

Phenotypic switch of CD8⁺ T cells reactivated under hypoxia toward IL-10 secreting, poorly proliferative effector cells

Romain Vuillefroy de Silly, Laura Ducimetière, Céline Yacoub Maroun, Pierre-Yves Dietrich, Madiha Derouazi and Paul R. Walker

Geneva University Hospitals and University of Geneva, Geneva, Switzerland

CD8⁺ T cells controlling pathogens or tumors must function at sites where oxygen tension is frequently low, and never as high as under atmospheric culture conditions. However, T-cell function *in vivo* is generally analyzed indirectly, or is extrapolated from *in vitro* studies under nonphysiologic oxygen tensions. In this study, we delineate the role of physiologic and pathologic oxygen tension *in vitro* during reactivation and differentiation of tumor-specific CD8⁺ T cells. Using CD8⁺ T cells from pmel-1 mice, we observed that the generation of CTLs under 5% O₂, which corresponds to physioxia in lymph nodes, gave rise to a higher effector signature than those generated under atmospheric oxygen fractions (21% O₂). Hypoxia (1% O₂) did not modify cytotoxicity, but decreasing O₂ tensions during CTL and CD8⁺ tumor-infiltrating lymphocyte reactivation dose-dependently decreased proliferation, induced secretion of the immunosuppressive cytokine IL-10, and upregulated the expression of CD137 (4-1BB) and CD25. Overall, our data indicate that oxygen tension is a key regulator of CD8⁺ T-cell function and fate and suggest that IL-10 release may be an unanticipated component of CD8⁺ T cell-mediated immune responses in most *in vivo* microenvironments.

Keywords: CD8⁺ T cell · Oxygen · Hypoxia · IL-10 · T-cell reactivation



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Introduction

In a healthy physiologic context, oxygen tensions in mammalian tissues are tightly regulated, but still show significant variation both between tissues and within the same tissue (Supporting Information Fig. 1A) [1–3]. However, oxygen tensions are far below physiologic values in different pathologic conditions, mostly involving inflammation [4], solid tumors [5, 6], and infections [7].

In contrast to sufficient oxygen supply (i.e. normoxia), oxygen deprivation (i.e. hypoxia) leads to cellular responses involving

stabilization of HIFs (hypoxia-inducible factors), transcription factors that are composed of an inducible α -subunit (i.e. HIF-1 α , HIF-2 α , or HIF-3 α) together with a constitutive β -subunit (i.e. HIF-1 β , HIF-2 β , or HIF-3 β) [8]. Upon stabilization, HIFs transcribe various genes with HIF-responsive elements in their promoter; these include those that increase angiogenesis (e.g. VEGF) and glucose metabolism (e.g. glucose transporters) [9]. Interestingly, HIF-1 α and HIF-2 α can have unique, redundant, or opposing roles [9, 10]. Furthermore, while HIF-1 α is described to be stabilized and active below 2% O₂ in an acute manner, HIF-2 α has been shown to be stable below 5% O₂ in a chronic manner [11, 12]. However, although HIFs are central to many hypoxia responses, certain effects have been shown to be HIF-independent [13–15]. Intriguingly, HIF-1 α can also be involved in immune cell activation

Correspondence: Dr. Paul R. Walker
e-mail: Paul.Walker@hcuge.ch

under atmospheric oxygen fractions (AtO₂; i.e. 21%), as reported for macrophages and T cells [16, 17].

Hypoxia is an essentially *in vivo* microenvironmental phenomenon that is challenging to study and recapitulate *in vitro*. When exploring hypoxia, one has to compare hypoxic to normoxic conditioning. However, most published studies consider AtO₂ as normoxia [1]. This could lead to misinterpretation, as, for example, physiological oxygen fraction (physioxia) found in secondary lymphoid organs is close to 5% O₂ and can induce differential T lymphocyte activation as compared to AtO₂ *in vitro* [18–20]. This highlights the necessity to not only use physioxia as a reference point to study hypoxia impact *in vitro*, but also to analyze cell physiology in general. Physioxia in tissues varies greatly: e.g. skin 1.1%, brain 4.4%, pulmonary alveoli 14.5% (Supporting Information Fig. 1A) [3]. As a baseline for physioxia in healthy tissues, 5% O₂ was previously used for assessing lymphocyte function [19]; we also selected this compromise value for our study.

The impact of hypoxia and HIF has been intensively studied in the context of malignancy or infection and often positively correlated with pathogenesis. In cancer, hypoxia promotes tumor cell stemness, migration, invasiveness, metastasis, and resistance to radiotherapy, chemotherapy, and to CTL-mediated killing [10, 21–25]. In viral infection, hypoxia increases viral replication and gene expression [26]. To date, the impact of hypoxia on T cells implicated in anti-tumor and anti-viral immunity is not extensively described. Most studies focused on CD4⁺ T cells, without consensus regarding the effects. Earlier studies suggested a positive correlation between hypoxia and viability or proliferation [27, 28], more recent studies showed the opposite [29, 30]. Hypoxia was also shown to increase Th17 and Treg polarization [31, 32]. Concerning cytokine production, there are conflicting results for secretion of IL-2, IFN- γ , IL-10, and IL-1 β [29, 30, 33–37]. Overall, despite certain contradictions in this emerging research area, the knowledge base for CD4⁺ T cells is becoming more comprehensive. The impact of hypoxia and oxygen tensions on CD8⁺ T cells is restricted to a few interesting studies [17, 18, 38–42]. However, there are many issues regarding the reactivation of already primed CD8⁺ T cells (hereafter considered as CTLs) that require further investigation. It is particularly important to understand CTL responses to hypoxia because, in contrast to certain Th subsets, these cells must function in the most oxygen-deprived sites in the body (e.g. tumor bed, sites of infection).

In this study, we explored hypoxia impact on effector CD8⁺ T cells reactivated either under 5% (physioxia in many healthy tissues) or 1% O₂ (hypoxia found in inflamed or tumoral sites) (Supporting Information Fig. 1B). Gene expression analysis of reactivated CTLs underlined the importance of using physioxia instead of AtO₂ as a normoxia reference, as many genes described to be modulated under hypoxia were already regulated under physioxia. Our investigations of immune function under oxygen deprivation revealed that whereas hypoxia did not modify CTL killing capacities, it potently induced IL-10 secretion upon reactivation, and dramatically decreased CTL expansion.

Results

Enhanced effector signature of CD8⁺ T cells primed under physioxia

We used CD8⁺ T cells from Pmel-1 mice that carry a transgenic TCR directed against the immunodominant epitope of the tumor/self-antigen gp100, allowing us to obtain a clonal population of naïve CD8⁺ T cells [43]. To model a physiologic effector CD8⁺ T-cell response, we first generated CTLs under physioxia (5% O₂), as found in LNs, and compared them to CTLs generated under AtO₂ (i.e. 21% O₂). We performed this comparison because 21% O₂ is commonly used during *in vitro* cell culture as a normoxic control to study hypoxia impact.

We analyzed the expression of a panel of 42 genes described to be either regulated under hypoxia, to be immune-function related, or to be involved in cell survival (Supporting Information Table 1). Relative to 21% O₂, the physioxia oxygen fraction (5% O₂) that was used during CTL generation upregulated RNA expression of different genes described to be increased by hypoxia. These included *hif1a* exon I.1 (albeit weakly expressed, as judged by Nanostring absolute RNA counts (data not shown)), *hif2a*, *vegfa*, *glut1*, *entpd1* (CD39), *galectin3*, *gelsolin*, and *tnfrsf9* (CD137). However, *hif1a*, *adora2a* (A2AR), and *hif1b* were not modulated, *hif3a* was not expressed, while *nt5e* (CD73) was downregulated [11, 44–50] (Fig. 1A). Several genes positively associated with CD8⁺ T cell survival and expansion were upregulated, including *gata3*, *il2ra*, and *cd28* [51–53]. However, *maf* and *ctla4*, associated with increased apoptosis and immune regulation [54, 55], were also upregulated. It was noteworthy that CTLs generated under physioxia showed a higher effector profile, evidenced by *blimp1* and *fasl* upregulation, and *tcf7* downregulation (Fig. 1B). Consistent with these observations, CTLs generated under normoxia showed increase of the CD44⁺ CD62L⁺ fraction (Fig. 1C) and a decrease of CD127 (IL-7R α) expression (Supporting Information Figs. 1C and 2A). This was not due to the CD8⁺ T-cell clone used, as OT-I CTLs and polyclonal CTLs from C57BL/6 gave the same results (Supporting Information Fig. 2B–D). Accordingly, CTLs generated under physioxia had increased killing capacities as compared to CTLs generated under AtO₂ (Supporting Information Fig. 2E). We next investigated whether hypoxia (1% O₂) could impact CTL lytic capacities. Independently of the oxygen fraction used during CTL generation, hypoxia during the cytotoxicity assay had no effect (Supporting Information Fig. 2E). As this assay implies a short-term hypoxia exposure (4 h), we also preconditioned the CTLs three days before carrying out the killing assay. Interestingly, CTLs generated under AtO₂ and that were preconditioned under hypoxia showed increased cytotoxicity (Fig. 1D). However, CTLs generated under physioxia and that were preconditioned under hypoxia did not augment their killing capacities. GranzymeB (GrB) was preferentially expressed by CTLs that were generated under physioxia as compared to those generated under AtO₂ (Supporting Information Fig. 2F–G), consistent with the increase in effector versus memory differentiation that we

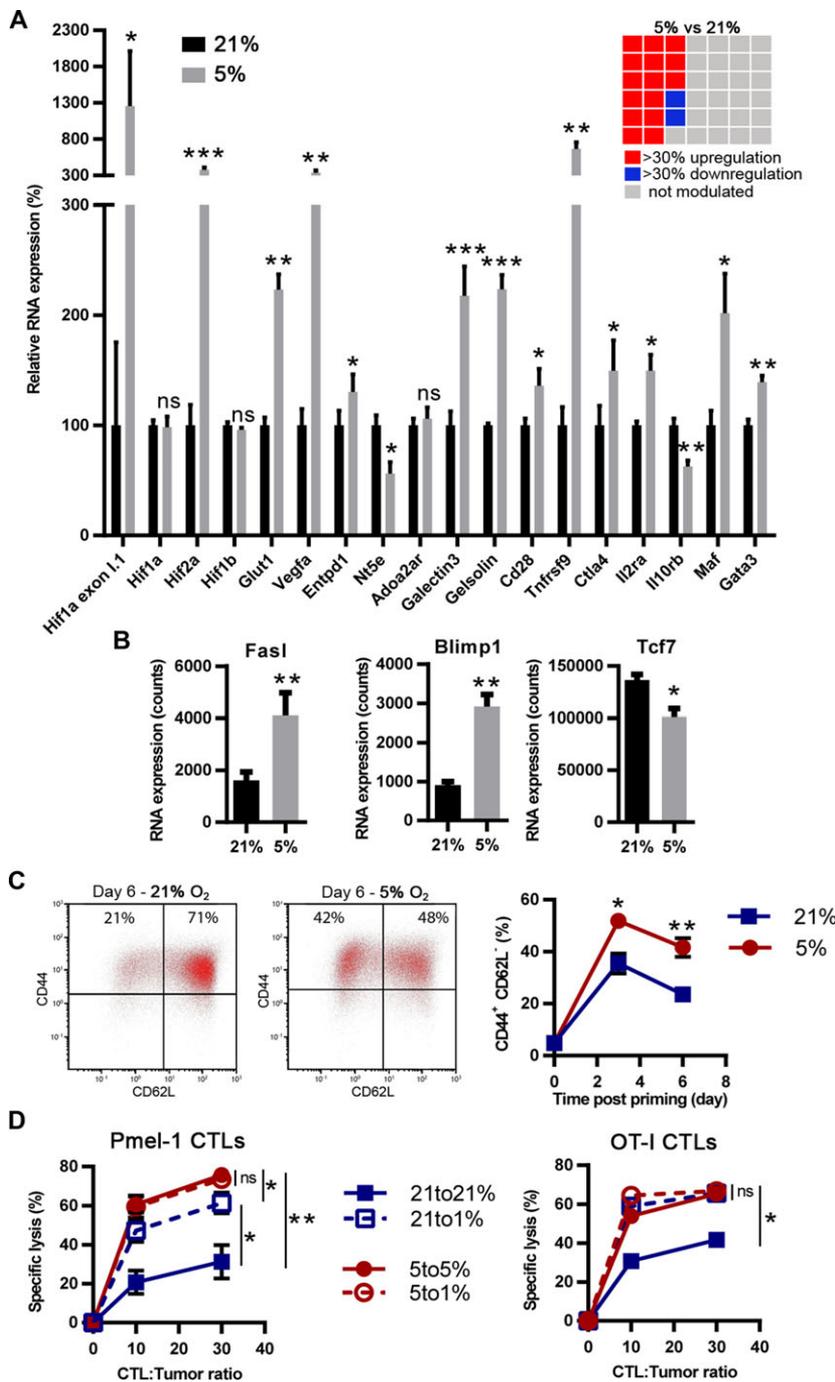


Figure 1. CTLs generated under physioxia have a higher effector profile than those generated under atmospheric oxygen fraction. (A and B) CTLs from Pmel-1 splenocytes were generated under 21% (black bars) or 5% O₂ (gray bars) and were analyzed for RNA expression. Results represent (A) the mean relative gene expression or (B) the RNA absolute count + SEM of five independent experiments (n = 5). The checkerboard represents the 42 genes analyzed (only genes modulated by more than 30% with a p < 0.05 are colored in red or blue). (C and D) CTLs from Pmel-1 splenocytes were generated under O₂ fractions as indicated and were analyzed for marker expression by flow cytometry. (C) CTLs were stained for CD62L and CD44, gated on live CD8⁺ cells, at day 0, day 3, and day 6 postpriming. Dot plots show one representative experiment at day 6. Line graph shows the mean percentage ± SEM CD44⁺ CD62L⁺ cells among CTLs from at least four independent experiments (n ≥ 4). (D) CTLs generated under 21 and 5% were preconditioned for 3 days under 21 (“21 to 21%”), 5 (“5 to 5%”) or 1% (“21 to 1%”) and “5 to 1%”) O₂. The resulting CTLs were assessed for their capacity to kill EL-4 tumor target cells pulsed with hgp100 (Pmel-1 CTLs; n ≥ 4) or Ova peptide (OT-I CTLs; n = 3) for 4 h under the oxygen fraction used for preconditioning. Results show the mean ± SEM specific lysis of tumor cells from at least three independent experiments. ns: not statistically significant, *p < 0.05, **p < 0.01, ***p < 0.001 (A, B: Three-way ANOVA; C, D: Student’s t-test).

observed. However, we did not observe any difference when CTLs were preconditioned or not under hypoxia. Therefore, hypoxia might improve CTL killing capacities of CTLs generated under AtO₂ through a mechanism independent of GrB production.

Hypoxia decreases expansion of reactivated CTLs

We next investigated the impact of oxygen tension on expansion of already primed CD8⁺ T cells. The impact of hypoxia on CD8⁺ T-cell antitumor or antiviral responses is most criti-

cal to examine during CTL reactivation, such as occurs when already primed cells have migrated to nonlymphoid tissues to exert their effector functions in a potentially hypoxic microenvironment. To model this, CTLs generated under physioxia were reactivated either under physioxia (5% O₂) or hypoxia (1% O₂). We also compared these results to CTLs generated and reactivated under AtO₂ (Supporting Information Fig. 1B). As expected, one day postrestimulation HIF-1α stabilization was regulated negatively by oxygen fraction (Supporting Information Fig. 3). However, two days postrestimulation its level decreased under low oxygen fraction while it was highly stabilized under AtO₂; thus

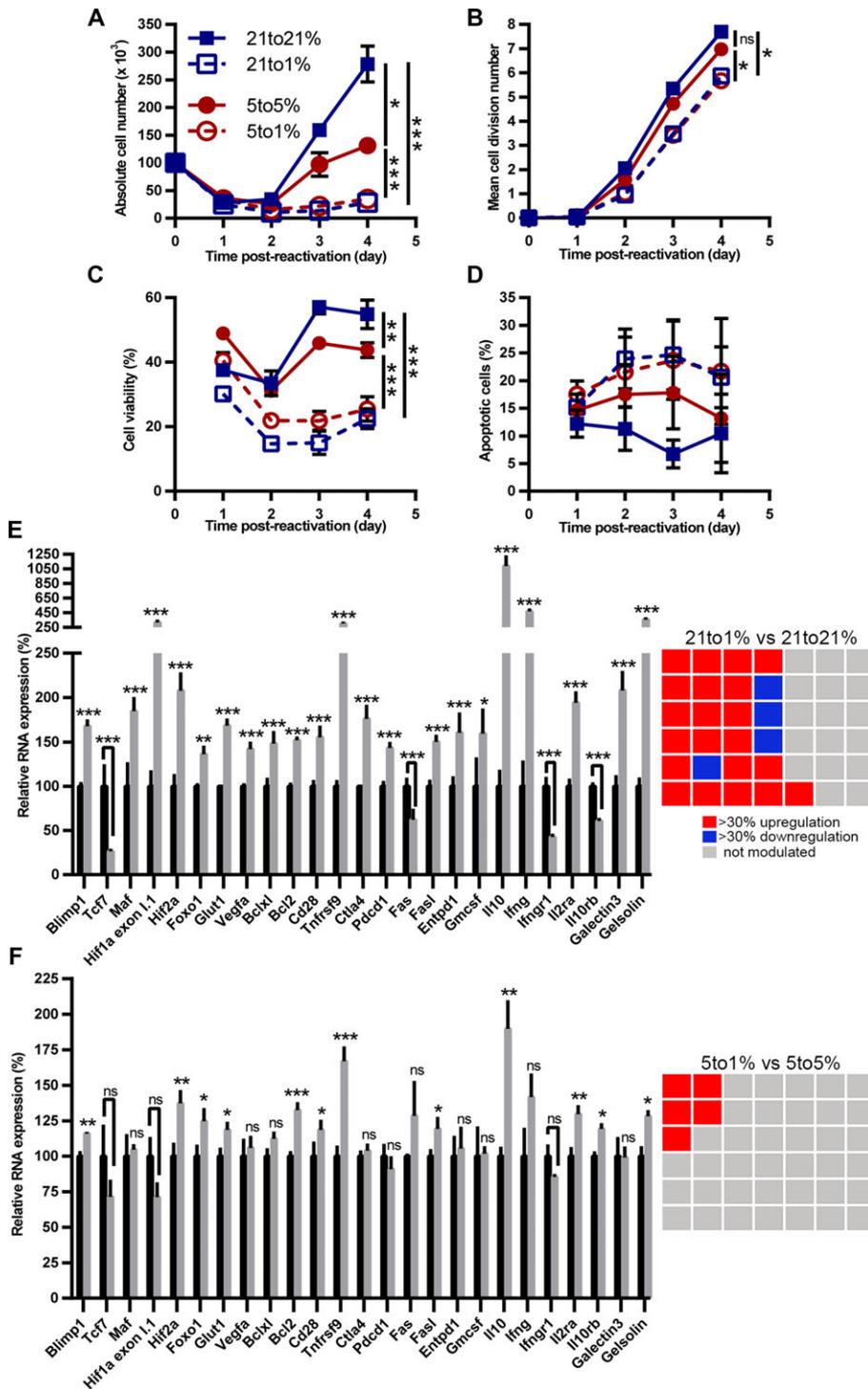


Figure 2. Hypoxia modulates expansion and RNA profile of reactivated CTLs. CTLs generated under 21% (squares) or 5% (circles) O₂ from Pmel-1 splenocytes were reactivated for indicated times under 21% O₂ (closed squares with solid line), 5% O₂ (closed circles with solid line) or 1% O₂ (open squares or open circles with dashed line). Results show (A) mean cell number, (B) mean cell division number, (C) mean cell viability, and (D) mean apoptotic cells \pm SEM out of at least three independent experiments ($n \geq 3$). CTLs generated under (E) 21% or (F) 5% O₂ from Pmel-1 splenocytes were reactivated for two days under indicated oxygen fractions. (E) RNA profile from CTLs reactivated under 21% (black bars) or 1% O₂ (gray bars). (F) RNA profile from CTLs reactivated under 5% (black histograms) or 1% O₂ (gray histograms). Results represent the mean relative gene expression + SEM out of four independent experiments ($n = 4$). The checkerboard represents the 42 genes analyzed (only genes modulated by more than 30% with a $p < 0.05$ are colored in red or blue). To display common genes modulated under each condition, genes composing the checkerboard are organized identically (in an arbitrary fashion). ns: not statistically significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (A–D: Student’s t-test; E, F: Three-way ANOVA).

confirming previous results showing that HIFs are involved in TCR signaling. As found for priming of CD8⁺ T cells (data not shown), hypoxia dramatically decreased CTL expansion after reactivation (Fig. 2A). This was associated with reduced cell divisions and viability (Fig. 2B, C), with a trend toward more apoptosis (Fig. 2D). Similar expansion was obtained using OT-I CD8⁺ T cells and also with polyclonal CTLs from C57BL/6 mice (Supporting Information Fig. 4A–D).

Hypoxia differentially modulates gene expression when comparing to physioxia or atmospheric oxygen

We next determined the impact of hypoxia on gene expression by reactivated CTLs. Hypoxia highly impacted numerous genes (25/42) as compared to AtO₂ (Fig. 2E). However, as compared to physioxia, hypoxia impacted one fifth of them (5/42), and to a lesser extent (Fig. 2F). CTLs reactivated for two days under

hypoxia showed increased expression of *il10* and *tnfrsf9* (CD137), and to a lesser extent *bcl2*, *hif2a*, and *il2ra* (CD25). Of all genes analyzed, *il10* was the most modulated under hypoxia, independently of the oxygen fraction used as normoxia reference. RNA expression kinetics for *il10*, *ifng*, *tnfrsf9* (CD137), *il2ra* (CD25), and *hif2a* (Supporting Information Fig. 5A–E) indicated a strong upregulation of RNA levels by two days postreactivation, then a decrease, although *hif2a* RNA expression was more stable. For all these genes there was a negative correlation between oxygen fractions and RNA expression. Concerning *bcl2* RNA (Supporting Information Fig. 5F), its expression was decreased from two days postreactivation, but it was more stable under hypoxia. In order to know whether these modulations were due to hypoxia itself or to a combination of hypoxia plus TCR stimulation, we analyzed expression of the same panel of genes by CTLs cultured for two days without stimulation (Supporting Information Fig. 6A–D). There was no upregulation of *il10*, *hif2a*, or *il2ra*, showing that hypoxia-driven upregulation of these molecules is dependent on TCR stimulation. Interestingly, *tnfrsf9* was upregulated when we compared CTLs cultured under 1% vs those cultured under 5% O₂.

IL-10 secretion by CTLs inversely correlates with oxygen fraction

We confirmed our results for key genes at the protein level. By flow cytometry, we observed an increase of CD137 (4-1BB) and CD25 (IL-2R α) under hypoxia on reactivated CTLs (from Pmel-1, OT-I, and C57BL/6 mice), as compared to physioxia or AtO₂ (Fig. 3A, B, Supporting Information Fig. 7A–D). Although Bcl-2 was upregulated under hypoxia when compared to AtO₂, there was no effect of hypoxia on Bcl-2 levels if the comparison was made with physioxia (Supporting information Fig. 5G).

To further explore the unexpected production of IL-10 by CD8⁺ T cells under low oxygen tensions, we analyzed intracellular IL-10 production by reactivated CTLs. Lowering oxygen tension increased the fraction of IL-10 producing CTLs (Fig. 3C, D), the IL-10-positive fraction also coexpressed IFN- γ . However, whereas hypoxia augmented the IFN- γ -positive fraction as compared to AtO₂, it did not significantly increase this fraction as compared to physioxia (Fig. 3D). Analysis of secreted IL-10 indicated weak secretion under AtO₂, moderate induction by physioxia, and potent induction by hypoxia (Fig. 3E). Using CD8⁺ T cells from OT-I and C57BL/6 mice yielded the same results (Supporting Information Fig. 7E and G). Thus, these data suggest that IL-10 is a typical CTL cytokine following reactivation under physioxia, and that its secretion is further increased under hypoxia. However, IL-10 is not a cytokine secreted after primary activation of naïve CD8⁺ T cells, even when primed under physioxia or hypoxia (data not shown). We next tested whether IL-10 secretion was responsible for the decreased cell expansion under hypoxia; using an IL-10-blocking antibody during CTL reactivation did not restore expansion under hypoxia (Fig. 3F). Thus, hypoxia impacts on the cytokine secretion profile of CTLs, but at least for IL-10, without

autocrine consequences. We did not observe any hypoxia-related increase in expression of IFN- γ , IL-2, or TNF- α (Fig. 3G–I, Supporting Information Fig. 7F and H). Of note, IL-4, IL-6, and IL-17 were undetectable after CTL restimulation (data not shown).

As IL-10, CD25, and CD137 were strongly upregulated in reactivated CTLs by hypoxia in vitro, we confirmed these results in vivo and ex vivo using the E.G7-OVA tumor model. To show that CD8⁺ tumor-infiltrating lymphocytes (TILs) indeed express CD25, CD137, and IL-10 in vivo, we purified CD8⁺ TILs and compared them to CD8⁺ T cells from spleen. Whereas in the spleen, CD25 and CD137 were not expressed, ~13% of CD8⁺ TILs were CD25⁺ and ~30% were CD137⁺ (Fig. 4A), with most CD25⁺ cells coexpressing CD137. As IL-10 protein cannot be observed easily without in vitro cell manipulation, we looked at *il10* RNA. Expression was higher in CD8⁺ TILs as compared to CD8⁺ splenocytes (Fig. 4B); moreover, *hif2a* was also overexpressed. As indicated by scatter graphs showing correlation between CD8⁺ purity and *il10* mRNA (Supporting Information Fig. 8C), *il10* mRNA expression was not from contaminating non-T cells such as tumor cells or macrophages. In order to confirm that oxygen fraction is a major regulator of CD8⁺ TIL expansion, and of CD25, CD137, and IL-10 expression, we reactivated CD8⁺ TILs ex vivo with anti-CD3/anti-CD28-coated beads under varying oxygen fractions (i.e. 21, 5, and 1%). As we previously described using in vitro generated CTLs, expansion, and viability correlated positively with oxygen levels (Fig. 4C). Finally, confirming our in vitro data, we observed that CD25, CD137 (Fig. 4D), and most importantly IL-10, were negatively correlated with oxygen levels (Fig. 4E). Importantly, upregulation of these molecules under hypoxia needed a combination of TCR-ligation plus hypoxia (Fig. 4D, E). In addition, IFN- γ secretion was slightly decreased under hypoxia (Fig. 4F).

Discussion

Understanding the impact of hypoxia on CD8⁺ T-cell responses represents an important step to predict in vivo immune function, notably in cancer and infection. Indeed, when developing immunotherapies based on T cells (e.g. vaccines, adoptive cell transfer), the effector cells will have to function in a hypoxic environment.

A central issue in judging the impact of hypoxia is choosing the appropriate physiologic oxygen reference point. Previous work on T cells [18] convincingly demonstrated that physioxia considered as 2% O₂ [56], or as 5% O₂ [19] significantly impacts naïve T-cell activation, as compared to AtO₂. Therefore it is unsurprising that there is little consensus on the effects of hypoxia and low oxygen tensions on T cells when most other studies chose to compare responses under hypoxia with AtO₂ (that could be considered as hyperoxia) [27, 29, 33–35]. Our study rationale was designed to allow a more comprehensive appreciation of hypoxia impact, by comparison with both AtO₂ and physioxia (5% O₂).

We observed that CTLs generated under physioxia had a predominantly effector rather than memory phenotype and had increased GrB content. Furthermore, *Blimp1*, a transcription

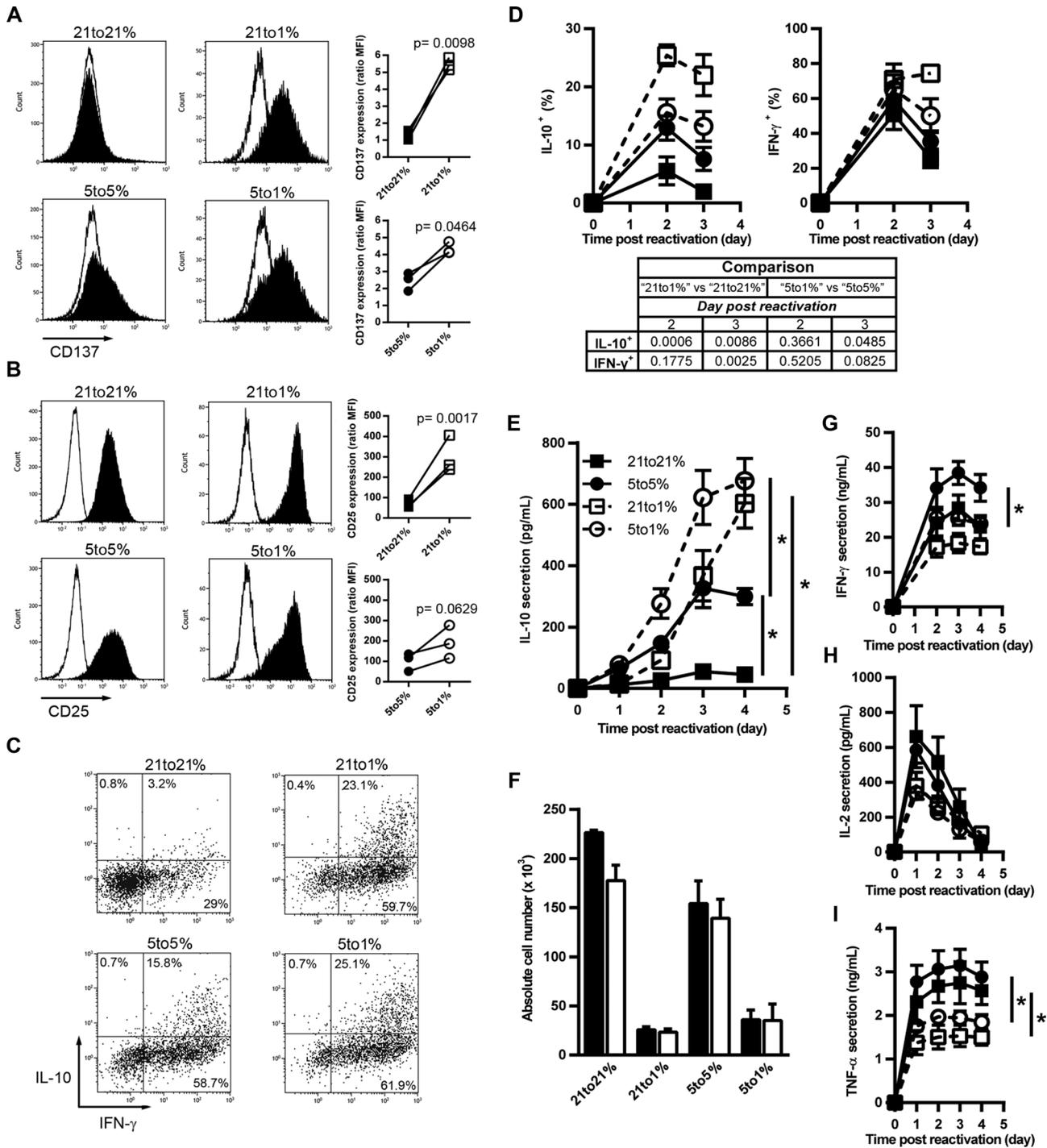


Figure 3. Decreasing oxygen fraction promotes IL-10 production by reactivated CTLs. CTLs were generated under 21% O₂ and reactivated under 21% (21 to 21%) or 1% O₂ (21 to 1%), or were generated under 5% O₂ and reactivated under 5% (5 to 5%) or 1% (5 to 1%) O₂. Four days post-reactivation cell surface expression of (A) CD137 or (B) CD25 was assessed by flow cytometry. (A and B) Open histograms: control isotype staining; closed histograms: staining for the molecule of interest. Line graphs: median fluorescence intensity from three independent experiments (n = 3). (C) Dot plots: IL-10 and IFN- γ intracellular staining from CTLs reactivated for two days. (D) Percentage of IL-10⁺ or IFN- γ ⁺ CTLs from CTLs generated under 21% (squares) or 5% O₂ (circles) that were reactivated under 21% (closed squares with solid line), 5% (closed circles with solid line) or 1% O₂ (open squares and open circles with dashed line), mean \pm SEM of 4–5 independent experiments (n \geq 4). The table represents the p-value obtained by two-tailed Student's t-test calculations. Comparison were made between CTLs generated at 21% and reactivated at 1% versus CTLs generated at 21% and reactivated at 21% O₂ ("21 to 1%" versus "21 to 21%") or between CTL generated at 5% and reactivated at 1% versus CTL generated at 5% and reactivated at 5% O₂ ("5 to 1%" versus "5 to 5%"). Mean cytokine secretion of (E) IL-10, (G) IFN- γ , (H) IL-2, and (I) TNF- α \pm SEM out of three independent experiments (n = 3). *p < 0.05. (F) Cell yield from CTLs reactivated for four days in the presence of IL-10 blocking antibody (open bars) or isotype control (closed bars), mean \pm SD of two independent experiments (n = 2). *p < 0.05 (Student's t-test).

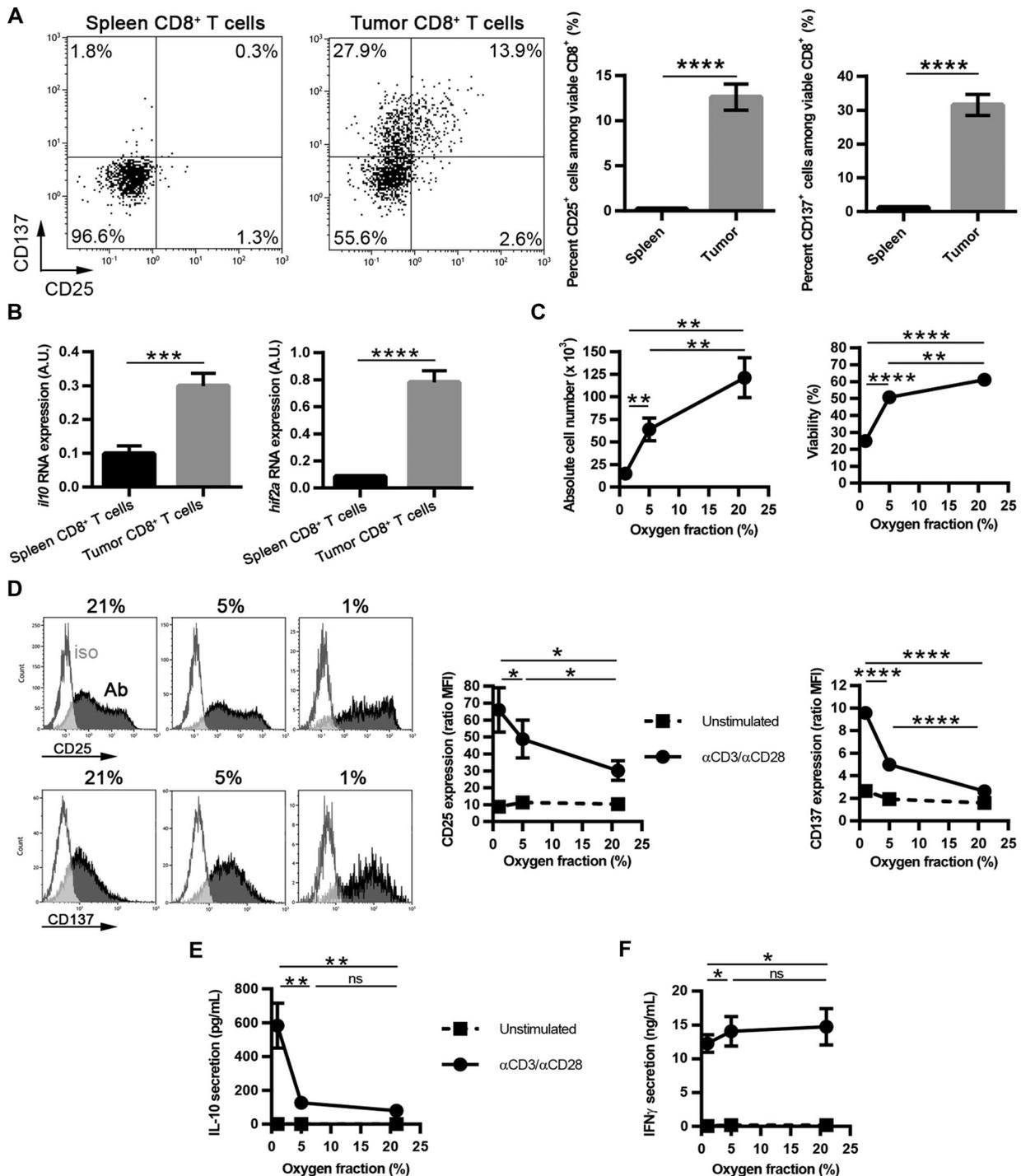


Figure 4. CD25, CD137, and IL-10 are expressed by CD8⁺ TILs ex vivo, and are positively regulated by hypoxia after reactivation. Spleen and tumor CD8⁺ T cells were purified from E.G7-OVA-bearing mice. (A) CD25 and CD137 expression was analyzed by flow cytometry. Dot plots show one representative experiment. Bar graphs represent the mean ± SEM percent CD25-positive or CD137-positive CD8⁺ T cells from six independent experiments (*n* = 17). (B) *il10* and *hif2a* expression were quantified by qPCR. Results show mean ± SEM expression from five independent experiments (*n* = 13). (C–F) CD8⁺ TILs were cultured for three days with or without αCD3/αCD28-coated beads. (C) Results show mean ± SEM absolute cell number and viability from four independent experiments (*n* = 8). (D) CD25 and CD137 were analyzed by flow cytometry. Open histograms: control isotype staining; closed histograms: staining for the molecule of interest. Line graphs (dashed lines: unstimulated; solid lines: αCD3/αCD28-stimulated): median fluorescence intensity ± SEM from four independent experiments (*n* = 8). (E) IL-10 and (F) IFN-γ secretion were quantified by ELISA. Results show mean ± SEM cytokine secretion from four independent experiments (*n* = 8). ns: not statistically significant, **p* < 0.05, ***p* < 0.01, *****p* < 0.0001 (Student's *t*-test).

factor associated with effector differentiation [57] was upregulated, while *Tcf7*, a transcription factor associated with differentiation and persistence of memory CD8⁺ T cells [58] was downregulated. Consistent with the effector versus memory profiling, CTLs generated under physioxia were more lytic than those generated at 21% O₂, suggesting that CTL generation under AtO₂ (e.g. for adoptive therapy) could be suboptimal. This is consistent with previously reported increased granzyme A expression and cytotoxicity of T cells preactivated under physiologic oxygen tensions [18, 59]. This might also be linked to increased HIF activity under physioxia, as HIFs were described to increase CTL effector function [17, 41]. Our results show that physioxia (as found in secondary lymphoid organs) facilitates efficacious CD8⁺ T-cell expansion and differentiation, and that in vitro analyses under AtO₂ poorly mimic these in vivo conditions. Of particular interest, eight important genes described to be hypoxia-regulated, including the widely reported *Vegfa* and *Glut1* genes, were regulated even under the physiologic oxygen value that we chose as physioxia (i.e. 5%), as compared to AtO₂. The impact of oxygen tensions is relevant to understand T-cell priming under physioxia [18, 19], even if naïve T cells only rarely enter hypoxic sites as in the tumor bed [60, 61]. Naïve and effector T cells will be expected to differ in their sensitivity to activation stimuli since they are phenotypically distinct, and possess unique metabolic patterns (e.g. glucose and cysteine requirements) [17, 62].

For effector CD8⁺ T-cells, their reactivation will frequently occur in poorly oxygenated, and often hypoxic tissues, at a lower oxygen fraction than during priming. However, the critical issues of their survival, expansion, and function under such in vivo relevant conditions were seldom addressed [17, 41]. In agreement with previous studies [38, 40], we observed that hypoxia did not modify CTL killing capacities in a short-term assay, regardless of the oxygen tension used for CTL generation. Nevertheless, we noticed that preconditioning for three days under hypoxia of CTLs generated under AtO₂ increased their lytic capacities. Importantly, we showed that CTL expansion following reactivation was strongly decreased under hypoxia, with both decreased viability and proliferation. We hypothesized that the underlying defect may be linked to disturbed IL-2R signaling, as recently shown in CD4⁺ T cells [30], but addition of exogenous IL-2 to CD8⁺ TILs did not restore proliferation under hypoxia (Supporting Information Fig. 8D), despite elevated expression of CD25. Another explanation could be hypoxia-induced immune regulation through adenosine-A2AR signaling [46, 63]. However, our results (with reactivated CD8⁺ T cells) are more in line with a recent publication showing a hypoxia-induced A2AR-independent decrease of cell expansion [42], since we observed neither CD25, nor *Fasl* downregulation that characterizes adenosinergic-induced immunosuppression [63, 64]. However, in an in vivo microenvironment where adenosine levels have been shown to be high, hypoxia-induced adenosinergic-dependent and independent inhibition of T-cell expansion might occur concomitantly.

As expected, hypoxia during CTL reactivation led to strong modulation of numerous genes (21 genes upregulated and four genes downregulated, of 42 tested) when comparing hypoxia to

AtO₂. However, as compared to physioxia, hypoxia was much less potent, modulating expression of only five of the 42 genes. Nevertheless, all of these genes overlapped with those identified as being modulated following switching cells from atmospheric to hypoxic conditions. Among these, two of the upregulated genes that we confirmed to be overexpressed at the protein level, *trf-srf9* (CD137) and *il2ra* (CD25), were previously reported to be hypoxia-regulated [18, 30, 44]. Of particular interest, from the whole panel of genes analyzed, *il10* was the most highly modulated gene by CTLs under hypoxia. We propose that identifying hypoxia impact relative to physioxia rather than AtO₂ is the best approach to identify truly hypoxia regulated gene expression that should be prioritized to further investigate for their in vivo significance in hypoxic tissues.

Concerning cytokine secretion, CTLs secreted less IFN- γ , TNF- α , and IL-2 under hypoxia; this might be a consequence of decreased CTL expansion under hypoxia. However, despite this (but consistent with RNA expression) CTLs and CD8⁺ TILs secreted increased amounts of the IL-10 immunomodulatory cytokine after restimulation under both physioxia and hypoxia, with hypoxia being the most potent inducer. This was only observed for effector CD8⁺ T cells, as the priming of naïve CD8⁺ T cells under hypoxia did not lead to IL-10 secretion (data not shown). Since we observed negligible secretion of IL-10 by CTLs reactivated under AtO₂, our results suggest that this capacity of CTLs is underestimated by conventional in vitro methodology, but will likely occur in vivo in many tissues, as we observed for *il10*-expressing CD8⁺ TILs. In the limited number of reports concerning IL-10 production by CD8⁺ T cells that we are aware of, most have been in the context of infection: coronavirus-induced acute encephalitis, chronic mycobacterium tuberculosis infection, and respiratory viral infection [65–68]. CTL-produced IL-10 has been suggested as a feedback mechanism to dampen immunopathology caused by excessive cytolytic and inflammatory activity [69]. Our results support such a function, since hypoxia is a feature of infected tissues [7]. However, although IL-10 is mainly known for its immunosuppressive activity, some studies have shown immunostimulatory effects [70]. We did not observe any stimulatory or inhibitory effects of IL-10 on CTL-expansion under hypoxia, but in a complex in vivo microenvironment, it could act in a paracrine manner on other immune cells, or on tumor cells. In malignancy, the role of IL-10 is still a matter of debate, with promotion of tumor immune surveillance being reported [71], but also correlation with poor prognosis, increased cancer recurrence, and metastasis [72]. It will be critical to determine in future studies whether IL-10 produced by tumor-specific CTLs can impact on their therapeutic efficacy.

Our study sheds light on specific effects of low oxygen tensions and hypoxia on the function and fate of effector CD8⁺ T cells in an in vitro model of tissue oxygenation. The use of monoclonal CD8⁺ T cells with identical sensitivity to ligands precluded the potential drawback of selection of certain clones within polyclonal T cells because of increased fitness or affinity at a given oxygen tension. Nevertheless, our major findings were similar in polyclonal C57BL/6-derived CD8⁺ CTLs and CD8⁺ TILs. Our data challenges

the use of AtO₂ as normoxia in order to determine the *in vivo* impact of hypoxia *in vitro*. Overall, we show that even if hypoxia does not disturb lytic capacities of CTLs, it could diminish antitumor or antiviral responses *in vivo* through a negative impact on CD8⁺ T cell fate and the induction of IL-10 secretion that could act in a paracrine manner on other immune cell types or tissues. If IL-10 released by CTLs is dampening inflammatory responses in hypoxic tissues *in vivo*, this may be a valuable feedback mechanism to limit immunopathology in infection, but it is likely to blunt tumor immunosurveillance or immunotherapy.

Materials and methods

Mice

Pmel-1 mice (Jackson Laboratory) carry a TCR transgene specific for human SILV (hgp100) cross-reactive with mouse gp100 (mgp100). OT-I mice (Charles River) carry a TCR transgene specific for ovalbumin. E.G7-OVA tumors were induced by subcutaneous injection of 3.10⁵ cells. Animal use was in accordance to Swiss federal law on animal protection (permission numbers: 1064/3717/2, GE/68/14, GE/80/14, GE/13/15).

Cell lines

The EL-4 and E.G7-OVA thymoma cell lines (ATCC) were grown in DMEM medium containing 4.5 g/L glucose, sodium pyruvate, and Glutamax (Gibco), supplemented with 10% fetal bovine serum, penicillin, and streptomycin.

Leukocyte isolation for CTL generation

Leukocytes were extracted from spleen and lymph nodes from Pmel-1, OT-I, or C57BL/6 mice. Pmel-1 and OT-I CD8⁺ T cells were primed with 10 nM hgp100 or 10 nM of OVA_{257–264}, respectively. For polyclonal CTL generation from C57BL/6, CD8⁺ T cells were negatively selected using the Miltenyi CD8⁺ T-cell isolation kit II (>94% purity), and were primed in a plate coated with 10 μg/mL of CD3 and CD28. Cells were cultured at 21% or at 5% oxygen, in DMEM medium containing 4.5 g/L glucose, sodium pyruvate, and Glutamax, supplemented with 6% fetal bovine serum, HEPES buffer, arginine, asparagine, penicillin, streptomycin, and 20 μM β-mercaptoethanol. Cells were split every two days with 50 IU/mL of human recombinant IL-2. The resulting CTLs were used 6–8 days postactivation.

Isolation of CD8⁺ TILs

E.G7-OVA tumors reaching 0.5 cm were harvested and processed to extract TILs. Briefly, tumors were digested by collagenase D,

leukocytes were isolated by Ficoll, then CD8-purified using the Miltenyi CD8a (Ly-2) kit (Supporting Information Fig. 8A, B).

CTL reactivation

CTLs were labeled with 10 μM CFSE (Invitrogen) to track cell proliferation. 10⁵ CTLs or 5.10⁴ CD8⁺ TILs were reactivated with anti-CD3 plus anti-CD28-coated beads (Dynabeads, Invitrogen) at a 1:1 (cell-to-bead) ratio under 21, 5, or 1% O₂. TIL reactivation was performed in the presence of 100 IU/mL IL-2. One-four days postreactivation, supernatants were collected for cytokine assessment. For IL-10 blocking, 10 μg/mL anti-IL-10 antibody (clone JES5-2A5; Biolegend) or corresponding isotype was added to the culture.

Staining for flow cytometry

For all experiments, viable cells were identified using “live/dead yellow” marker (Invitrogen) (Supporting Information Fig. 1C). For surface marker expression, cells were stained with CD8α-FITC, -PECy7, -APC, or -APCCy7 (clone 53-6.7; Biolegend), CD127-PECy7 (clone A7R34; eBioscience), CD44-AF647 (clone IM7; BD Pharmingen), CD62L-PE (clone Mel-14; Biolegend), CD137-APC (clone 17B5; eBioscience), CD25-PECy7 (clone PC61; Biolegend), GrB-PECy5.5 (clone GB11; Invitrogen), or Bcl-2-AF647 (clone BCL/10C4; Biolegend) antibodies, or appropriate isotype controls. For intracellular cytokine staining, CTLs were activated for 2–3 days with anti-CD3 plus anti-CD28-coated beads and were incubated overnight with GolgiPlug (BD Biosciences). Resulting cells were stained for CD8α, followed by intracellular staining (Fix/Perm kit; BD Biosciences) for IFN-γ-PECy7 (clone XMG1.2; Biolegend) and IL10-APC (clone JES5-16E3; Biolegend), or appropriate isotype controls. For apoptosis determination, cells were stained with AnnexinV-APC (ImmunoTools) and propidium iodide (PI; BD Biosciences). PI AnnexinV⁺ cells were considered as apoptotic. Median fluorescence intensity (MedFI) was determined using the Gallios flow cytometer (Beckman Coulter) and marker expression was calculated by MedFI marker/MedFI control isotype. Absolute cell numbers were determined using cell/bead number ratio normalization.

Cytotoxicity assay

EL-4 cells were pulsed for 1 h with different concentrations of hgp100 and labeled with 2.5 μM CFSE for subsequent EL-4 discrimination. Resulting cells were cocultured with CTLs at 21, 5, or 1% of O₂ for 4 h. Two assays were performed: either by coculture with the CTLs directly obtained 6 days after priming, or by coculture with CTLs that were beforehand preconditioned 3 days at 21, 5, or 1% O₂. EL-4 cell death was quantified by flow cytometry with the “live/dead fixable yellow dead cell stain kit.” CTL killing was calculated as follows: % specific

lysis = (% CTL-induced cell death—% spontaneous cell death)/(100—% spontaneous cell death).

Cell culture under specific oxygen fractions

Cells cultured at 21% O₂ were placed in a classical incubator under AtO₂ at 37°C and 8% CO₂. For CTL generation at 5% O₂, cells were incubated in a Ruskinn In Vivo₂ 300 Workstation at 37°C, 5% O₂ and 8% CO₂. All cellular manipulations were carried out under these conditions to prevent any reoxygenation of the cells. Hypoxic chambers (Billups-Rothenberg, Inc.) flushed with gas mixtures composed of 87% N₂/8% CO₂/5% O₂, or 91% N₂/8% CO₂/1% O₂ were used for culture of CTLs reactivated under 5% O₂ or 1% O₂, respectively. All culture medium and buffers for cell manipulation were preequilibrated overnight at 4°C in gas concentrations corresponding to subsequent culture/experimental conditions.

RNA expression analyses by qPCR

RNA was extracted using the Qiagen RNeasy kit and was DNase-treated according to the manufacturer's instructions. Equal amounts of RNA (53 ng) were used to synthesize cDNA (PrimerScript RT; Takara Bio Inc.) that was then assessed for *il10* (forward: CCAGAGCCACATGCTCC-TAGA; reverse: AGCTGGTCCTTTGTTGAAAGAA) and *hif2a* (forward: TCCCAGTTCCAGGACTACGGT; reverse: GGCCACGC-CTGACACCT) expressions by normalization with *actb* (forward: CTAAGGCCAACCGTGAAAAGAT; reverse: CACAGCCTGGATGGC-TAGGT) and *ef1a1* (forward: TCCACTTGGTCGCTTTGCT; reverse: CTTCTGTCCACAGCTTTGATGA) genes through real-time quantitative PCR (SDS 7900 HT instrument; Applied Biosystems) using the SYBR Green master mix (Applied Biosystems). The following parameters were used: 50°C for 2 min, 95°C for 10 min, and 45 cycles of 95°C 15 s—60°C 1 min. Each reaction was performed in three replicates. Raw Ct values were obtained with SDS 2.2 (Applied Biosystems). The 2^{ΔCt} method was used to normalize and linearize the results.

NanoString nCounter RNA Expression analysis

RNA from dry cell pellets was extracted using the RNeasy kit (Qiagen). Two hundred eighty nanogram of RNA was hybridized with multiplexed Nanostring probes and samples were processed according to published procedures [73]. Probes for the analysis of 47 different genes (Nanostring technologies) included five normalization genes (Supporting Information Table 1). Background correction was done by subtracting from the raw counts the mean +2 SD of counts obtained with negative controls. Values <1 were fixed to 1. Positive controls were used as quality assessment: we checked that the ratio between the highest and the lowest positive controls average among the samples was below 3. Then, counts

for target genes were normalized with the geometric mean of the three reference genes (*actb*, *tbp*, *ef1a1*) selected as the most stable using the geNorm algorithm [74]. Counts for each RNA species were analyzed using an in house Excel macro, and then expressed as counts (molecules of RNA/sample) [75].

Cytokine secretion analysis

Cytokine content (IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ, and TNF) from naive and effector CD8⁺ T-cell cultures was determined using the "mouse Th1/Th2/Th17 kit" cytometric bead assay or BD OptEIA sets for IFN-γ, IL-2, or IL-10 (BD Biosciences).

Statistical analyses

Statistical significance was evaluated using the two-tailed student's *t*-test for comparison of two groups using the GraphPad Software (Prism), or a three-way ANOVA test for RNA analysis.

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Abbreviations: AtO₂: atmospheric oxygen fraction · HIF: hypoxia-inducible factor · TIL: tumor-infiltrating lymphocyte

Full correspondence: Dr. Paul R. Walker, Geneva University Hospitals, Oncology Division, Rue Gabriel-Perret-Gentil 4, 1211 Geneva 14, Switzerland
Fax: +41-22-372-9858
e-mai: Paul.Walker@hcuge.ch

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