Oxygen-generating cryogels restore T cell-mediated cytotoxicity in hypoxic tumors

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Abstract

Solid tumors are protected from antitumor immune responses due to their hypoxic microenvironments. Weakening hypoxia-driven immunosuppression by hyperoxic breathing of 60% oxygen has shown to be effective in unleashing antitumor immune cells against solid tumors. However, efficacy of systemic oxygenation is limited against solid tumors outside of lungs. Therefore, it is essential to develop targeted oxygenation alternatives to weaken tumor hypoxia as novel approaches to cancer immunotherapies. Herein, we report on injectable oxygen-generating cryogels (O₂-cryogels) to reverse tumor-induced hypoxia. These macroporous biomaterials were designed to locally deliver oxygen, inhibit the expression of hypoxia-inducible genes in hypoxic melanoma cells, and reduce the accumulation of immunosuppressive extracellular adenosine. O₂-cryogels enhance T cell-mediated secretion of cytotoxic proteins, restoring the killing ability of tumor-specific CTLs, both in vitro and in vivo. In summary, O₂-cryogels provide a unique and safe platform to supply oxygen as a co-adjuvant in hypoxic tumors and improve cancer immunotherapies.

Introduction

Over the last decade, immunotherapy has evolved into a very promising new frontier for fighting various types of cancer.(1) A number of these immunotherapies are either approved for use or are under clinical trials, particularly immune checkpoint inhibitors (e.g., anti-PD-1, anti-PD-L1, and/or anti-CTLA-4 antibodies) and T cell therapy (e.g., chimeric antigen receptor [CAR] T cells).(2-4) However, their limited success in solid tumors has been attributed to tumor-protecting mechanisms that lead to immune escape.(5) Low oxygen tension ($< 3\% \text{ O}_2$), or hypoxia, is a common feature of solid tumors.(6) Hypoxia within the tumor microenvironment (TME) is a major impediment to effective cancer therapies and has been associated with poor clinical outcomes.(7) Hypoxic tumors often exhibit poor responses to systemic therapies(8) and radiotherapy,(9, 10) resistance to apoptosis,(11) and an aggressive phenotype that leads to rapid disease progression and poor prognosis.(12, 13)

Hypoxic stress is a key factor involved in creating an immunosuppressive environment and as a result, inhibits incoming cytotoxic T lymphocytes (CTLs).(14-17) These immunosuppressive effects are primarily mediated by hypoxia-inducible factors (HIFs)(18-20) and A2A adenosine receptors (A2ARs).(21, 22) Tumor hypoxia stabilizes the transcription factor HIF1 α ,(23) leading to increased expression of the adenosine-generating ecto-enzymes CD39 and CD73.(24) This results in the accumulation of extracellular adenosine in the TME, which signals through cAMP-elevating A2ARs on the surface of immune cells.(25-27) Adenosine signaling suppresses the antitumor activity of T cells and natural killer (NK) cells, particularly their cytotoxic effector functions and secretion of important proinflammatory cytokines such as interferon gamma (IFN γ).(28, 29)

Several classes of therapeutics can reverse hypoxia-induced immunosuppression,(30) including A2AR antagonists,(31, 32) HIF inhibitors,(30, 33) and supplemental oxygenation therapy.(24, 34) Recent clinical studies using A2AR antagonists have shown encouraging results in targeting the hypoxia-adenosinergic

pathway.(35) Similarly, systemic oxygen therapy such as respiratory hyperoxia has proven effective to enhance antitumor immunity in preclinical studies.(22) However, respiratory hyperoxia ($> 60\% \text{ O}_2$) has been associated with inflammatory changes, alveolar infiltration, and, eventually, pulmonary fibrosis.(36) Furthermore, although safer, respiratory hyperoxia with lower oxygen concentrations ($\leq 60\% \text{ O}_2$) is less effective against solid tumors in tissues distant from the lungs and it may exacerbate inflammatory events in patients.(22, 36) Therefore, there is a critical need to develop new strategies to target oxygen delivery and minimize off-target toxicities. Such approaches would be useful to reduce or eliminate local tumor hypoxia, create an immunopermissive TME, and ultimately enhance the effectiveness of cancer treatments.

Over the past decade, innovative biomaterials have gained great momentum as a means to study or modulate biological environments.(37, 38) Recent studies have shown that incorporating peroxides, percarbonate, or hydrogen peroxide (H_2O_2)-based particles in biomaterials allowed oxygen delivery within tissues.(39, 40) These biomaterials release oxygen in water(41, 42) and are typically coupled with an antioxidant to attenuate harmful free radical byproducts.(39, 40, 43) However, the administration of biomaterials is often invasive, which limits their clinical feasibility.(44-46) Furthermore, biomaterials that release oxygen require a highly interconnected macroporous network to tackle mass transport and diffusion limitations,(47) but such structures are challenging to engineer.(48) Recently, cryogel scaffolds have emerged as promising biomaterials that can be injected through conventional small-bore needles for minimally invasive delivery.(49, 50) This new class of injectable hydrogels possesses unique features such as shape-memory properties, mechanical flexibility, and a highly interconnected macroporous architecture essential for oxygen diffusion and availability throughout the construct.

We hypothesized that an oxygen-generating biomaterial capable of reversing tumor-induced hypoxia may reactivate inhibited T-cell function and activity, and subsequently reinforce T cell-mediated cytolytic activity against tumor cells. In this work, we engineered injectable macroporous oxygen-generating

cryogels (O_2 -cryogels) that can reoxygenate O_2 -deprived TME, reverse hypoxia-driven immunosuppression, and ultimately restore antitumor CTL activity. The cryogels were fabricated with the naturally occurring glycosaminoglycan hyaluronic acid (HA), one of the major components of the tumor extracellular matrix (ECM). The three-dimensional (3D) cryogels were hybridized with calcium peroxide (CaO_2) microparticles and conjugated to catalase (CAT) to boost local oxygen release while minimizing cytotoxicity. We first characterized the physical properties of O_2 -cryogels, including their unique interconnected macroporous networks, shape memory properties, and syringe injectability. Next, we investigated the ability of O_2 -cryogels to release oxygen in a controlled and sustained fashion under normoxic (20% O_2) and hypoxic (1% O_2) conditions while being inherently cytocompatible and biocompatible. We also studied the potential of O_2 -cryogels to reverse tumor hypoxia, downregulate expression levels of hypoxia-induced proteins, increase proinflammatory cytokines, and decrease immunosuppressive molecules. Finally, in comparison to respiratory hyperoxia (60% O_2), we evaluated the ability of O_2 -cryogels to restore the antitumor activity of inhibited hypoxic T cells in hypoxia and their use as an immunological co-adjuvant in a highly aggressive and advanced-stage murine melanoma model. This study reports on (i) a strategy to re-establish T cell function when subjected to hypoxic stress and (ii) the potential of biomaterial-mediated intratumoral oxygen delivery as a safe co-adjuvant to reinforce pre-existing antitumoral immune response in vivo.

Results

Fabrication of O_2 -cryogels

O_2 -cryogels were fabricated by free radical polymerization of HAGM, APR, and APC at $-20\text{ }^{\circ}\text{C}$ in the presence of various concentrations of CaO_2 (Figure 1a). HAGM was incorporated into the cryogel

backbone to mimic aspects of the TME,(51) APR to prevent anoikis and promote cell–cryogel interactions,(52) and CaO₂ and APC to release oxygen and deplete H₂O₂ byproduct, respectively. During cryopolymerization, the macromonomers, CaO₂, and initiators are phase-separated from ice crystals (step 1), enabling polymerization within the unfrozen liquid phase and CaO₂ particle entrapment in the newly formed polymer walls (step 2). Once the reaction is complete, the cryogels are thawed at RT to melt the ice crystals, leaving behind a polymer network with large and interconnected pores (step 3).

Unlike other large-scale oxygen-generating biomaterials, O₂-cryogels were designed to withstand substantial mechanical deformation and compression, resulting in a minimally invasive biomaterial that can overcome the shear stress experienced during syringe injection (step 4). O₂-cryogels were engineered to controllably release oxygen via CaO₂ decomposition in water. We hypothesized that local oxygen delivery would relieve the hypoxic stress and as a result, (1) reverse the immunosuppressive TME and (2) restore impaired effector T-cell function. This strategy is intended to facilitate CTLs to infiltrate solid tumors and ultimately kill tumor cells (Figure 1b).

Characterization of O₂-cryogels

The controlled and sustained release of oxygen from O₂-cryogels hinges on the efficient encapsulation and distribution of CaO₂ particles throughout the polymer network. To demonstrate the successful and homogeneous incorporation of the particles into the polymer walls, cryogels and O₂-cryogels containing different amounts of CaO₂ (0.1–1% wt/vol) were stained with Alizarin Red S before and after syringe injection through a 16-gauge needle (Figure 2a). The red staining intensity increased with increasing CaO₂ concentration, confirming the efficient incorporation of these particles within O₂-cryogels. Furthermore, O₂-cryogels exhibited the same shape-memory properties as those of their cryogel analogues (Supplementary Video 1), and no significant changes in Alizarin Red S staining were observed following injection. Confocal microscopy (Figure 2b–g) combined with scanning electron microscopy imaging (Figure 2 h–i and Supplementary Figure 1) and EDX analysis (Figure 2j–k and Supplementary Figure 2)

validated the Alizarin Red S staining results. Specifically, the particle entrapment within O₂-cryogel polymer walls increased proportionally to the initial CaO₂ loading. Additionally, the particles were homogeneously distributed throughout the O₂-cryogel construct. Altogether, these results suggest that CaO₂ particles remain physically entrapped within polymer walls even under an applied shear stress.

Cryogels consist of a highly interconnected polymer network.⁴⁹ However, the impact of CaO₂ encapsulation on the physical properties of cryogels has never been investigated. Therefore, we evaluated the change in the cryogel macrostructure as a function of CaO₂ concentration, using scanning electron microscopy (Figure 2l–m). O₂-cryogels displayed large pores ranging from 20 to 100 μm in diameter, and the pore size decreased proportionally with increasing CaO₂ concentration—from 70.51 ± 10.7 μm (0% CaO₂) to 47.43 ± 6.7 μm (1% CaO₂). Next, the impact of the network microstructure of O₂-cryogels on mechanical properties was assessed (Figure 2n–p). The addition of CaO₂ did not change their pore connectivity (80 ± 3%) (Figure 2n). However, an increase in CaO₂ concentration slightly enhanced their swelling ratio, from 23.9 ± 0.9 (0% CaO₂) to 26.6 ± 0.6 (1% CaO₂) (Figure 2o). Additionally, CaO₂ encapsulation dramatically affected the Young's modulus of the cryogels during compression (Figure 2p). While cryogels displayed a modulus of 7 ± 2 kPa, an increase in CaO₂ concentration to 1% CaO₂ reduced the Young's modulus value down to 1.5 ± 0.2 kPa. Collectively, these results indicate that CaO₂ encapsulation slightly impacted the physical properties of the cryogels without altering their unique interconnected macroporous structure, injectability, and shape-memory properties.

Controlled and sustained release of oxygen from O₂-cryogels

In water, CaO₂ slowly decomposes to form calcium hydroxide (Ca(OH)₂) and oxygen as depicted in Equations 2 and 3:

However, this reaction also produces the reactive oxygen species H_2O_2 as a byproduct, which can be harmful to cells, especially at concentrations beyond $10\ \mu\text{M}$.⁽⁵³⁾ To prevent H_2O_2 -related cytotoxicity, CAT was chemically modified with ACRL-PEG-NHS to form APC. Next, APC was covalently incorporated into O_2 -cryogel during cryopolymerization to sustainably degrade H_2O_2 into H_2O and O_2 . Compared to non-modified CAT, the enzymatic activity of modified CAT (i.e., APC) was decreased by $70 \pm 3\%$ (Supplementary Figure 3a). Next, the ability of APC to deplete H_2O_2 following cryogelation was investigated. APC-containing or APC-free O_2 -cryogels were incubated in water at $37\ ^\circ\text{C}$ for 15 min, after which the production of H_2O_2 was evaluated. Incorporating APC in O_2 -cryogels containing 0.1% to 0.3% CaO_2 effectively prevented any residual H_2O_2 release (Figure 3a). Similarly, APC-grafted O_2 -cryogels containing 0.5% and 1% CaO_2 achieved low H_2O_2 levels of 4 ± 1 and $11 \pm 2.5\ \mu\text{M}$, respectively. APC also promoted oxygen production from H_2O_2 (Supplementary Figure 3b). While no oxygen release occurred when APC-free cryogels were immersed in 50 mM H_2O_2 (Supplementary Video 2), cryogels containing 1% APC produced small gas bubbles under the same conditions, suggesting rapid conversion of H_2O_2 to oxygen (Supplementary Video 3).

Next, we characterized the kinetics of oxygen release from cryogels containing varying amounts of CaO_2 (0 to 1% wt/vol) by measuring the concentration of dissolved oxygen in the medium at $37\ ^\circ\text{C}$ every 5 min for two days (Figure 3b and Supplementary Figure 3c). The amount of oxygen generated by O_2 -cryogels was proportional to the CaO_2 content under normoxic (Figure 3c) and hypoxic conditions (Figure 3d). In normoxic conditions, O_2 -cryogels induced a maximum oxygen concentration increase in the medium (up to 215%) and achieved a sustained release of oxygen for up to 48 h (Figure 3c). As expected, cryogels did not release any oxygen. Similar results were also observed with preconditioned hypoxic (1% O_2) media (Figure 3d). O_2 -cryogels increased the oxygen concentration in the medium up to 2000% of the initial concentration and allowed a sustained oxygen release for up to 48 h. In hypoxia, O_2 -cryogels released effectively oxygen and were able to reach oxygen levels above physioxia (5–8% O_2 , i.e., $80\ \mu\text{M}$ of O_2 at $37\ ^\circ\text{C}$, 1013 hPa) for 8 h (with 0.3% CaO_2) and 40 h (with 1% CaO_2).

These O₂-cryogels were designed to be injectable. Therefore, their capacity to efficiently release oxygen upon injection was also evaluated. Because our experimental design required large cryogel cylinders, each O₂-cryogel was compressed by up to 90% of their volume and subjected to shear stress to mimic the injection process (Supplementary Figure 4). Regardless of the CaO₂ concentration, the cryogels showed the same oxygen release kinetics before and after compression, consistent with the CaO₂ entrapment measurements (Figure 2a). Collectively, these results indicate that injectable oxygen-releasing cryogels were successfully fabricated and can sustainably deliver oxygen for up to 48 h under hypoxic and normoxic conditions.

Cytocompatibility (in vitro) and biocompatibility (in vivo) of O₂-cryogels

We next examined cell viability within O₂-cryogels as an indication of cytocompatibility. Cryogels and O₂-cryogels were partially dehydrated, seeded with 1×10^5 B16-F10 mouse melanoma cells, and incubated at 37 °C for 24 h in normoxia (Figure 3e and 3g) or hypoxia (Figure 3f and 3g). In normoxia (Figure 3e), B16-F10 cell-laden cryogels containing up to 0.5% CaO₂ displayed high viability (~97%), similar to their cryogel analogues, indicating their cytocompatibility. Cryogels containing 1% CaO₂ showed a slight decrease in viability ($85 \pm 2\%$). However, regardless of the O₂-cryogel composition, cells homogeneously attached and spread throughout the constructs (Figure 3g). When incubated in hypoxia, similar results were obtained in terms of cell attachment and spreading (Figure 3g), but the viability of B16-F10 cells remained high ($95 \pm 2\%$) throughout the assessed CaO₂ concentration range (Figure 3f). These results suggest that the O₂-cryogels developed in this study are cytocompatible, promote cell adhesion, and support cell viability under both normoxic and hypoxic conditions.

To further investigate the biocompatibility of O₂-cryogels, APC-free and APC-functionalized O₂-cryogels containing 1% CaO₂ were injected into the subcutaneous space of the dorsal flanks of C57BL/6J mice. Cryogels functionalized with APC were used as controls. Seven days post-injection, the cryogels and O₂-cryogels were excised with the surrounding tissue and stained using H&E and MT for histological analysis

(Figure 3h). All explanted biomaterials retained their initial size. Only a few neutrophils were observed in the APC-functionalized cryogels and O₂-cryogels, suggesting minimal inflammation. Furthermore, the junction between the connective tissue stroma and these cryogels consisted mainly of fibrin, suggesting that the biomaterials were well integrated within the native tissue. In contrast, APC-free O₂-cryogels showed a high number of neutrophils in the connective tissue stroma and within the implant, indicating an inflammatory reaction. Collectively, these results suggest that O₂-cryogels are biocompatible and that the enzyme CAT contributes to preventing a host inflammatory response.

Reversing local cellular tumor hypoxia and associated immunosuppression

O₂-cryogels were next analyzed for their capacity to reverse hypoxia at the cellular level. Cellular hypoxia of B16-F10 in cryogels at various amounts of CaO₂ was assessed in the presence of HypoxyprobeTM-1 (Figure 4a,b). B16-F10 cells (1×10^5) were seeded in cryogels or O₂-cryogels and incubated at 37 °C for 24 h in hypoxia. After incubation, $96 \pm 1.2\%$ of the cells within cryogels became hypoxic. Higher CaO₂ concentrations within O₂-cryogels were associated with lower percentages of hypoxic cells, between 84.5% (with 0.1% CaO₂) and 10.6% (with 1% CaO₂). Additionally, no significant difference was observed between cells within O₂-cryogels containing 1% CaO₂ cultured in hypoxia and cells within cryogels cultured in normoxia, suggesting effective reversal of cellular hypoxia.

In addition to analyzing cellular hypoxia, the concentration of extracellular adenosine – downstream of hypoxia – was measured within the supernatant of cell-laden cryogels and O₂-cryogels after 24 h of incubation (Figure 4c). As expected, cells cultured within cryogels and subjected to hypoxia showed increased levels of extracellular adenosine ($0.85 \pm 0.05 \mu\text{M}$) compared to those cultured in normoxic conditions ($0.68 \pm 0.08 \mu\text{M}$). However, in hypoxia, O₂-cryogels were associated with significant decreased levels of extracellular adenosine, with values similar to those in normoxic conditions obtained with 0.5% CaO₂ ($0.67 \pm 0.05 \mu\text{M}$) and 1% CaO₂ ($0.65 \pm 0.06 \mu\text{M}$).

The effect of local oxygenation on hypoxic B16-F10 cell phenotype was also investigated. A sudden change in oxygen concentration or cell toxicity can impact cell phenotype. In this context, only O₂-cryogels containing 0.5% CaO₂, a formulation allowing sustained release of oxygen over 30 h, were tested and compared to their CaO₂-free cryogel analogues. B16-F10 cells were seeded within these cryogels and cultured under normoxic or hypoxic conditions for 24 h. Total mRNA was then extracted from the cells, and the expression profile of the hypoxia-inducible genes *HIF1α*, *Wnt11*, *CD73*, *VEGFα*, *CD44*, *CD73*, and *CD133* was analyzed by RT-qPCR (Figure 4d). As expected, when seeded in cryogels under hypoxic conditions, B16-F10 cells demonstrated increased expression of *HIF1α* (+45%), *Wnt11* (+123%), *CD73* (+75%), *VEGFα* (+125%), *CD44* (+140%), and *CD133* (+246%) compared to the expression levels under normoxic conditions. In contrast, no changes in gene expression were observed in cells seeded in O₂-cryogels containing 0.5% CaO₂ under hypoxic conditions. Additionally, in comparison to cells cultured in hypoxia with cryogels, *CD44* and *CD133* gene expression levels decreased in B16-F10 cells cultured in O₂-cryogels (by 65% and 48%, respectively). Collectively, these results suggest that O₂-cryogels can reverse cellular hypoxia, thereby preventing the accumulation of immunosuppressive adenosine and the induction of a hypoxic and aggressive phenotype in cancer cells.

Restored cytotoxicity of hypoxic OT-1 T cells in an antigen-specific B16-OVA model

Several studies have established that the hypoxic TME dramatically impairs the ability of tumor-reactive T cells to eliminate tumor cells.(16, 17, 54) Thus, we investigated the effect of O₂-cryogels on the tumoricidal capacity of spleen-derived OVA-specific OT-1 T cells to eliminate B16-OVA cells in normoxic and hypoxic culture (Figure 5a). Cytotoxic T-cell function was first assessed in real time by time-lapse microscopy (Figure 5b and Supplementary Videos 4,5). B16-OVA cells were cultured for 3 h in 24-well plates in normoxia to allow adhesion and subsequently cocultured with OT-1 T cells for 4 h in hypoxia in the presence of cryogels or O₂-cryogels containing 0.5% CaO₂. As expected, under hypoxic

conditions, antigen-specific OT-1 T cells did not induce tumor cell death when exposed to cryogels (Figure 5b and Supplementary Video 4). However, when incubated with O₂-cryogels, the defective cytotoxic function of hypoxic OT-1 T cells was rescued, leading to the rapid killing (< 4 h) of B16-OVA tumor cells (Figure 5b and Supplementary Video 5).

To substantiate these observations, we compared the effect of the CaO₂ concentration on T-cell cytotoxicity in hypoxic and normoxic conditions (Figure 5c–d and Supplementary Figures 5,6a). To this end, B16-OVA cells were first cultured in cryogels containing different CaO₂ concentrations for 3 h in normoxia and then cocultured with OT-1 splenocytes for 4 and 24 h in normoxia or hypoxia. Under normoxic conditions, cytotoxic OT-1 T cells infiltrated O₂-cryogels and killed B16-OVA cells ($85 \pm 5\%$ cytotoxicity) within 4 h (Supplementary Figure 6a), regardless of the CaO₂ concentration. In hypoxic conditions, the cytotoxic activity of OT-1 T cells within cryogels decreased substantially (4 h = $3.55 \pm 1\%$; 24 h = $34.38 \pm 11\%$ cytotoxicity), confirming that low oxygen tension inhibits the cytotoxicity of T cells in this model (Figure 5c,d and Supplementary Figure 6a); this is consistent with the time-lapse microscopy results. After 4 h of cocultivation in hypoxia, O₂-cryogels restored the OT-1 T-cell cytotoxicity by up to 50% in a CaO₂ concentration-dependent manner (Supplementary Figure 6a). Moreover, O₂-cryogels containing 0.5% and 1% CaO₂ particles fully restored the cytotoxicity of OT-1 T cells after 24 h in hypoxia to similar levels to those in cells cultured in normoxia (Figure 5c,d).

This finding was further confirmed by evaluating the concentrations of IFN γ , perforin, and granzyme B secreted by functional OT-1 T cells after 4 h (Supplementary Figure 5b–d) and 24 h (Figure 5e–g). IFN γ is a key mediator of cytotoxic T cell motility and cytotoxicity,⁽⁵⁵⁾ and perforin and granzyme B are the main molecules involved in inducing apoptosis and mediating cytotoxicity of the B16-OVA target cells.⁽⁵⁶⁾ The IFN γ , perforin, and granzyme B concentrations were dramatically lower in the supernatant of cell-laden cryogels in hypoxia (60% to 95% inhibition) than in their normoxic counterparts, in agreement with the cytotoxicity assessments. While O₂-cryogels containing 0.1% CaO₂ had a minimal impact on hypoxic OT-1 T cells within the first 4 h, they were able to partially relieve the hypoxic stress

after 24 h, resulting in a slight increase in perforin and granzyme B production. Strikingly, cryogels containing higher amounts of CaO₂ completely restored the ability of OT-1 T cells to secrete perforin and granzyme B within 24 h. Overall, these results indicate that O₂-cryogels can locally reverse hypoxia, overcome hypoxia-driven T-cell inhibition, and restore the killing ability of OT-1 T cells via the secretion of proinflammatory cytokines and cytotoxic proteins fatal to B16-OVA cells (Figure 5h).

Boosting antitumor immune responses in an aggressive and advanced murine melanoma model

After demonstrating that O₂-cryogels reversed hypoxia-induced immunosuppression in vitro, we next tested their capacity to promote antitumor immunity as a co-adjuvant in a highly aggressive and advanced-stage murine melanoma model (Figure 6a). Advanced tumors were first established via subcutaneous inoculation of 2x10⁵ B16-F10 tumor cells on both flanks, and treatments were administered once advanced-stage tumors reached > 150 mm³ in volume (on day 17). PBS or O₂-cryogel were injected in each tumor (2 tumors/mouse), and as a control group, mice were placed in a hyperoxic chamber at 60% oxygen to promote immune-mediated tumor rejection.⁽¹⁷⁾ At day 24, photographs of subcutaneous tumors and excised tumors showed that the size of O₂-cryogel-treated tumors was significantly decreased compared to tumors in mice treated with PBS (sham) or respiratory hyperoxia. Strikingly, we observed local eradication of tumor tissue around O₂-cryogels after excision. Tumor growth for mice treated with hyperoxia was slightly reduced compared to PBS, whereas tumor sizes were markedly smaller following intratumoral injections of O₂-cryogels (Figure 6b). As a result, O₂-cryogel treatment extended mouse survival up to 35 days, with a median survival time of 31 days as opposed to 23 days for PBS or respiratory hyperoxia (Figure 6c). Overall, in comparison to PBS, the median lifespan of treated mice with O₂-cryogels was extended by nearly 35%.

To understand the mechanisms underlying the improved antitumor immune responses in O₂-cryogel-treated mice, immune cell types and fractions were quantified in tumors, tumor-draining LNs, and spleens at day 24 (Figure 6d and Supplementary Figure 7). While the fraction of viable immune cells was only 31

$\pm 8\%$ in PBS-treated tumors, O₂-cryogels greatly increased the viable immune cell fraction to $72 \pm 4\%$ (Figure 6d). Surprisingly, immune cell viability in the inguinal draining LNs was reduced in hyperoxia-treated mice. Furthermore, O₂-cryogels reduced the fraction of hypoxic immune cells in tumors, LNs, and spleens by 50% relative to PBS-injected tumors (Figure 6d, Supplementary Figure 7). The capacity of O₂-cryogels to promote antitumor immunity is further highlighted by the increased fractions of tumor-infiltrating NK cells from 9% to 15% and CD4⁺ T cells from 18% to 33%. In tumor-draining LNs, the number of NK cells was also increased (1.2% to 1.9%) with O₂-cryogels, whereas the number of CD4⁺ T cells did not. In contrast, hyperoxia significantly reduced CD4⁺ T-cell numbers in the LNs. Surprisingly, the fractions of CD8⁺ T cells in tumors, LNs, and spleens were not impacted by either O₂-cryogel or respiratory hyperoxia. O₂-cryogels were also shown to counteract immunosuppression in tumors and LNs by considerably reducing the expression of co-inhibitory receptor PD-1 on intratumoral T cells from 42% to 19%. Although O₂-cryogel injection did not alter PD-1 expression on T cells in the LNs and spleens, we observed an increase in the fraction of PD-1⁺ T cells in the LNs and spleens from hyperoxia-treated mice. Unlike with PBS, mice treated with hyperoxia and O₂-cryogels exhibited a substantial reduction in the fractions of CD4⁺CD25⁺FoxP3⁺ regulatory T cells in tumors and LNs, leading to higher ratios of cytotoxic CD8⁺ T cell to regulatory T cell by three- to four-fold, respectively.

To further characterize the oxygen-mediated immune response, serum cytokine levels were assessed at day 24 (Figure 6e). Compared to PBS, hyperoxia and O₂-cryogel treatment resulted in a two-fold increase in the concentrations of pro-inflammatory cytokines IL-5 and IL-6. There was also a marked increase in serum TNF α from 10.8 ± 1 to 19.6 ± 1.1 pg/mL with O₂-cryogels. Levels of several anti-inflammatory or tumor-promoting cytokines were also altered after treatments. Interestingly, intratumoral O₂-cryogel injection reduced serum levels of tumor-promoting cytokine IL-13 from 23.7 ± 1.1 to 20.4 ± 0.6 pg/mL. Moreover, IL-17 concentrations were increased in hyperoxia-treated mice. Serum levels of IFN γ , IL-2, IL-4, IL-9, and IL-22 were unaltered after hyperoxia or O₂-cryogel treatment (Supplementary Figure 8). Taken together, these data support a model in which O₂-cryogels act as a co-adjuvant to reverse intratumor

hypoxia-mediated immunosuppression in vivo (Figure 6f). This strategy reduces the number of regulatory T cells and expression of immune checkpoints. Furthermore, local oxygenation increases infiltration of cancer-fighting immune cells, including T cells and NK cells, leading to local elimination of tumor tissues.

Discussion

Hypoxia plays a key role in both the development and maintenance of tumor immune privilege. To overcome hypoxia-mediated tumor resistance, approaches targeting hypoxia, such as systemic hyperbaric oxygen therapy, and hypoxia-inducible signaling pathways such as HIF inhibitors and A2AR antagonists, have shown encouraging results in preclinical and clinical studies.⁽³⁰⁻³³⁾ These approaches have been reported to boost tumor-specific cytotoxicity of T cells and NK cells.⁽¹⁷⁾ However, to date, the modest efficacy in melanoma coupled with potential treatment-related toxicities have prevented their implementation as a standard of care.⁽⁵⁷⁾ Therefore, it is important to have an alternative, non-systemic anti-hypoxic strategy to reduce tumor hypoxia and enhance cancer immunotherapies. To this end, we developed a new and promising approach by engineering O₂-cryogels to target oxygen delivery in oxygen-deprived environments.

We created a new class of sponge-like and syringe-injectable O₂-cryogels that can produce oxygen in a controlled and sustained fashion, enabling reversal of local tumor hypoxia and restoration of T cell-mediated cytotoxicity. These O₂-cryogels were fabricated by physically caging solid CaO₂ particles within the cross-linked polymer network of cryogels. CaO₂ particles were homogeneously distributed throughout the cryogel structure, providing predictable and spatiotemporally controllable release of oxygen. To prevent H₂O₂-related toxicity, chemically modified CAT (APC) was covalently incorporated to the cryogel backbone.

Oxygen diffusion is often geometrically restricted in conventional (i.e., mesoporous) hydrogels,⁽⁵⁸⁾ altering both the cellular and local tissue oxygenation.^(40, 43) However, from O₂-cryogels, oxygen is

freely released to the surrounding environment, reaching up to 800 μM after 2 h, and in a sustained fashion for 2 days. O_2 -cryogel-mediated oxygenation reversed cellular hypoxia for at least 24 h, both through the entire construct and to distant locations. The highly interconnected macroporous channels of O_2 -cryogels facilitate oxygen transport into the surrounding environment, resulting in increased oxygen levels. In this scenario, oxygen release relies on the diffusion of free water through the polymer network to hydrolyze CaO_2 particles. However, in body tissues, bound water may result in slower and more sustained release of oxygen. Therefore, oxygen release kinetics could be further optimized for in vivo applications.⁽⁵⁹⁻⁶¹⁾ For instance, encapsulation of perfluorocarbon or H_2O_2 /polyvinylpyrrolidone (PVP)^(62, 63) particles in O_2 -cryogels could facilitate oxygen production in body tissues. Additionally, other peroxides (e.g., magnesium peroxide, zinc peroxide), oxides (e.g., manganese dioxide and zinc oxide), and percarbonates (e.g., sodium percarbonate) could be used as alternative oxygen-generating agents.⁽⁴³⁾

O_2 -cryogels were conjugated with CAT to decompose H_2O_2 into additional oxygen. This feature resulted in a safe biomaterial when subcutaneously injected in mice, and cells cultured within these constructs remained viable under both normoxic and hypoxic conditions. However, cell viability was slightly altered when B16-F10 cells were cultured in O_2 -cryogels containing 1% CaO_2 . This could be attributed to a partial loss of enzymatic activity during the chemical modification of CAT and its successive cryogenic treatments. Furthermore, once anchored within the polymer walls, the steric hindrance could also alter CAT activity in depleting H_2O_2 . To protect CAT, several techniques could be investigated. For instance, optimizing the chemical modification of CAT, introducing different PEG spacers to reduce steric hindrance, and/or incorporating lyoprotectants (e.g., trehalose) to prevent freeze-drying induced protein denaturation should help preserve CAT to deplete H_2O_2 more effectively.⁽⁶⁴⁾ Alternatively, conjugating CAT to cryogels post-fabrication could also be used as a means to retain its enzymatic activity.

Oxygen-generating biomaterials have the potential to restore the function of impaired pre-existing T cells when subjected to hypoxia. In this work, we showed that O_2 -cryogel-induced oxygenation can reinstate T

cell-mediated antitumor activity in hypoxia to levels comparable to those observed in normoxia. Although there was only a slight increase in IFN γ secretion, hypoxia reduction substantially enhanced T cell-mediated secretion of cytotoxic proteins such as perforin and granzyme B. Strikingly, the tumoricidal function of T cells was restored after only 4 h, and as little as 0.1% CaO₂ was sufficient to boost cytotoxic protein secretion after 24 h. Overcoming cellular hypoxia most likely reinforced the killing ability of OT-1 T cells against tumor cells. Mechanistically, O₂-cryogels appeared to prevent stabilization of hypoxia-dependent HIF1 α , a major orchestrator of tumor cell adaptation to hypoxia and aggressiveness. O₂-cryogels also induce the downregulation of VEGF α , Wnt11, CD44, and CD133, which have been associated with angiogenesis as well as tumor cell proliferation, migration, and invasion in solid tumors.(65-69) Additionally, O₂-cryogels inhibited the expression of adenosine-producing ecto-enzyme CD73. This was further confirmed with reduced concentrations of extracellular adenosine, a key immunosuppressive metabolite that may allow “cold” tumors to escape immune surveillance.(25-27, 70) Collectively, our data suggest that O₂-cryogels contribute to oxygen-mediated restoration of T-cell effector function within a hypoxic TME. This is in agreement with previous studies where Sitkovsky et al. showed that oxygenation increases the density of peptide-major histocompatibility complex (MHC) class I molecules at the surface of tumor cells, enhancing their recognition and elimination by tumor-specific T cells.(71)

O₂-cryogels developed in this study can withstand the injection process without releasing CaO₂ particles. Furthermore, these gels can overcome shear stress during injection while retaining their oxygen-generating feature. This characteristic is a significant advantage over previously reported oxygen-generating preformed hydrogels that require invasive surgical implantations.(41, 42, 46) We believe that, similarly to systemic oxygenation,(72) local delivery of oxygen from O₂-cryogels can be used as co-adjuvant therapy in the treatment of malignancies. However, unlike systemic oxygenation, local oxygen delivery from O₂-cryogels has the potential to target any solid tumors and prevent potential side effects

(e.g., pulmonary edema).(73) In this work, we showed that respiratory hyperoxia, our positive control, and O₂-cryogel co-adjuvant therapies can both slow down tumor growth and increase mice survival in a highly aggressive and advanced-stage murine melanoma model. Both strategies reduced the fractions of hypoxic immune cells in tumors, allowing these cells to overcome immunosuppression(74), as indicated with the decrease of tumor-infiltrating regulatory T cells. However, in our study, respiratory hyperoxia was associated with reduced immune cell viability in the tumor-draining inguinal LNs, and PD-1 expression on T cells was increased, suggesting that long-term systemic oxygenation and/or poor oxygenation of subcutaneous tumors could impair immune cell function. In addition, this treatment led to high serum levels of IL-17 and IL-13, two cytokines promoting cancer progression,(75, 76) which is in agreement with the moderate effect seen with respiratory hyperoxia on curbing tumor growth. In contrast, O₂-cryogel therapy increased CD4⁺ T cell number and the CD8⁺/regulatory T cell ratio in tumors, suggesting a significant shift in the immune balance from immunosuppression to active antitumor immunity. This is further demonstrated by elevated serum level of TNF- α , indicating that local oxygen delivery mediated a strong pro-inflammatory immune response. Interestingly, O₂-cryogels also enhanced immune cell survival within the tumors and promoted infiltration of NK cells, important contributors to antibody-dependent cell-mediated toxicity and cell-mediated immune responses against cancer.(77) These observations might explain why O₂-cryogel-mediated co-adjuvanted oxygen therapy induced tumor regression locally, delayed overall tumor growth, and prolonged survival. Collectively, our data suggest that O₂-cryogel therapy outperformed respiratory hyperoxia, used here as our gold standard. Furthermore, local oxygen delivery may provide a therapeutic strategy to reduce hypoxic regions within solid tumors across various cancers and drive an influx of cancer-fighting effector cells(17) to induce tumor regression or rejection.(16, 22, 46)

Recently, injectable cryogel vaccines have shown to be effective as a prophylactic treatment against melanoma.(78, 79) These vaccines elicit strong and long-lasting antitumor immune responses. Yet, their

efficacy in a therapeutic setting is limited and requires further optimization. In this context, O₂-cryogels could potentially be used as co-adjuvant to reinforce DC activation and ultimately induce downstream stimulation of cytotoxic T cells. Additionally, further in vivo testing is required to fully assess the clinical potential of our approach and investigate whether O₂-cryogels can rescue other oxygen-deprived antitumor immune cells, such as macrophages and dendritic cells.

Moving forward, innovative biomaterials such as O₂-cryogels hold great potential to advance the future of cancer immunotherapy.(49, 50, 78-82) Considering the recent clinical approval of several immune checkpoint inhibitors for cancer treatment(83) and the recent advances in targeting the hypoxia-adenosinergic signaling pathway,(84) the combination of O₂-cryogels with immune checkpoint blockade (e.g., CTLA-4 or PD-1)(17) should be investigated for a synergistic effect. This is further supported with recent studies suggesting hypoxia as a potential obstacle on immune checkpoint inhibitors to treat cancer.(17, 85) Additionally, O₂-cryogel-mediated hypoxia reduction could be combined with other strategies under development to tackle hypoxia-driven immunosuppression such as A2AR antagonists,(31, 86) HIF inhibitors,(30, 33) and hyperoxia(16, 34) to cooperatively bypass hypoxia-driven T-cell immunosuppression and restore tumor-reactive T-cell function.

Methods

Materials

Sodium salt of HA, phosphate-buffered saline (PBS), dimethylformamide (DMF), glycidyl methacrylate (GM), triethylamine (TEA), adipic acid dihydrazide (AAD), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), CAT, calcium peroxide (CaO₂) particles, tetramethylethylenediamine (TEMED), ammonium persulfate (APS), Alizarin Red S, fluorimetric hydrogen peroxide assay kit, fetal bovine serum (FBS), paraformaldehyde (PFA), Triton X-100, and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). NHS-rhodamine, 5/6-carboxy-tetramethyl-rhodamine succinimidyl ester (NHS-rhodamine), penicillin, streptomycin, gentamycin, rabbit anti-mouse IgG (H+L) Alexa Fluor 488 secondary antibody, PureLink™ RNA Mini Kit, High Capacity cDNA Reverse Transcription Kit gene expression assays were purchased from Thermo Fisher Scientific (Waltham, MA, USA). IFN γ , TNF α , and granzyme B DuoSet ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA). Carboxyfluorescein succinimidyl ester (CFSE), CD45 (PE/Cy7, clone 104), NK1.1 (APC, clone PK136), PD-1 (BV421, clone 29F.1A12), CD3 (BV605, clone 17A2), CD4 (PerCP/Cy5.5, clone GK1.5), CD8 (Alexa Fluor 700, clone 53-6.7), CD25 (APC/Cy7, clone 3C7) and FoxP3 (PE, clone MF-14) antibodies, and LEGENDplex Mouse Th Cytokine Panel were purchased from Biolegend (San Diego, CA, USA). OVA 257-264 (SIINFEKL peptides) was purchased from InvivoGen (San Diego, CA, USA). Amine-terminated GGGGRGDSP (G₄RGDSP) peptide was purchased from Peptide 2.0 (Chantilly, VA, USA). Acrylate-PEG-N-hydroxysuccinimide (3.5 kDa) was purchased from JenKem Technology (Plano, TX, USA). ViaQuant™ Fixable Far-Red Dead Cell Staining Kit was purchased from GeneCopoeia (Rockville, MD, USA). Alexa Fluor 488-phalloidin was purchased from Cell Signaling Technology (Danvers, MA, USA). The Hypoxyprobe Plus kit was purchased from Hypoxyprobe Inc. (Burlington, MA, USA). A fluorometric adenosine assay kit was purchased from Biovision (San Francisco, California, USA). The Perforin ELISA kit was purchased from G-Biosciences

(St. Louis, MO, USA). Melanoma cells (B16-F10, CRL-6475) were purchased from ATCC (Rockville, MD, USA).

Chemical modification of hyaluronic acid

HA was conjugated with GM as previously described.⁽⁸⁷⁾ Briefly, HA salt (5 g) was dissolved in PBS (1 L, pH 7.4) and mixed with DMF (335 mL), GM (62 mL), and TEA (46 mL). The reaction was allowed to proceed for ten days at room temperature (RT), then the mixture was precipitated in a large excess of acetone, filtered using grade 4 Whatman paper, and dried overnight at RT in a vacuum oven. The resulting product, hyaluronic acid glycidyl methacrylate (HAGM), was characterized by ¹H nuclear magnetic resonance (NMR).

Rhodamine-labeled HAGM (R-HAGM) was prepared by coupling NHS-Rhodamine with amine-terminated HAGM (NH₂-HAGM) as previously described.⁽⁸⁷⁾ To synthesize NH₂-HAGM, HAGM (1 g) was dissolved in 0.1 M 2-(N-morpholino) ethanesulfonic acid (MES) (100 mL) at pH 5.5. Next, AAD (4 g) and EDC (90 mg) were added to the solution. The reaction mixture was stirred at RT for 4 h to allow the amination of HAGM to proceed. NH₂-HAGM was then precipitated in a large excess of acetone, filtered, and dried in a vacuum oven overnight at RT. NH₂-HAGM (1 g) was dissolved in 0.1 M NaHCO₃ buffer solution (10 mL) at pH 8.5 and mixed with NHS-Rhodamine (10 mg). The reaction was allowed to proceed for 16 h at RT, and R-HAGM was precipitated in a large excess of acetone, filtered, and then dried overnight at RT in a vacuum oven.

Chemical modification of G₄RGDSP peptide and catalase

Acrylate-PEG-G₄RGDSP (APR) was synthesized by coupling the amine-terminated G₄RGDSP peptide to acrylate-PEG-N-hydroxysuccinimide (molar ratio, 1:1). Briefly, acrylate-PEG-N-hydroxysuccinimide (100 mg) and G₄RGDSP (22.3 mg) were mixed in 1 mL 0.1 M NaHCO₃ buffer solution at pH 8.5, allowed

to react for 4 h at RT, and freeze dried overnight. Similarly, acrylate-PEG-CAT (APC) was synthesized by coupling the CAT enzyme to acrylate-PEG-N-hydroxysuccinimide comonomers (molar ratio, 1:3).

Fabrication of O₂-cryogels

CaO₂-containing cryogels (i.e., O₂-cryogels) were fabricated by redox-induced free radical cryopolymerization in dH₂O at -20 °C. Briefly, HAGM (40 mg, 4% wt/vol) was mixed with APR (8 mg, 0.8% wt/vol), APC (10 mg, 1% wt/vol), and various concentrations of CaO₂ (0–1% wt/vol) in dH₂O (1 mL). The mixture was precooled at 4 °C, and then TEMED (0.42% wt/vol) and APS (0.84% wt/vol) were added to the solution. The solution was poured into Teflon® molds, transferred to a freezer preset at -20 °C, and cryopolymerized for 16 h. Finally, O₂-cryogels were brought to RT to remove ice crystals and washed with dH₂O. For microscopy imaging, a fraction of HAGM (0.3% wt/vol) in each formulation was substituted with R-HAGM to fluorescently stain the polymer network of cryogels.

Characterization of CaO₂ encapsulation and distribution, and O₂-cryogel microstructural features

The encapsulation efficiency of CaO₂ within the polymer walls of O₂-cryogels was determined by Alizarin Red S staining, which stains red on reaction with inorganic calcium. Specifically, a 2% wt/vol Alizarin Red S solution was freshly prepared in dH₂O and adjusted to pH 4.2. O₂-cryogels were incubated with Alizarin Red S for 20 min, rinsed with dH₂O several times until the washing solution was clear, and photographed with a Canon camera or imaged using a Leica TCS SP5 X WLL confocal microscope (Buffalo Grove, IL, USA). The CaO₂ distribution in the polymer walls as well as the microstructural features of O₂-cryogels were characterized by scanning electron microscopy and energy-dispersive X-ray analysis (EDX). First, O₂-cryogels were freeze dried for 24 h, mounted on a sample holder using carbon tape, and sputter coated with platinum/palladium up to a thickness of 5 nm. The resulting samples were imaged by secondary electron detection on a Hitachi S-4800 scanning electron microscope at a voltage of

25 kV and a current of 10 μ A. Pore sizes were determined using the Fiji(88), Analyze Particles, and Diameter J software plugins.

Physical characterization of O₂-cryogels

The swelling ratio was determined using a conventional gravimetric procedure as previously described.(49) Briefly, O₂-cryogel cylinders (6-mm diameter, 6-mm height) were prepared, immersed in PBS for 24 h at 37 °C, and weighed (m_s). Next, O₂-cryogel cylinders were washed in dH₂O, freeze dried, and weighed again (m_d). Then, as shown in Equation 1, the equilibrium mass swelling ratio (Q_m) was calculated by dividing the mass of fully swollen O₂-cryogel (m_s) by the mass of freeze-dried O₂-cryogel (m_d):

$$Q_m = \frac{m_s}{m_d} \quad (\text{Equation 1})$$

The degree of pore connectivity was evaluated using a water wicking technique. Specifically, fully hydrated O₂-cryogel disks (6-mm diameter, 1-mm height) were first weighed on an analytical scale. Next, a Kimwipe® was used to wick away free water within interconnected pores, and gels were weighed a second time. The degree of pore connectivity was then calculated as the volume of water (%) wicked away from the gels.

Young's modulus values were determined using an Instron 5944 universal testing system (Instron, Norwood, MA, USA). Cylindrical cryogels (6-mm diameter, 8-mm height) were dynamically deformed at a constant rate between two parallel plates for 10 cycles (strain rate of 10% per min) under constant hydration in PBS (pH 7.4). Compressive strain (mm) and load (N) values were then quantified at the 8th cycle using Instron's Bluehill 3 software. Young's modulus values were determined by measuring the tangent of the slope of the linear region on the loading stress-strain curve.

Enzymatic activity of catalase and its derivatives (H₂O₂ depletion)

The enzymatic activities of CAT and APC were assessed using a fluorimetric hydrogen peroxide assay kit. Aqueous enzyme solutions were prepared at different concentrations (0.1–5 u/mL), mixed with 30 μ M H₂O₂, and incubated at 37 °C for 30 min. Then, the fluorescence intensity (Ex/Em = 540/590 nm) was recorded using a Synergy HT plate reader (BioTek, Winooski, Vermont, USA) to quantify the residual H₂O₂ concentration in the solution. To evaluate potential H₂O₂ depletion from O₂-cryogels, cryogel samples were incubated in dH₂O at 37 °C for 15 min, transferred to 96-well plates, and assessed using the fluorimetric hydrogen peroxide assay kit, as described above.

Oxygen release kinetics from O₂-cryogels

The oxygen release kinetics of O₂-cryogels were determined using needle-type optical oxygen microprobes (PyroScience GmbH, Aachen, Germany) under normoxic (20% O₂) and hypoxic conditions (1% O₂). Briefly, O₂-cryogel cylinders (6-mm diameter, 8-mm height) were individually placed into 2 mL Eppendorf tubes containing Dulbecco's modified Eagle's medium (1.8 mL, DMEM) (Corning, Corning, NY, USA) and incubated at 37 °C under normoxic or hypoxic conditions using a Napco CO₂ 1000 hypoxic incubator (Thermo Fisher Scientific). The needle-type probe was positioned in the center of the cylinders, and the dissolved oxygen concentration (μ mol/L, 1 point every 300 seconds) was recorded for up to 2 days to monitor oxygen release. Additionally, to mimic syringe injection conditions, O₂-cryogels were compressed by up to 90% of their volume prior to additional oxygen release measurements. Briefly, cryogel cylinders were allowed to swell in PBS until reaching equilibrium and were mechanically compressed between two parallel plates. The compressed cryogels were allowed to swell in DMEM until they returned to their initial shape and size, and oxygen release was monitored for up to 2 days at 37 °C under normoxic conditions.

Cell seeding and culture in O₂-cryogels

Melanoma cells (B16-F10) were cultured in complete DMEM (DMEM supplemented with 10% FBS, 100 µg/mL penicillin, and 100 µg/mL streptomycin). B16-F10 ovalbumin (OVA)-expressing cells (B16-OVA, kindly provided by Prof. M. Sitkovsky) were cultured in complete DMEM supplemented with 50 µg/mL gentamycin. Cells were incubated at 37 °C in either humidified 5% CO₂/95% air (normoxia) or humidified 5% CO₂/1% O₂/94% N₂ (hypoxia). Prior to cell seeding, square-shaped O₂-cryogels (dimensions: 4 mm × 4 mm × 1 mm) were sanitized with 70% ethanol for 15 min, washed several times with sterile PBS, and mechanically compressed on sterile gauze to partially remove water from the pores under sterile conditions. Finally, a B16-F10 or B16-OVA cell suspension in complete DMEM (10⁷ cells/mL, 10 µL) was added dropwise to the top of each square-shaped cryogel and incubated for 1 h to allow cell adhesion. The cell-laden O₂-cryogels were then supplemented with additional complete DMEM (2 mL) for the duration of the experiment. For experiments requiring a hypoxic environment, complete DMEM was preincubated (conditioned) for at least 1 h under hypoxic conditions.

Cell viability assay

Cell viability within O₂-cryogels was evaluated by a live/dead assay. After a 1-day incubation, cell-laden O₂-cryogels were incubated for 15 min in the presence of a ViaQuant™ Fixable Far-Red Dead Cell Staining Kit according to the manufacturer's instructions. Next, the cryogels were rinsed with PBS (1 mL, 2 times), fixed with 4% PFA (0.5 mL) for 20 min at RT, and washed with PBS (1 mL, 2 times). The cells were permeabilized in PBS supplemented with 0.1% Triton X-100 (0.5 mL) for 5 min, successively stained with Alexa Fluor 488-phalloidin and DAPI (1 mL) according to the manufacturer's protocols and imaged by confocal microscopy using a Leica TCS SP5 X WLL instrument. Tile images were recreated from five individual O₂-cryogel samples to visualize all the dimensions of the gel, and cell viability was calculated by Fiji software(88) as the ratio between the dead cell number and the total cell number, which

correspond to surface areas positive for far-red and blue or green fluorescence, respectively. For each condition, one representative high-resolution stacked image was collected with a 2- μ m separation between slices (z-stacks).

Minimally invasive delivery and biocompatibility assessment

Seven-week-old female C57BL/6J mice (n = 4; The Jackson Laboratory, Bar Harbor, Maine, USA) were anesthetized with isoflurane (4–5% for induction and 1–3% for maintenance) in oxygen using an inhalation anesthesia system (300 SERIES vaporizer, VSS, Rockmart, GA, USA). Cryogels (i.e., CaO₂-free cryogels) and O₂-cryogels (i.e., CaO₂-containing cryogels) were suspended in PBS (0.2 mL) and subcutaneously injected into both dorsal flanks of each mouse using a 16-gauge hypodermic needle. After 7 days, mice were euthanized, and cryogels and O₂-cryogels were explanted from the surrounding tissues. Next, each sample was fixed for 48 h in 4% PFA (2 mL), embedded in paraffin, cryosectioned into 5- μ m-thick slices, and stained with hematoxylin and eosin (H&E) as well as Masson's trichrome (MT) for histological analysis.

Hypoxia detection assays

Cellular hypoxia within O₂-cryogels was assessed using a HypoxyprobeTM-1 kit according to the manufacturer's recommendations. Briefly, after 24 h of incubation in normoxia or hypoxia, cell-laden O₂-cryogels were incubated for 2 h in 200 μ M HypoxyprobeTM-1 in complete DMEM (2 mL), fixed, permeabilized, and immunostained with mouse MAb1 antibody and rabbit anti-mouse IgG (H+L) Alexa Fluor 488 secondary antibody, as well as DAPI. Cryogel samples (cryogels and O₂-cryogels) were then imaged by confocal microscopy using the Leica TCS SP5 X WLL instrument. Cellular hypoxia fractions were calculated using Fiji software(88) based on the ratio between the number of hypoxic cells (green fluorescence) and the total number of cells (blue fluorescence).

Adenosine concentration was quantified using a fluorometric adenosine assay kit following the manufacturer's protocol. After 24 h of incubation under either normoxic or hypoxic conditions, supernatants from each cell-laden cryogel samples (cryogels and O₂-cryogels) were collected and mixed with the assay kit. The adenosine concentration was determined based on the fluorescence intensity (Ex/Em = 535 nm/587 nm), which was measured using a Synergy HT plate reader (BioTek) and normalized using an adenosine standard curve.

The gene expression of HIF1 α , wingless-type MMTV integration site family member 11 (Wnt11), vascular endothelial growth factor α (VEGF α), cluster of differentiation 44 (CD44), CD73, CD133, and hypoxanthine guanine phosphoribosyl transferase (HPRT) were assessed by RT-qPCR. After 24 h of incubation in either normoxia or hypoxia, total RNA was extracted from cell-laden cryogel samples using a PureLinkTM RNA Mini Kit according to the manufacturer's protocol. For RT-qPCR, we first used a High Capacity cDNA Reverse Transcription Kit on a MyCycler (Bio-Rad, Hampton, NH, USA). Then, gene expression was measured using the following TaqMan[®] Gene Expression Assays on an Mx3005P QPCR System (Agilent, Santa Clara, CA, USA): *HIF1 α* : Mm00468869_m1, *Wnt11*: Mm00437327_g1, *VEGF α* : Mm00437306_m1, *CD44*: Mm01277163_m1, *CD73*: Mm00501915_m1, *CD133*: Mm00477115_m1, and *HPRT* (housekeeping gene): Mm03024075_m1.

Cytotoxicity assay in a B16-OVA melanoma model

B16-OVA cells were stained with 10 μ M CFSE for 15 min at 37 °C according to the manufacturer's protocol. Next, 1 $\times$ 10⁵ cells in complete Roswell Park Memorial Institute (RPMI) medium (RPMI medium supplemented with 10% FBS, 100 μ g/mL penicillin, and 100 μ g/mL streptomycin) were seeded onto square-shaped cryogels and incubated in normoxia. After 3 h of incubation, splenocytes isolated from OT-1 RAG mice (The Jackson Laboratory, Bar Harbor, ME, USA) in complete RPMI (2 $\times$ 10⁵ cells, 1:2 target/effector ratio) were added to each well. Splenocytes were prestimulated with 2 μ g/mL SIINFEKL

peptides for 24 h in complete RPMI supplemented with 50 μ M 2-mercaptoethanol. For experiments requiring a hypoxic environment, complete RPMI, splenocyte suspensions, and B16-OVA-laden O₂-cryogels were preincubated (conditioned) for 1 h in hypoxia prior to coculture. After 24 h of B16-OVA/splenocyte coculture, (i) supernatants were collected to quantify IFN γ , TNF α , granzyme B, and perforin secretion by ELISA; and (ii) the cytotoxicity of OT-1 cells was assessed by the cell viability assay described above.

The cytolytic activity of OT-1 T cells towards B16-OVA cells was monitored by time-lapse microscopy. First, under normoxic conditions, 1×10^5 B16-OVA cells in complete RPMI were seeded in a 24-well plate for 3 h to allow adhesion. Next, 2×10^5 activated splenocytes in complete RPMI (1:2 target/effector ratio) with either square-shaped cryogels or O₂-cryogels containing 0.5% CaO₂ were added to each well (total volume = 500 μ L). Complete RPMI, splenocyte suspensions, and the 24-well plate seeded with B16-OVA cells were preincubated (conditioned) for 1 h in hypoxia prior to coculture. Next, time-lapse experiments were performed in a closed chamber using a bright field inverted microscope (Zeiss Axio Observer Z1, Zeiss, Oberkochen, Germany) at 37 °C under hypoxic conditions (5% CO₂ + 1% O₂ + 94% N₂). One preselected region of each well (350 $\times$ 350 μ m) was imaged every 10 min for 4 h.

Intratumoral oxygen delivery as a therapeutic co-adjuvant in advanced-stage tumor-bearing mice

Murine melanoma tumors (two tumors/mouse) were established in C57BL/6 mice by subcutaneous injection of B16-F10 cells (2×10^5 cells in 100 μ L PBS) in both flanks. Seventeen days following tumor inoculation (tumor volume > 150 mm³), mice were treated twice a week with either intratumoral injection of PBS (100 μ L, sham), cylindrical-shaped 1% O₂-cryogel (height: 35 mm, diameter: 1.2 mm; in 100 μ L PBS) or placed in chambers with controlled oxygen content (60%) to mimic supplemental oxygen therapy to humans.⁽⁸⁹⁾ Tumor volume was measured twice a week using a caliper. Once tumors exceeded 1 cm³, or severe ulceration, bleeding, or weight variation were observed, mice were euthanized. To assess the

antitumor immune response, cryogels, spleens, and lymph nodes (LNs) were explanted and dissociated at day 24, and the immune cell populations were analyzed by flow cytometry (AttuneNxT, Thermo Fisher Scientific). The following markers were used to determine the T cell, NK cell, and hypoxic cell subpopulations: CD45 (immune cells), HypoxyprobeTM-1 (hypoxic cells), NK1.1 (natural killer cells), PD-1 (checkpoint inhibitor), CD3 (T cells), CD4 (T helper cells), CD8 (cytotoxic T cells), CD25 and FoxP3 (regulatory T cells). To evaluate the systemic inflammatory response, mouse blood was collected at day 24 (50 μ L), and the level of IL-2, IL-4, IL-5, IL-6, IL-9, IL-13, IL-17A, IL-17F, IL-22, IFN- γ , and TNF- α in serum was analyzed using LEGENDplex Mouse Th Cytokine Panel.

Animal studies

All experiments requiring mice were conducted in compliance with the National Institutes of Health (NIH) guidelines and approved by the Division of Laboratory Animal Medicine and Northeastern University Institutional Animal Care and Use Committee (protocol number 17-0828R).

Statistical analysis

All data are presented as the mean $\pm$ standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism software (La Jolla, CA, USA). Significant differences between groups were analyzed by one-way analysis of variance (ANOVA) and Dunnett's or Bonferroni's post-hoc test, two-tailed Pearson test, or a two-tailed Mantel-Cox test. Survival analysis was performed with the Kaplan-Meier test. As a proxy for life expectancy, the median survival time was determined as the time when a survival curve reaches 50% on the y-axis. Differences were considered statistically significant at $P < 0.05$.

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Author contributions

T.C. and S.A.B. conceived and designed the experiments. T.C., L.J.E., and M.R. performed the experiments. T.C., S.H., L.J.E., A.M., M.V.S and S.A.B. analyzed the data and wrote the manuscript. T.C., L.J.E., A.M. and S.A.B. conceived the figures. All authors discussed the results and commented the manuscript. The principal investigator is S.A.B.

Conflicts of Interest

The authors declare no conflicts of interest.

Figures

a

b

Figure 1. Schematic describing the fabrication process of O₂-cryogels and their ability to restore T cell-mediated cytotoxicity in a hypoxic tumor microenvironment. **a)** To fabricate O₂-cryogels, hyaluronic acid grafted with methacrylate residues (HAGM), calcium peroxide CaO₂ particles, acrylate-PEG-catalase (APC), acrylate-PEG₃₅₀₀-G₄RGDSP (APR), and ammonium persulfate/tetramethylethylenediamine (initiator system) were mixed and incubated at -20 °C for 16 h to allow cryopolymerization. Steps 1,2: This process involves a phase separation with ice crystal formation, solutes concentration (polymer, APC, APR, CaO₂), and crosslinking within a non-frozen liquid phase leading to CaO₂ particle entrapment within the dense polymer walls. Step 3: A thawing process was performed to remove ice crystals and leave an open interconnected macroporous network. Step 4: The mechanically robust O₂-cryogels sustained syringe injection using a conventional small-bore needle and released oxygen in a controlled manner when hydrated. The schematic shows the active release of oxygen bubbles by O₂-cryogels in water. **b)** In hypoxia, tumor cell-laden cryogels can generate an immunosuppressive environment that inhibits cytotoxic T cells and prevents their cytotoxic function (left panel). Oxygen-releasing O₂-cryogels can reverse local hypoxia and restore the ability of T cells to target tumor cells, T cell-tumor cell interactions, and the secretion of cytotoxic proteins (e.g., perforin and granzyme B), leading to apoptotic tumor cell death and tumor cell debris (right panel).

Figure 2. Physical characteristics of O₂-cryogels. O₂-cryogels were prepared with 0.1, 0.3, 0.5, or 1% wt/v CaO₂ and their physical features compared to cryogels. **a–i)** Entrapped calcium peroxide particles within the O₂-cryogel polymer walls. **a)** Photographs showing cryogels and O₂-cryogels stained with Alizarin Red S before and after syringe injection. **b–g)** Confocal images showing CaO₂ particles (stained with Alizarin Red S) physically entrapped within O₂-cryogel polymer walls at various concentrations (**c–g**) and compared to cryogels (**b**). The confocal image (**f**) highlights the hierarchical macroporous structure of O₂-cryogels containing 0.5% CaO₂ (entrapped CaO₂ particles, crosslinked polymer walls, and interconnected macropores). **h,i)** Scanning electron microscopy images showing cryogels (**h**) and entrapped particles within the polymer walls of O₂-cryogels containing 0.5% CaO₂ (**i**). Pseudocoloring in pink is used to highlight CaO₂. **j, k)** Elemental analysis of cryogels and O₂-cryogels containing 0.5% CaO₂. **l)** Pore size of cryogels and O₂-cryogels. **m)** Scanning electron microscopy images showing the interconnected macroporous structure of cryogels and O₂-cryogels. **n–p)** Physical characterization. **n)** Pore connectivity, **o)** Mass swelling ratios, and **p)** Young's modulus values of cryogels and O₂-cryogels. Each image or photograph is representative of n = 5 samples. Values represent the mean ± SEM (n = 6 cryogels per condition). Data were analyzed using ANOVA and Dunnett's post-hoc test (compared to cryogels); *P < 0.05, **P < 0.01. Scale bars = 4 mm (**a**), 50 μm (**b–g**), 25 μm (**i**), and 200 μm (**m**).

Figure 3. O₂-cryogels enable controlled and sustained release of oxygen and are cytocompatible (in vitro) and biocompatible (in vivo). **a)** H₂O₂ release from catalase-free (white) and (red, catalase-containing) O₂-cryogels. **b)** Oxygen measurement setup. Cryogels and O₂-cryogels were individually immersed in 2-mL Eppendorf tubes containing cell culture medium and incubated at 37 °C under normoxic (20% O₂) or hypoxic (1% O₂) conditions. A needle-type optical oxygen microprobe was inserted in the center of each cryogel, and changes in the dissolved oxygen concentration were recorded during the course of the experiment. **c, d)** Controlled oxygen release from cryogels and O₂-cryogels under normoxic (c) and hypoxic conditions (d). **e, f)** B16-F10 cell viability after 24 h of incubation within cryogels and O₂-cryogels under (e) normoxic (20% O₂) and (f) hypoxic (1% O₂) conditions. **g)** Representative confocal images of B16-F10 cell viability after 24 h of incubation within cryogels and O₂-cryogels under normoxic (top) and hypoxic (bottom) conditions. Blue = nuclei stained with DAPI, red = dead cells stained with ViaQuant Far Red, green = actin cytoskeleton stained with Alexa Fluor 488 phalloidin, yellow = polymer walls stained with rhodamine. **h)** Histological analysis of explanted cryogels (cryogel) containing 1% catalase, O₂-cryogels containing 1% CaO₂ (1% O₂-cryogel) and 1% catalase, and catalase-free 1% O₂-cryogels on day 7 post-subcutaneous injection using H&E and MT staining. The arrows indicate the boundary between the cryogel and host tissue. Confocal and histological images are representative of n = 5 samples per condition. Values represent the mean ± SEM (n = 6 cryogels). Data in panels (a, e, f) were analyzed using ANOVA and Dunnett's post-hoc test (compared to catalase-free O₂-cryogels); *P < 0.05, **P < 0.01, ***P < 0.001. Scale bars = 50 μm (g) and 200 μm (h).

Figure 4. Cellular hypoxia reversal in melanoma cells via O₂-cryogel-mediated oxygenation. **a)** Detection of hypoxia in B16-F10 cells in cryogels and O₂-cryogels cultured in 1% O₂ for 24 h. Blue staining = nuclei (stained with DAPI), green = hypoxic cells stained with HypoxyprobeTM-1. The data are representative of n = 4 samples per condition. **b)** Quantification of cellular hypoxia following a 24-h incubation period in cryogels and O₂-cryogels under normoxic or hypoxic conditions. **c)** Adenosine concentration in B16-F10 cell supernatants after 24 h of incubation in cryogels and O₂-cryogels under normoxic and hypoxic conditions. **d)** HIF1 α , Wnt11, CD73, VEGF α , CD44, and CD133 expression levels in B16-F10 cells after 24 h of incubation in cryogels and O₂-cryogels containing 0.5% CaO₂ under normoxic and hypoxic conditions. Gene expression was determined by RT-qPCR, normalized against the expression level of HPRT (housekeeping gene) and compared to that in cell-laden cryogels in normoxia ($2^{-\Delta\Delta CT} = 1$). Values represent the mean $\pm$ SEM (n = 3–5). Data were analyzed using ANOVA and Dunnett's post-hoc test (compared to cryogels in normoxia); *P < 0.05, **P < 0.01. Scale bar = 100 μ m.

Figure 5. O₂-cryogels restore T cell-mediated cytotoxicity against hypoxic tumor cells. a) Restored T cell activity in O₂-cryogels. **b)** Time-lapse images showing OT-1 T cells attacking B16-OVA cells in cryogels and O₂-cryogels containing 0.5% CaO₂ after incubation for 1 h (top) and 4 h (bottom) under hypoxic conditions (1% O₂). B16-OVA cells and OT-1 T cells are pseudocolored in green and red, respectively. **c)** Confocal images showing cell viability of B16-OVA in O₂-cryogels containing 0% or 0.5% CaO₂ after 24 h of co-culture with OT-1 T cells under hypoxic conditions (1% O₂). Red = dead cells stained with ViaQuant Far Red, blue = nuclei stained with DAPI. The data are representative of n = 5 samples per condition. **d-g)** B16-OVA cells were cocultured with OT-1 T cells for 24 h under normoxic (20% O₂, red background) or hypoxic (1% O₂, blue background) conditions. **d)** Quantification of T cell-mediated cytotoxicity. **e–g)** Secretion of IFN γ (e), granzyme B (f), and perforin (g) from OT-1 T cells. **h)** Schematic depicting an O₂-cryogel (i) inducing local oxygenation in an oxygen-deprived solid tumor, (ii) reversing hypoxia-driven immunosuppression, (iii) restoring the tumoricidal function of cytotoxic T cells fatal to tumor cells. Values represent the mean $\pm$ SEM (n = 4). Data were analyzed using ANOVA and Dunnett's post-hoc test (compared to cryogels), *P < 0.5, **P < 0.01. Scale bars = 20 μ m (b) and 100 μ m (c).

Figure 6: Antitumor immune response and survival in advanced-stage tumor-bearing mice treated with O₂-cryogel therapy as co-adjuvant. **a)** Mice were inoculated at day 0 with 2×10^5 B16-F10 cells in both flanks. On day 17, when the tumor volume reached $> 150 \text{ mm}^3$, tumor-bearing mice were injected intratumorally with PBS or O₂-cryogels twice a week until the end of the study ($n = 10/\text{group}$). Another group of tumor-bearing mice ($n = 10$) was placed into a hyperoxic chamber at 60% O₂. On day 24, half of the mice were sacrificed to perform mechanistic studies ($n = 5/\text{group}$, mouse back and tumor photographs), and the other half ($n = 5/\text{group}$) continued treatment. **b)** Melanoma tumor growth curve. **c)** Kaplan-Meier survival curves based on a $1,500 \text{ mm}^3$ tumor volume as the endpoint criteria. **d)** Flow cytometry quantification of the number of immune cells (CD45⁺), hypoxic immune cells (CD45⁺ Hypoxia⁺), natural killer cells (CD45⁺ NK1.1⁺), PD-1 positive T cells (CD45⁺ CD3⁺ PD-1⁺), T helper cells (CD45⁺ CD3⁺ CD4⁺), cytotoxic T cells (CD45⁺ CD3⁺ CD8⁺), and regulatory T cells (CD45⁺ CD3⁺ CD4⁺ CD25⁺ FoxP3⁺), as well as the ratio of CD8⁺/regulatory T cells in the tumor-draining inguinal lymph nodes (LNs) and tumors at day 24 (i.e., day 7 post-treatment). **e)** Quantification of anti-tumor (IL-5, IL-6, TNF- α) and pro-tumor (IL-13, IL-17a, IL-17f) serum cytokines at day 7 post-treatment. **f)** Schematic illustrating the O₂-cryogel therapeutic approach in solid tumors. In hypoxic tumors (bottom), the immunosuppressive environment prevents cytotoxic T cell and NK cell recruitment and function. O₂-cryogels (top) reverse local hypoxia, leading to increased NK, T helper, and cytotoxic T cell infiltration and function. Values represent the mean $\pm$ SEM ($n = 5$ mice per group). Data were analyzed using a two-tailed Pearson test (b), a two-tailed Mantel-Cox test (c), and an ANOVA with a Dunnett's post-hoc test (compared to PBS), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.