Editorial

Oxygen starvation during T cell priming boosts cancer-killing potential

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Immune-based tumor-therapy has seen substantial progress in recent years and has made important inroads in the fight against cancer. Checkpoint inhibitors are a clinical success and the use of cell-based therapy models is rapidly expanding. T cells, such as chimeric antigen receptor T cells (CARs) and tumor infiltrating lymphocytes (TILs), are now routinely cultured and activated in labs and subsequently adoptively transferred to patients with the aim of reducing and eradicating tumors. However, although positive results have been obtained in clinical trials, immune rejection of tumors is often not successful. This has focused attention on improving delivery and cytotoxic potential of transferred cells. Gropper *et al.* (1), now show that culturing CD8 T cells under hypoxic conditions, during *in vitro* priming and prior to adoptive transfer, enhances their cytolytic capacity resulting in more robust anti-tumor activity in a murine cancer model. These findings could have implications for the future of T cell-based tumor therapies and may improve the design of protocols aimed at potentiating cytotoxicity prior to adoptive transfer of T cells.

Immunotherapy with adoptive T cells has become one of the leading forces in the fight against cancer. Advanced clinical trials demonstrated positive results in treating a variety of cancers including melanoma, cervical cancer, lymphoma, leukemia and neuroblastoma (2). Although clinical trials show promising results, the efficiency of those therapies needs further improvement with a main goal to increase T cell survival and anti-tumor activity (3). The success of cell-based cancer immunotherapy is thought, at least in part, to be determined at the tumor site and by the tumor environment (4). Hypoxia, a state of low oxygen tension caused by a reduction in blood supply, is one of the environmental determinants that can influence the effectiveness of T cell mediated tumor rejection. Hypoxia is a particular characteristic of solid tumors due to their often limited vascularization. This is associated with poor prognosis in many types of cancers, including cervical carcinoma, head and neck cancer and sarcoma (5). Hypoxia is reported to promote tumor cell stemness, migration, metastasis, invasiveness, and resistance to radio- and chemotherapy (6).

The impact of hypoxia on T cells and their function in anti-tumor immunity is not fully understood (7). T cells migrate in the oxygen-rich bloodstream and are thought to be primed in oxygen sufficient lymph nodes (8), while encountering hypoxic conditions when migrating into the tissues. An early study of activated human T cells suggested a positive correlation between hypoxia, T cell viability and proliferation (9). However, a study by Gaber *et al.* (10) showed that hypoxia decreases the proliferation of mitogen stimulated human $CD4^+$ T cells. Tumor-associated hypoxia has been reported to promote the recruitment of regulatory T (T_{reg}) cells through induction of the CC-chemokine ligand 28, which could reduce tumor rejection (11). In addition, hypoxia can alter the differentiation of $CD4^+$ T cells, thereby altering their functional capacities and reducing T cell migration toward tumors (12,13). Furthermore, increased use of anaerobic glycosylation in hypoxia and

the subsequent secretion of lactate causes acidification of the tumor environment that can decrease T cell activation, proliferation and cytotoxicity (14). Collectively, the influence of hypoxia on T cells can be manifold with direct and indirect consequences on tumor rejection. This has hampered our understanding of the impact of hypoxia on T cell-mediated tumor cell cytotoxicity and apparent contradictory results have been reported, with both T cell inhibitory and boosting observations (15-17).

Gropper *et al.* took a reductionist approach to explore the direct impact of hypoxia during murine CD8 T cell priming (1). They used ovalbumin (OVA)-specific $CD8^+$ T cells from OT-I T cell receptor (TCR) transgenic mice. OT-I T cells were activated *in vitro* using the SIINFEKL peptide and interleukin (IL)-2 under hypoxic (1% O_2) or atmospheric (20% O_2) conditions for 5 days. Of note, the concentration of oxygen within lymph nodes, where T cells are primed, are reported to be between 0.5% and 6% (8). T cells cultured under hypoxic conditions were viable, larger in size and contained more mitochondrial mass compared with T cells cultured under atmospheric oxygen pressure. T cell proliferation assayed with CFSE-dilution showed less proliferation of CD8 T cells primed under hypoxic condition compared with atmospheric oxygen pressure, which reduced the yield of T cells harvested under reduced oxygen pressure. This can be of concern for adoptive T cell therapies, which require substantial T cell numbers. However, although the maturation status of hypoxic cytotoxic T cells (CTLs) up to day 3 post-activation was indiscriminate to those cultured under atmospheric oxygen pressure, 5-day post-activation cells primed under hypoxic conditions harbored an increased percentage of highly activated $CD44^{hi}CD62L^{lo}$ cells. Transcriptome analysis of CTLs generated under different oxygen pressures revealed increased glycolysis capacity of cells primed under hypoxia, such as higher levels of transcripts for glucose transporter (Glut) 1 and hypoxia-inducible factor (HIFs) family members. Together, the data showed that priming of CD8 T cells under hypoxic conditions results in reduced yield of CTLs, but with an increased mature phenotype such as previously reported for TILs *in vivo* (18).

To characterize the CTLs generated, markers associated with T cells exhaustion and inhibition, CTLA-4, PD-1, TIM3, LAG3 were compared between CD8 T cells primed under atmospheric and hypoxic conditions. Especially the co-expression of PD-1 with TIM3 and LAG3 on TILs has been used as a marker of CTL dysfunction, demonstrated by a reduced activation profile and cytokine production (19,20). Neutralizing monoclonal antibodies against PD-1 and CTLA-4 are approved for clinical use as checkpoint inhibitors and show very promising results in late stage clinical trials (21). The expression levels of PD-1 and CTLA-4 were, however, similar between CTLs generated under hypoxic or atmospheric oxygen conditions. Instead, the expression of TIM3 and LAG3 was increased on hypoxic CTLs. In contrast to PD-1, the increased expression of LAG3 and TIM3 on CTLs generated under hypoxic conditions is in common with TILs found in different tumors (19,20). These data indicated that CTLs generated under conditions of low or high oxygen pressure are not approaching senescence or exhaustion, but that priming under low oxygen pressure infers a phenotype more resembling TILs.

The increase in size and glycolytic capacity suggested an increased fitness of CTLs primed under hypoxia. This was strengthened by the observation that CTLs primed under hypoxic conditions contain higher granzyme B protein levels per granule. However, compared with CTLs primed under atmospheric oxygen levels, those generated under hypoxia do not contain more granules, perforin protein or degranulation capacity. *In vitro* cultures of CTLs and OVA-expressing B16 tumor cells revealed increased target killing of cells generated under hypoxic conditions compared with those stimulated under atmospheric oxygen levels. The increased level of granzyme B in CTLs generate under limited oxygen levels is in line with a previous report and suggests that these cells might also exhibit more efficient tumor killing *in vivo* (15). CTLs generated under high or low oxygen pressure adoptively transferred into B16-OVA tumor bearing mice were indistinguishable with respect to their migration pattern, speed and depth of tissue penetration. However, mice treated with CTLs primed under hypoxia showed increased regression of the tumors and prolonged survival compared with those treated with CTLs generated under high oxygen levels. Collectively the authors argue that these results may have beneficial implications for adoptive T cell therapies using either tumor isolated antigen-specific TILs or engineered antigen-specific CAR T cells. Priming or re-stimulation of CTLs under hypoxic conditions may be an effective way of enhancing anti-tumor efficiency.

Gropper *et al.*, show that the priming of mouse CD8 T cells under reduced oxygen levels results in slower proliferation but enhanced maturation with respect to glycolysis potential, mitochondrial mass and storage of the cytolytic protein granzyme B. The data suggest that

suboptimal T cell proliferation results in a qualitatively better T cell. This may be explained by the large energy levels required during clonal expansion, sustaining rapid proliferation as well as the anabolic processes of generating new cellular structures to be equally divided among daughter cells, such as mitochondria, and the synthesis of effector molecules such as granzymes. It remains to be determined if human CD8 T cells show a similar phenotype when cultured under different oxygen pressures. T cell therapy depends on the generation of sufficiently large amounts of T cells in often a short time and hence optimal T cell proliferation has been a prime focus. The results from Gropper *et al.* suggest a qualitative measure of T cell functionality is important. Although reducing T cell proliferation, such as achieved under limited oxygen conditions, comes at a cost of total T cell numbers, the efficacy of T cell therapy may be improved. Upon fast clonal expansion, resources or metabolic wiring may be insufficient to service both proliferation and T cell maturation optimally. If the enhanced quality of T cells is able to sustain the anti-tumor response for longer compared with those generated under atmospheric conditions remains to be determined. The CTLs transferred by Gropper *et al.*, do not show signs of exhaustion or senescence prior to transfer. However, the cells might well acquire this phenotype after entering to the immunosuppressive tumor environment. The immunosuppressive tumor environment remains a significant hurdle to overcome for the success of adoptive transfer therapies resulting in tumor elimination and not only temporal control. Combinations of adoptive transfer of CTLs cultured under hypoxic conditions and checkpoint blockade, as suggested before (3), could be a potential strategy to further increase the success of tumor therapies.

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Footnote

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