and increased activity and dependency of aerobic glycolysis in TEXP compared with that in Tα and TCM. TEXP are insensitive to mitochondrial inhibition, while a reduction of glycolytic activity abrogates their functional advantage in hypoxia. Of note, neutrophils and macrophages, which are immune cells specialized to function within hypoxic inflammatory sites, show similar glycolysis–dominant metabolic profiles (6, 43), suggesting that the observed hypoxia adaptation signature in TEXP may represent an evolutionarily conserved pathway for cells that function in low-O2 conditions. This is further supported by findings in cancer cells that exclusively rely on aerobic glycolysis for their growth (25) and the overexpression of HIF1α in many solid tumors, even in the absence of related genetic mutations (2, 30).

How and whether HIF1α contributes to the regulation of T cell responses remains controversial. Earlier studies demonstrated that targeted deletion of HIF1α in T cells enhanced their proliferation and antibacterial responses, suggesting a negative regulatory function of HIF1α in T cells that might protect tissues from immune damage (41). In contrast, recent findings suggest that HIF1α is critical for the differentiation and function of Tα (12, 13). These discrepancies can be attributed to differences in infection models and uncharacterized roles of HIF1α during the development of T cells in HIF1α-deleted mice. Our data in human T cells are consistent with those from more recent studies in mice showing that HIF1α and hypoxia promote TEXP functions (12, 13). This functional correlation is further supported by the mechanistic link we have identified between HIF1α expression and glycolysis. We discovered that HIF1α can be regulated at the translational level by the glycolytic enzyme GAPDH. In Tα and TCM, GAPDH is disengaged from the glycolytic pathway, since these cells are characterized by low glycolytic activity, and GAPDH can bind to the AREs in the 3′-UTR of HIF1A mRNA, suppressing its translation. In contrast, GAPDH binding is absent in TEXP, which have enhanced glycolytic
activity, and GAPDH is fully engaged in glycolysis. This role of
GAPDH mimics the mechanism that was previously observed for
IFNG mRNA, which is also regulated at the translational level by
GAPDH in a glycolysis-related manner (39). Unfortunately, we
could not perform RNA IP experiments using freshly isolated TEXP
because of the scarceness of this subset in the peripheral blood,
and thus we cannot formally prove that GAPDH binds to HIF1A
mRNA in TEXP. Although we also acknowledge that TEXP cannot be
considered identical to TEM freshly isolated from the peripheral
blood, we showed that both TEM and TEXP have similar upregulation
of HIF1α protein expression and phenotypic behavior in hypoxia.
It is also likely that additional mechanisms may also contribute to
the enhanced expression of HIF1α in TEM. Our findings also suggest
a positive feedback loop between HIF1α expression and glycolysis
in T cells. While the inhibition of HIF1α expression by GAPDH is
relieved upon the elevation of glycolytic activity, HIF1α transacti-
vates its target genes that further facilitate glycolysis. When and
how this loop is initiated during T cell differentiation remains to
be investigated. Previous studies showed that Myc mediates a gly-
colytic switch upon activation in TN (17). It is thus possible that the
effect of Myc is still persisting in T cells differentiating in culture
upon stimulation and this could partially explain the glycolytic
activity of TEM in steady-state conditions under normoxia.

We believe our experiments using adoptively transferred
tumor-specific T cells also provide significant insight to the
field of cancer immunotherapy. The microenvironment in solid
tumors is hypoxic due to dysfunctional angiogenesis (4, 25). The
“hypoxia adaptation signature” we found in TEM can be potentially
exploited through genetic manipulations in subsets of tumor-spe-
cific T cells to enhance their functions in hypoxic tumor sites. On
the other hand, the tumor microenvironment is also deprived of
glucose and other nutrients because of the high metabolic rate of
tumor cells (44). T cells must compete with tumor cells for glucose
to support their effector functions and proliferation in hypoxia,
and T cells in metabolite-restricted conditions express high levels
of the inhibitory receptor programmed death 1 (PD1) and produce
less IFN-γ upon activation (39); thus, strategies aimed at enhanc-
ing the capacity of T cells to compete for glucose may enhance their
antitumor effects.

Overall, we believe our findings redefine the role of hypoxia
in regulating T cell responses, especially in pathological periph-
eral tissues. The translational regulation of HIF1α by the glycolytic
enzyme GAPDH represents a novel mechanism in the control of
T cell functions via cellular metabolism.

Methods

Cell line

The CHLA-255 neuroblastoma cell line was provided by L.S. Metelitsa
of Baylor College of Medicine (45) and was maintained in IMDM
(Gibco) supplemented with 10% FBS (HyClone) and 2 mM Glutamax
(Invitrogen). The LA-N-1 neuroblastoma cell line was obtained from
M. Brenner at Baylor College of Medicine. The LA-N-1 cell line and
EBV-transformed lymphoblastoid cell lines (LLCs) were cultured in
RPMI 1640 (HyClone) supplemented with 10% FBS (HyClone) and
2 mM Glutamax (Invitrogen). All cell lines were routinely tested for
mycoplasma and for surface expression of target antigens.

Isolation of human peripheral blood T cells and expansion of TEM
Human PBMCs were obtained from healthy volunteer donors (Gulf
Coast Regional Blood Center) by Ficoll, and peripheral blood T cells
were isolated by magnetic sorting using a pan-T cell selection kit from
Miltenyi Biotec. TEM were expanded by stimulation with plate-bound
OKT3 (1 ng/ml) and CD28 Abs (BD Biosciences) and then cultured in
media containing 45% Click’s media (Irvine Scientific); 45% RPMI
1640 (HyClone); 10% FBS (HyClone); 1% L-glutamine (Invitrogen);
and 100 U/ml IL-2 (Teceluekin) for 10 to 12 days. For CAR-modified
TEM, stimulated PBMCs were transduced with a γ retroviral vector
encoding a second-generation (CD28) CAR targeting the GD2 antigen
(CAR.GD2) and maintained in 50 U/ml IL-2 for 10 days.

Cell sorting

For isolation of TEM, TEM, and TEXP subsets, peripheral blood T cells
were labeled with FITC-conjugated anti-CCR7 (R&D Systems) and
APC-conjugated anti-CD45RA Abs (BD Biosciences). CCR7+CD45RA−
(naïve), CCR7−CD45RA+ (central memory), and CCR7−CD45RA−
effector memory subsets were then sorted by FACS. The purity of
sorted subsets was greater than 95%.

In vitro hypoxia incubation

Most of the hypoxia experiments were conducted using the hypoxia
incubator (Thermo Scientific). For protein analysis and other time-
sensitive experiments, a hypoxia glove box (Coy Lab Products) was
used so that protein extraction was performed under hypoxic condi-
tions. To activate T cells in hypoxia, cells were preincubated in hypoxia
(1% O2) for 24 hours and activated with plate-bound OKT3/α-CD28
Abs. Anti-CAR.GD2 Ab 1A7 was used to activate CAR.GD2-T cells.

Flow cytometry

Cells were immunostained and acquired on a BD FACSCalibur, Fortessa
and Gallios cytometer (Beckman Coulter). The following fluorophore-
conjugated Abs were used for flow cytometric analysis: anti-Ki67 (B56);
anti-CD3 (SK7); anti-CD4 (SK3); anti-CD8 (DX2); anti-
CD25 (2A3); anti-CD27 (M-T271); anti-CD28 (CD28.2); anti-CD45RO
(UCHL-1); anti-CD62L (DREG-56); anti-CD69 (L78); anti-IL-2 (MQ1-
H12); anti-IFN-γ (B27); anti–TNF-α (MAB11); anti-perforin (514-16; BioLegend); and anti-HP1 (4.3.11.3;
Hypoxyprobe). Caspase activity was measured with a Vybrant FAM Poly
Caspase Kit (Life Technologies). Flow cytometric data were analyzed by
FlowJo software, version 9.3.2 (Tree Star).

51Cr release assay

The cytotoxic activity of CAR-T was evaluated in a standard 6-hour
51Cr release assay. Normoxia-cultured or hypoxia-cultured LA-N-1
cells were labeled for 1 hour at 37°C in 5% CO2, with 51Cr (PerkinElmer),
was washed 3 times, and plated at 5 x 10^5 cells per well with CAR-T to give
effector/target (E:T) ratios of 40:1, 20:1, 10:1, and 5:1. After 6 hours
of incubation in normoxia or hypoxia, the supernatant was harvested
and for surface expression of target antigens.
In vivo hypoxia mouse model

Intratumoral injection model. Eight-week-old NSG mice (The Jackson Laboratory) were s.c. engrafted with 1 × 10⁶ CHLA-255 neuroblastoma cells. Twenty-one days after tumor engraftment, 1 × 10⁶ CAR.GD2-T cells or control CAR-T cells (CAR.CD19) were injected intratumorally. Three days after T cell inoculation, mice were injected with 1.5 mg HP1 per mouse for hypoxia labeling. Sixty minutes after HP1 injection, mice were euthanized and tumor tissues collected for FACS analysis.

Systemic injection model. Eight-week-old NSG mice were s.c. engrafted with a 1 × 10⁶ EBV-transformed human B cell line (CD19⁺). Twenty-one days after engraftment, 1 × 10⁶ CAR.CD19-T cells were injected i.v. Subsequent hypoxia labeling by HP1 and FACS analysis was performed as described for the intratumoral injection model.

Staining procedure. Single-cell suspensions were prepared from the explanted tumor tissues and surface stained with Viability Dye (catalog 423101; BioLegend), anti-human CD3, anti-human CD4, anti-human CD45, anti-human CD4, and anti-human CD8. Cells were then fixed, permeabilized, and intracellularly stained with anti-HP1 and anti-Ki67.

Quantitative real-time RT-PCR

Quantitative real-time PCR (qPCR) was performed using TaqMan Gene Expression Master Mix (Life Technologies). TaqMan qPCR primers for HIF1A, SLC2A1 (GLUT1), LDHA, BCL2, BNIP3, BCL2L1, FAS, FASL, TP53, BAX, BAK, BAD, and EPAS1 (HIF2A), and the loading control 18S RNA were designed by Life Technologies.

Immunoblot analysis

Proteins were extracted from more than 2 × 10⁶ T cells using RIPA buffer (Thermo Fisher Scientific) supplemented with protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). Protein samples were separated by electrophoresis using 10% Mini-PROTEAN precast gels (Bio-Rad) and transferred to PVDF membranes (Bio-Rad). Membranes were blocked with 5% BSA in TBS-5% Tween and blotted with primary Abs against HIF1α, LDHA/C (Cell Signaling Technologies), and GLUT1 (catalog ab115730; Abcam) and goat anti-rabbit secondary Abs (Santa Cruz Biotechnology Inc.). β-actin (I3E5) was used as a loading control.

Quantitative HIF1α ELISA

Proteins were extracted from more than 2 × 10⁶ T cells, and HIF1α protein levels were measured directly using quantitative HIF1α ELISA (DYC1935-2; R&D Systems).

3′-UTR GFP reporter assay

HIF1α 3′-UTR or HIF1α 3′-UTR ARE mutant (T-to-G mutations for all predicted AREs) were cloned after a GFP reporter gene driven by a CMV promoter. A 3′-UTR sequence lacking AREs was used as a control. Reporter vectors were introduced into freshly isolated PB-Ts or T⁰ex⁰ via nucleofection (P3 Primary Cell 4D-Nucleofector Kit; Lonza), and GFP expression was determined 8 hours after nucleofection by flow cytometry. As for the 3′-UTR assay on 2-DG-treated T⁰ex⁰, the T⁰ex⁰ were treated with 1 mM 2-DG 48 hours before nucleofection and cultured for 8 hours with 1 mM 2-DG-containing media after nucleofection.

RNA IP

Matched donor PB-Ts or T⁰ex⁰ were UV-crosslinked with 150 mJ/cm² at 254 nm using a UV Crosslinker (Spectroline) and were lysed with lysis buffer containing 100 mm KCl; 5 mm MgCl₂; 10 mm HEPES, pH 7.0; 0.5% Nonidet P-40 detergent supplemented with fresh 1 mm DTT; 1,000 units/ml RNAsin (Promega); and Mini Protease Inhibitor Cocktail (Thermo Fisher Scientific). The post-nuclear cytosolic content was collected and removed for input samples (10%). The remaining lysates were immunoprecipitated with 10 μg anti-GAPDH Abs (Life Technologies) or with control mouse IgG Abs (The Jackson Laboratory) using a Pierce Crosslink IP Kit (Thermo Fisher Scientific). The rest of the immunoprecipitated complexes were digested with 30 μg proteinase K. The immunoprecipitates and input samples were subjected to RNA extraction using TRIzol LS Reagent (Life Technologies). RNA isolates were used for first-strand cDNA synthesis using a SuperScript VILO cDNA Synthesis Kit (Life Technologies). cDNA was used for qPCR quantification, with HIF1α or β-actin as a control. The Ct for each RNA IP sample was normalized to that of total input to account for the differences in sample preparation using the following calculation: ΔCt (normalized) = Ct (sample) – (Ct [input] – log2 [10]).

The ΔCt (normalized) of the GAPDH IP sample was further normalized to the IgG IP sample as follows: ΔΔCt = ΔCt (normalized)GAPDH – ΔCt (normalized)IgG.

The immunoprecipitation fold enrichment above the sample-specific background was calculated as follows: fold enrichment = 2ΔΔCt.

Statistics

A paired, 2-tailed Student’s t test was used to determine statistically significant differences between 2 samples. When multiple comparison analyses were required, statistical significance was evaluated by ANOVA, followed by Bonferroni’s post-hoc analysis. If the data reflected measurement of 1 sample over time or under different conditions, repeated-measures ANOVA was used, followed by Bonferroni’s post-hoc analysis. Graph generation and statistical analyses were performed using GraphPad Prism, version 5.0d (GraphPad Software). A P value of less than 0.05 was considered statistically significant.

Study approval

The present studies in animals were reviewed and approved by the IACUCs of Baylor College of Medicine and the University of North Carolina.

Author contributions

YX, JR, JRN, and GD designed the experiments. YX, AC, MZ, BS, and JRN conducted the experiments and/or analyzed the data. LSM and JTY provided equipment for the hypoxia experiments. YX and GD wrote the manuscript, and all authors edited the manuscript.

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