Tumor hypoxia drives suppressor function in exhausted T cells limiting antitumor immunity

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Biological Sciences - Article

Keywords: tumor hypoxia, T cells, immunotherapy, CD39, tumor microenvironment

Posted Date: September 29th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-924657/v1>

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Tumor hypoxia drives suppressor function in exhausted T cells limiting antitumor immunity

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ABSTRACT

While CD8⁺ cytotoxic T cells are clearly critical for identification and elimination of cancer cells, factors concentrated within the tumor microenvironment drive their altered differentiation to a hypofunctional, short-lived state termed exhaustion¹. Exhaustion is a progressive lineage, and it is now clear that the most terminally exhausted T (tT_{exh}) cells are not the targets of checkpoint blockade immunotherapy but rather serve as factors that limit immunotherapeutic efficacy²⁻⁴. Compared directly, tumor-infiltrating CD8⁺ tT_{exh} cells bear notable phenotypic similarity to CD4⁺Foxp3⁺ regulatory T (T_{reg}) cells, suggesting beyond loss of proinflammatory function, tT_{exh} cells may be directly anti-functional and constrain tumor specific immunity. Here we show, when isolated from the same tumor microenvironment, terminally exhausted CD8⁺ T cells carried similar capacity to suppress T cell proliferation as CD4⁺Foxp3⁺ T_{reg} cells. Unlike T_{reg} cells, tT_{exh} cells suppress solely via the ectonucleotidase CD39, which serves to deplete extracellular ATP (eATP) and produce an adenosinergic environment. CD39 expression in tT_{exh} cells is driven by exposure to tumor hypoxia, such that therapeutic targeting of hypoxia limits tT_{exh} suppression and support responses to immunotherapy. Specific deletion of CD39 in CD8⁺ T cells slows tumor progression and improves response to checkpoint blockade, highlighting a major role for immunosuppression by exhausted T cells in cancer. Thus, tT_{exh} cells are an unappreciated immunoregulatory population and for full immunotherapeutic efficacy, strategies should be designed to either limit their generation, reprogram their immunosuppressive nature, or remove them from the tumor microenvironment.

MAIN TEXT

T cell exhaustion is a terminally differentiated state defined by low potential for self-renewal, loss of polyfunctionality, and an absence of progression to memory¹. Terminal exhaustion is driven by continuous T cell receptor (TCR) signaling and metabolic stress and is characterized by expression of the transcription factors (Tox, Blimp, and Eomesodermin) and co-inhibitory receptors (PD-1, Lag-3, Tim-3, and Tigit)⁵. While it was once hypothesized that checkpoint blockade therapy could restore function to terminally exhausted CD8⁺ T (tT_{exh}) cells, we now understand PD-1 blockade preferentially acts upon less differentiated progenitor exhausted T (pT_{exh}) cells (PD-1⁺Tim-3⁻)^{2-4,6-8} and improves their functionality. Numerous reports have

associated tT_{exh} cell enrichment with poor patient outcomes and predictive of resistance to immunotherapy^{9,10}. In immunotherapy (IMT)-resistant murine tumor models (**Fig. 1a**) and treatment naïve melanoma samples from patients (**Fig. 1b**), PD-1⁺Tim-3⁺ tT_{exh} cells are numerically enriched, and significantly outnumber other T cell populations, including regulatory T cells (**Fig. 1c-d; Extended Fig. 1a**). Notably, tT_{exh} cells express several lineage-defining factors in common with T_{reg} cells (**Fig. 1e; Extended Fig. 1b**). While murine tT_{exh} cells do not significantly upregulate Foxp3 (**Fig. 1e; Extended Fig. 1b**), tT_{exh} cells display elevated expression of the T_{reg} cell-associated transcription factor Helios, as well as effector molecules that mediate T_{reg} cell suppressor functions: neuropilin 1 (Nrp1), interleukin 2 receptor alpha (*Il2ra*, CD25), Fas ligand (FasL), CD39, and numerous co-inhibitory and co-stimulatory receptors (cytotoxic T lymphocyte antigen 4 [CTLA-4], lymphocyte activating 3 [Lag-3], T cell immunoreceptor with Ig and ITIM domains [TIGIT], and inducible T cell co-stimulator [ICOS]; **Fig. 1e; Extended Fig. 1b**). These similarities between CD4⁺Foxp3⁺ T_{reg} cells and CD8⁺ tT_{exh} cells suggest some overlap in transcriptional programming that may drive CD8⁺ T cells to adopt a regulatory phenotype upon terminal differentiation.

To determine if tT_{exh} cells possess any intrinsic regulatory capabilities within the tumor microenvironment (TME), we compared tumor-infiltrating CD8⁺ T cell populations with T_{reg} cells directly *ex vivo* in miniaturized T cell suppression assays as previously described¹¹. T cell subpopulations were isolated from C57/BL6 mice expressing FlpO and mAmetrine concomitant with *Foxp3* (hereafter, B6.*Foxp3^{flpo-mAmetrine}*) carrying day 14 orthotopically implanted B16-F10 melanoma tumors (~8x8 mm)(**Extended Fig. 1c**). These putative suppressor populations were then co-cultured at diminishing ratios with proliferation dye-labeled, congenically mismatched naïve CD4⁺ T cells (responder T cells) and T cell-depleted splenocytes coated with anti-CD3. Strikingly, on a per-cell basis, tT_{exh} cells and T_{reg} cells equivalently inhibit responding T cell proliferation in *ex vivo* microsuppression assays (**Fig. 1f**) whereas CD8⁺PD-1⁺Tim-3⁻ pT_{exh} cells (**Fig. 1f**) and CD8⁺PD-1⁻ T cells (data not shown) did not, suggesting tT_{exh} cells harbored a unique regulatory functionality among CD8⁺ TIL. To validate these findings, we repeated these data in another melanoma line, clone 24, derived from the autochthonous melanoma mouse model, *Pten^{fl/fl}Braf^{LSL.V600E}Tyr2^{Cre}.ER*^{12,13}. Clone 24, like B16-F10, fails to respond to single agent checkpoint blockade therapy and aggressively progresses *in vivo*. Clone 24-infiltrating tT_{exh} cells and T_{reg} cells, like those sorted from B16-F10 tumors, were equivalently suppressive *ex vivo*, while the more stem-like pT_{exh} cells were not (**Extended Fig. 1d**). These findings of suppressor functions in tT_{exh} cells were also repeated in size-matched anti-PD-1 therapy-resistant head and neck carcinoma, MEER^{PD1res}, which we have recently reported to produce highly suppressive T_{reg} cells (**Fig. 1g; Extended Fig. 1c**)¹⁴. However, when isolated from the well-characterized anti-PD-1-sensitive colorectal carcinoma line MC38, tT_{exh} cells failed to control responding T cell effector functions (**Fig. 1h; Extended Fig. 2e**). Notably, T_{reg} cells infiltrating MC38 tumors retained suppressor capacity (**Fig. 1h**). These data suggest co-inhibitory receptor expression is not requisite for suppressor functions in tT_{exh} cells and, importantly, differing tumor microenvironments result in different functional fates for infiltrating CD8⁺ T cells. We then sought to delineate the mechanism of tT_{exh} cell suppression and the metabolic or environmental factors that generate this suppressor population.

tT_{exh} cell suppression requires CD39

CD4⁺Foxp3⁺ T_{reg} cells are potent suppressors that employ multiple, sometimes redundant means to suppress immune function in the local environment. Thus, we sought to dissect which inhibitory mechanism(s) were employed by terminally exhausted CD8⁺ T cells. Several reports have noted CD8⁺ T cell-derived IL-10 promotes autoimmune disease¹⁵, chronic viral infection⁶, and cancer^{16,17}. Therefore, we initially interrogated the contribution of tT_{exh} cell-secreted IL-10 by directly comparing B16-F10-derived tT_{exh} cells from either wild-type or IL-10 deficient C57/BL6 mice (*Il10*^{-/-}). Though baseline tumor growth was unaffected by the germline absence of IL-10 (**Extended Fig. 2a**), TIL from IL-10-deficient mice displayed a less exhausted signature (**Extended Fig. 2c**), consistent with previous reports that have shown IL-10 can enforce T cell exhaustion^{13,17}. Despite this, purified IL-10-deficient PD-1⁺Tim-3⁺ tT_{exh} cells retained significant suppressor functionality compared to their corresponding pT_{exh} cell counterparts (**Fig. 2a-b**). In concurrent experiments, titration of a well characterized IL-10 neutralizing antibody¹⁸ failed to prevent suppression by tT_{exh} cells (**Extended Fig. 2c**).

We next investigated whether tT_{exh} cell cytotoxicity could explain their suppressor activity. Host antigen specific CD8⁺ T cells have been shown to induce tolerance in autoimmune disease through granzyme- and perforin-mediated lysis of autoreactive CD4 T cells^{19,20}. Indeed, cytotoxicity is also a critical feature of CD4⁺Foxp3⁺ T_{reg} cell suppression^{21,22}. Tumor-resident tT_{exh} cells maintain elevated expression of multiple cytolytic effector molecules—granzymes, perforins, and FasL. Thus, we interrogated the role for tT_{exh} cell cytotoxicity by deriving the responder pool from the well-established model of apoptosis-resistance via Bcl2 overexpression (*Bcl2*^{tg}). Bcl2 overexpression did not prevent responder T cell suppression by tT_{exh} cells, in fact, by improving cell viability (**Extended Fig. 2d**), *Bcl2*^{tg} T cells were more sensitive to tT_{exh} cell suppression (**Fig. 2c**). These data suggest tT_{exh} suppression was not mediated through direct cytotoxicity of the responding T cells. However, it is well established that tT_{exh} cells are themselves apoptosis prone^{3,5,6} and indeed suppressor tT_{exh} cells from B16-F10 have higher *ex vivo* cleaved caspase 3 staining versus non-suppressive MC38-derived tT_{exh} cells (**Extended Fig. 2e**). Intriguingly, by improving tT_{exh} cell viability by overexpressing Bcl2, B16-F10-derived tT_{exh} cells displayed markedly reduced capacity to suppress *ex vivo* (**Extended Fig. 2f**). Evidence suggests CD4⁺Foxp3⁺ T_{reg} cells not only maintain suppressive capabilities as they undergo cell death but suppression can be augmented by the process²³. Thus, tT_{exh} cells may not suppress local immunity by killing, but rather further exert their regulatory capabilities by dying.

Apoptosis augments T_{reg} cell suppressive function through the release and subsequent CD39/CD73-mediated hydrolysis of extracellular ATP (eATP) to adenosine^{23–26}. CD39 has previously been shown to faithfully mark the most terminally exhausted CD8⁺ T cells^{27–29}, and in our system, higher CD39 expression corresponds to increased *ex vivo* suppression (9.64% increase per 0.1 log CD39 MFI; ****p* < 0.001)(**Fig. 2e**). However, unlike regulatory T cells, CD8⁺ tT_{exh} cells do not co-express CD73, and during differentiation to exhaustion ‘switch’ from being CD73⁺ to CD39⁺ (**Fig. 2f, Extended Fig. 3a**). Indeed, CD39 expression in peripheral and tumor-infiltrating CD8⁺ T cells is restricted to tT_{exh} cells (**Extended Fig. 3b**). Beyond the

immunoregulatory effects of adenosine, CD39 may also restrict T cell function by limiting available eATP, aids efficient T cell activation^{30–32}. Thus, while tT_{exh} cells may not be capable of producing adenosine in isolation, as they are CD73^{lo}, elevated CD39 expression may contribute to immune suppression either by limiting eATP or by supporting an adenosinergic environment with CD73⁺ cells. Indeed, tT_{exh} failed to mediate suppressor activity when the suppression assay was conducted using stimulatory magnetic beads rather than live or fixed antigen presenting cells (**Extended Fig. 3c**), suggesting interaction with local immune cells may be critical to tT_{exh} cell suppression.

To determine whether local CD73 expression facilitates tT_{exh} cell suppression, we assayed responder T cell and APC populations from CD73-deficient (*Nt5e*^{-/-}) mice in microsuppression experiments. Indeed, CD73-deficient T cells resist tT_{exh} cell-mediated suppression, supporting the notion that local adenosine production is at least partially responsible for the observed regulatory role of tT_{exh} cells (**Extended Fig. 3d**). Concurrently, we treated our microsuppression assays with a small molecule inhibitor specific to adenosine receptors, A2AR and A2BR (AB928), and found adenosine receptor blockade completely ablated tT_{exh} cell suppression (**Extended Fig. 3e**). Neither CD73 deficiency (**Extended Fig. 3d**) nor adenosine receptor blockade (**Extended Fig. 3e**) prevented CD4⁺Foxp3⁺ T_{reg} cell-mediated suppression, suggesting that unlike the numerous inhibitory mechanisms employed by T_{reg} cells, tT_{exh} cells employ a single method of constraining local immune response through CD39. To undermine CD39 activity on tT_{exh} cells, we utilized a *Cd4* promoter/enhancer driven Cre (*Cd4*^{Cre}) crossed with mice harboring a floxed *Entpd1* (encoding CD39³³; hereafter, *Cd4*^{Cre}*Entpd1*^{fl/fl}), resulting in CD39 deficiency in both CD4⁺ and CD8⁺ T cells (**Extended Fig. 3f**). In B16-F10 tumors, CD39 expression was dispensable in T cells for expression of coinhibitory receptors, PD-1 and Tim-3 (**Extended Fig. 3g**), however, when interrogated *ex vivo*, CD39-deficient tT_{exh} cells displayed a dramatic loss of suppressor capacity (**Fig. 2h**). T cell-specific deletion of CD39 was sufficient to slow tumor growth (**Extended Fig. 3h**); however, with this model we cannot distinguish the contributions of CD4⁺ T_{reg} cells and CD8⁺ tT_{exh} cells to the improved tumor response. In human disease, CD39 expression in CD8⁺ T cells is associated with tumor antigen specificity and a terminally exhausted signature^{27–29}. Intriguingly, CD39 has been reported as being both positively prognostic²⁸ and conversely, as supporting tumor progression^{34–36}, encouraging active exploration of CD39 as a therapeutic target^{37,38}. Thus, our data suggest CD39 is required for the suppressor activity of tT_{exh} cells, which generates a local adenosinergic environment in which CD73⁺ cells are immunosuppressed.

CD39 expression limits T cell activation

We next sought to better understand the mechanism by which CD39 may be limiting anti-tumor immunity with a gain-of-function approach. We retrovirally overexpressed CD39 on polyclonal, peripheral CD8⁺ T cells, (pMSCV-*Entpd1*-IRES-mCherry; **Extended Fig. 4a**), achieving high surface expression (**Fig. 2h**) and efficient enzymatic activity (**Extended Fig. 4b**). As eATP has been defined as a crucial autocrine signaling molecule in activated T cells^{30,39}, we first investigated whether enforced expression of CD39 on effector T cells would diminish signaling through the T cell receptor (TCR). Using anti-CD3/anti-CD28 antibody-bound microbeads, we

stimulated CD39-overexpressing T cells *in vitro* and probed events proximal and distal to TCR ligation. Compared to empty vector control CD8⁺ T cells (pMSCV T cells), CD39-overexpressing T cells displayed significant reduction in the phosphorylation of Zap70 (Y319) and Akt (S473) (**Fig. 2i-j**). Metabolic reprogramming to glycolysis⁴⁰ (**Extended Fig. 4c-f**) and calcium flux (**Extended Fig. 4g**) were also diminished in CD39-overexpressing cells, suggesting local CD39 expression was impacting TCR signaling. Further, when restimulated by anti-CD3/anti-CD28 antibody-bound microbeads, CD39-overexpressing T cells secreted significantly less interferon gamma (IFN γ) and tumor necrosis factor (TNF) (**Extended Fig. 4h**). Notably, CD39-overexpressing T cells were not inherently deficient in cytokine production, as TCR signaling-independent activation of these cells by phorbol 12-myristate 13-acetate (PMA) and ionomycin produced equivalent cytokine production as to controls (**Extended Fig. 4i**). These data highlight the functional consequences of CD39 expression on effector T cells *in cis*, although confounded by CD73 co-expression in these cells (unlike tT_{exh} cells). We then interrogated the effect of CD39 overexpression on neighboring cells, as CD39 expression would presumably still generate an adenosinergic environment. We repeated restimulation experiments in 1:1 coculture using congenically marked T cells. Compared to pMSCV T cells cultured alone, CD39-overexpressing T cells showed significant reduction in cytokine production (**Fig. 2k-l**) as well as lower proliferation (**Fig. 2m**). However, wild-type T cells cultured in the presence of CD39-overexpressing T cells also suffered a loss of cytokine and proliferation following TCR stimulation (**Fig. 2k-m**). These data suggest CD39 is sufficient to limit effector functions both within the cell expressing CD39, but also those in the local environment.

Hypoxia maintains CD39 on tT_{exh} cells

While the hypoxia-Hif1 α -VHL axis is implicated in T cell activation and effector functions^{41,42}, we and others have shown hypoxia exposure in the tumor microenvironment restricts effective anti-tumor immunity and accelerates differentiation of CD8⁺ T cells to terminal exhaustion^{5,43,44}. Disruption of this pathway with genetic or pharmacologic approaches reinvigorates anti-tumor immunity and sensitizes immunotherapy-resistant tumors^{43–46}. Hypoxia, oxidative stress, and pro-inflammatory mediators can also induce CD39 expression^{47–50}. Notably, tT_{exh} cells are significantly enriched in hypoxic zones within solid tumors (**Fig. 3a-b**) and have correspondingly higher expression of CD39 (**Fig. 3c**)⁵. Further, deletion of Hif1 α (*Cd4^{Cre}Hif1 α ^{ff}*) in T cells prevents CD39 upregulation when activated *ex vivo* in tumor hypoxic conditions (1.5% O₂) (**Extended Fig. 5a**), supporting the notion that tumor hypoxia may be a key contributor to the suppressor fate of CD39⁺ tT_{exh} cells. To further examine the role of hypoxia in suppressor tT_{exh} generation, we asked whether our recently published⁵ *in vitro* protocol for generating tT_{exh}-like cells *de novo* could also recapitulate our observed suppressor functions. In brief, naïve T cells are continuously stimulated with anti-CD3/CD28-coated beads in tumor hypoxic conditions, yielding tT_{exh}-like cells that possess many features of terminal exhaustion⁵ (**Extended Fig. 5a-b**). *In vitro* generated tT_{exh}-like cells (continuously stimulated under hypoxia) were capable of suppressing effector T cell functions, while effector T cell controls displayed no negative effect (**Extended Fig. 5c**). Further, when comparing CD8⁺ T cell infiltrate from B16-F10, that generates suppressive tT_{exh} cells, and MC38, that does not, we find MC38 TIL experience

significantly less tumor hypoxia⁴⁴ (**Extended Fig. 5d**) and consequentially display reduced CD39 expression in tT_{exh} cells (**Extended Fig. 5e**).

Our lab has previously reported that disruption of tumor oxygen consumption through deletion of mitochondrial complex I subunit, NADH:ubiquinone oxidoreductase subunit S4 (Ndufs4; B16-F10^{ND4-}; **Extended Fig. 5f**) significantly decreases tumor hypoxia (**Fig. 3d**)^{5,12}. B16-F10^{ND4-}-infiltrating CD8⁺ T cells display a more activated and less exhausted signature (**Extended Fig. 5g**) and have significantly reduced surface CD39 expression (**Fig. 3e**). Further, when directly compared *ex vivo* to either Foxp3⁺ T_{reg} cells from the same environment (**Extended Fig. 5h**) or from tT_{exh} cells isolated from wild-type B16-F10 (**Fig. 3f-g**), B16-F10^{ND4-}-infiltrating tT_{exh} cells display no detectable suppressor activity. These data suggest targeting the hypoxic tumor microenvironment may limit tT_{exh}-mediated suppression of anti-tumor immunity. To further explore how alleviation of tumor hypoxia might reverse tT_{exh} cell suppressor functions, we treated *B6.Foxp3^{flpo-mAmetrine}* mice bearing B16-F10 tumors with one of two established tumor hypoxia reducing agents—axitinib, a small molecule tyrosine kinase inhibitor that blocks activity of VEGF and normalizes tumor vasculature, and metformin, a type 2 diabetes drug targeting mitochondrial complex I that inhibits tumor mitochondrial respiration akin to our B16-F10^{ND4-} model^{5,44}. We found that a short course of either axitinib or metformin treatment in an advanced B16-F10 melanoma tumor could effectively resolve tumor hypoxia (**Extended Fig. 6a-b**), restrict CD39 expression on tT_{exh} cells (**Extended Fig. 6c**), and limit differentiation to terminal exhaustion (**Extended Fig. 6d**). Isolated tT_{exh} cells from treated mice also displayed significant loss of suppressor capacity (**Fig. 3h-i**) without disrupting CD4⁺Foxp3⁺ T_{reg} cell function (**Extended Fig. 6e-f**). Thus, hypoxia reversibly drives suppressor function in tT_{exh} cells by upregulating CD39, such that therapeutic mitigation of tumor hypoxia reduces the immunosuppressive functions of terminally exhausted T cells.

tT_{exh} cells are functionally significant suppressors in tumors

Numerous immune cells within the TME, as well as some tumor cells and stroma, express CD39. This is, in part, due to the hypoxic environment created by tumor metabolism as well as local circulating inflammatory mediators. But what was the contribution of tT_{exh}-mediated immunosuppression to the tumor microenvironment? Within the CD8⁺ compartment of a tumor-bearing mouse, CD39 expression is essentially restricted to tT_{exh} cells (**Extended Data Fig. 3b**). Thus, to determine the significance of CD39 expression of tT_{exh} cells alone, we employed a tamoxifen-inducible, CD8-specific system, utilizing a transgenic Cre-ERT² fusion protein under control of the CD8a enhancer, *E8i*, and a *Rosa26*-driven recombination reporter, crossed with *Entpd1^{fl/fl}* (*E8i^{GFP-Cre-ERT2-Rosa26-LSL-TdTomato}Entpd1^{fl/fl}*; hereafter, *E8i^{Cre.ERT2}Entpd1^{fl/fl}*). These mice were implanted with B16-F10 orthotopically as before and treated with oral tamoxifen (TAM) when tumors were palpable. We then treated half of our cohort carrying advanced tumors—where combination immunotherapy would be ineffective—with anti-PD-1 and anti-CTLA-4. Remarkably, CD8 T cell-restricted deletion of CD39 was itself sufficient to significantly slow tumor progression (**Fig. 4a-c**), highlighting an unappreciated immunosuppressive role within tumors of exhausted T cells. The combination of CD39 deletion with checkpoint blockade further improved response, resulting in 43% partial response and 14% complete remission and a prolonging

survival of 13 days (improvement of 72%) from baseline (**Fig. 4a-c**). To understand the functional consequence of CD39 deletion in this system, we isolated TIL from B16-F10 tumors after three consecutive days of TAM treatment (day 8 post-tumor inoculation), when tumors were still similarly sized (~5x5 mm²). At this timepoint, CD39 expression was significantly depleted in *E8i^{Cre,ERT2}Entpd1^{ff}* TIL as compared to controls (**Fig. 4d**) and consequentially displayed a less exhausted (**Fig. 4e,f**) and more effector-like signature (**Fig. 4g**). Indeed, even at this early timepoint, *E8i^{Cre,ERT2}Entpd1^{ff}* TIL secreted significantly more proinflammatory cytokines, IL-2 and interferon gamma (IFN γ) upon *ex vivo* restimulation (**Fig. 4g**). Thus, by removing the ectoenzyme driving the tolerogenic functions within the tT_{exh} cell lineage, the tumor microenvironment is remodeled in a way that improves anti-tumor immune functions in aggressive, therapy-resistant tumors.

Our understanding of terminal exhaustion in tumor-infiltrating CD8⁺ T cells is evolving^{1,51}. We now appreciate how metabolic features of tumor cells¹², and the resulting hypoxic^{5,14} and nutrient dearth microenvironment^{52–54} accelerate CD8⁺ T cell dysfunction and diminish efficacy of checkpoint blockade therapy. Here, we demonstrate that terminally exhausted T cells encountering tumor hypoxia adopt a regulatory phenotype via expression of the ectoenzyme CD39, enforcing local immune suppression and limiting tumor control in a manner independent of CD39 expression on other cells within the tumor. These findings not only improve our understanding of the breadth of functions attributed to exhausted T cells, but aid in tackling a major problem in resistance to cancer immunotherapy.

A rather surprising result was that IL-10 was dispensable for tT_{exh} cell suppression, although the diversity of roles of IL-10 within the tumor microenvironment continues to widen. Numerous reports have highlighted a role for this pleiotropic cytokine both in the progression of cancer and restriction of anti-tumor immunity^{55–58}, and in the enhancement of immune surveillance and cytotoxicity of CD8⁺ T cells^{17,59–63}. In our study, we investigated whether exposure to IL-10 within the tumor or secretion of IL-10 by tT_{exh} cells¹⁵ could contribute to suppression of T cell responses. Contrary to studies highlighting a role for IL-10 in CD8⁺ T cell-mediated control of inflammation^{15,16}, neither genetic deletion of IL-10 nor *in vitro* blockade diminished tT_{exh} cell-mediated suppression in our system. Thus, it does not appear to be a dominant tolerogenic mechanism utilized by tT_{exh} cells in cancer. Notably, recent reports have highlighted that engineered IL-10 molecules can metabolically reprogram tT_{exh} cells, improving their oxidative capacity and inflammatory functions^{17,60}.

Several other groups have identified other immunosuppressive CD8⁺ T cell populations in cancer⁶⁴ and autoimmune disease^{65,66}, highlighting the importance of delineating the diverse roles of CD8⁺ T cells and directing therapeutic efforts to curtail their pathologic deficits. In this study, we clearly demonstrate terminally exhausted CD8⁺ T cells, a common cellular fate of CD8⁺ T cells in solid tumors, upregulate the ectonucleotidase CD39 upon hypoxia exposure to levels sufficient to create a tolerogenic microenvironment. CD39 is a well characterized immunosuppressive molecule^{25,35,48,67,68}, and indeed recent efforts to therapeutically block CD39 and CD73 in combination with checkpoint blockade have yielded impressive results^{37,38,69}. Here, we delineate a specific role of CD39 on tT_{exh} cells, revealing that CD8⁺ T cell-restricted deletion

of CD39 alone results in retained polyfunctionality in tumor-infiltrating T cells and a slowed tumor progression. Deletion or blockade of CD39, CD73, or the adenosine receptors, A2AR/A2BR abolishes the suppressive function of tT_{exh} cells, but not of their $CD4^+Foxp3^+$ T_{reg} cell counterparts. Notably, targeting this pathway is insufficient to prevent T cell exhaustion in cancer. However, our data suggest by limiting CD39 activity on pre-existing tT_{exh} cells, other cells, including those newly infiltrating, within the tumor microenvironment are able to retain functionality.

We have recently reported exposure to hypoxia accelerates T cell exhaustion through byproducts like reactive oxygen species (ROS) produced from dysfunctional mitochondria, and that alleviation of hypoxia sensitizes tumors otherwise resistant to immunotherapy and reinvigorates the infiltrating immune response^{5,44,70}. Here, we posit that differentiation to terminal exhaustion results not only in dysfunctional but rather antifunctional cells, coordinated primarily through CD39 expression and adenosine-mediated suppression of T cell activation. Importantly, we show that this suppressor state is plastic: while T cell exhaustion may not be capable of being fully reversed by checkpoint blockade immunotherapy, the negative function of tT_{exh} cells may be diminished through targeting the hypoxic nature of the tumor microenvironment or through direct targeting of CD39-mediated suppression.

EXPERIMENTAL PROCEDURES

Mice

All experiments requiring live animals or *ex vivo* immune cells were performed in accordance with the Institutional Animal Care and Use Committee of the University of Pittsburgh. All mice were housed in specific pathogen-free conditions prior to use. Both male and female mice were used, and mice were 6–8 weeks old at the start of experimentation. C57BL/6, $Cd4^{\text{Cre}}$, $Hif1\alpha^{\text{fl/fl}}$, $Nt5e^{\text{ko}}$, $Bcl2^{\text{tg}}$, mice were obtained from The Jackson Laboratory. $Entpd1^{\text{fl/fl}}$ mice were obtained from SC Robson (Beth Israel Deaconess Medical Center). $E8i^{\text{Cre.ERT2}}$ and $Foxp3^{\text{flpo.mAmetrine}}$ mice were generated by DAA Vignali (University of Pittsburgh).

In vivo treatments and immunotherapy were performed as previously described^{5,44,71} with Bio X Cell-supplied anti-PD-1 (clone J43), anti-CTLA-4 (clone 9H10), and host-appropriate isotype controls. Cayman Chemical supplied Axitinib (2 mg/kg IP; suspended in DMSO (10%), Tween 80 (10%), and 30% Captisol (80%)) and Metformin (50 mg/kg IP; saline (100%)). Sigma supplied tamoxifen (50 mg/kg PO; 100% EtOH (5%), corn oil (95%)).

Tissue Culture and T cell Isolation

B16-F10, MC38, and MEER were originally purchased from American Type Culture Collection (ATCC). B16-F0^{OVA} was obtained from P Basse and L Falo (University of Pittsburgh). Platinum-E (Plat-E) was obtained from L Kane (University of Pittsburgh). Some immortalized tumor lines were generated in-house as previously described; B16-F10^{ND4-5}, Clone 24 (CL24)¹², MEER^{PD-1res}¹⁴. All cell lines were maintained in complete DMEM (10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 5 mM HEPES Buffer, 100 U penicillin, 100 µg/mL streptomycin, 500 µg/mL gentamycin) at no greater than 80% confluence.

Tumor, tumor-draining lymph nodes, and spleens were surgically resected, then enzymatically and mechanically digested to a single cell suspension. Cells were purified by either magnetic bead isolation as previously described⁷⁰, or fluorescence-activated cell sorting (FACS; on Beckman Coulter Mo-Flo Astrios High Speed Cell Sorter or Sony MA900 Cell Sorter), or a combination of the two. All T cells were cultured in complete RPMI (10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 5 mM HEPES Buffer, 100 U penicillin, 100 µg/mL streptomycin, 500 µg/mL gentamycin, 5 nM 2-mercaptoethanol) plus 100-25 U of murine IL-2 (PeproTech).

Ex vivo microsuppression assays were performed as previously described¹¹. To measure cytokine production, cells were incubated at 37°C for 6 hours or overnight in GolgiPlug Protein Transport Inhibitor (BD) with either 2:1 bead:cell ratio of Dynabeads T-Activator CD3/CD28 beads (ThermoFisher) or phorbol 12-myristate 13-acetate (PMA) and ionomycin (Sigma). Metabolic assays were performed as previously described^{5,40}. *In vitro* continuous stimulation under hypoxia protocol was performed as previously described⁵.

Human Peripheral Blood or Tumor Infiltrating Lymphocyte Isolation

Human blood was obtained from Vitalant; tumor preparations were obtained via UPMC Hillman Cancer Center IRB protocol 96-099. Each patient was consented as outlined in our protocol, then subject to peripheral blood collection and tumor biopsy. Tumor samples were mechanically digested to a single cell suspension, then passaged through 70 µm cell strainers (BD). Lymphocytes were purified from bulk suspension by density centrifugation and/or cell sorting.

Flow Cytometry and FACS

Viability staining with Zombie dyes (Biolegend) and surface staining (see below) were performed in PBS at 4°C for 20 minutes. Samples were then washed and immediately analyzed on a BD LSRFortessa Flow Cytometer. Fixation of samples for intracellular hypoxia/transcription factor staining: following viability/surface staining, cells were incubated in 4% paraformaldehyde at room temperature for 5 minutes, washed in PBS, permeabilized with eBioscience Foxp3/Transcription Factor Staining Kit (ThermoFisher) per manufacturers protocol, washed in permeabilization buffer, then stained in permeabilization buffer at 4°C overnight. Fixation of samples for intracellular cytokine staining: following viability/surface staining, cells were fixed in BD Cytofix/Cytoperm Fixation/Permeabilization Kit per manufacturers protocol, washed in permeabilization buffer, then stained in permeabilization buffer at 4°C overnight.

Biolegend supplied: Zombie Fixable Viability Dyes (UV, Aqua, Green, NIR); anti-mouse antibodies including, anti-CD8a (clone 53.6.7), anti-CD4 (GK1.5), anti-PD-1 (29F.1A12), anti-Tim-3 (RMT3-23), anti-Helios (22F6), anti-Nrp1 (3E12), anti-CD25 (PC61), anti-CTLA-4 (UC10-4B9), anti-Lag-3 (C9B7W), anti-CD39 (Duha59), anti-FasL (MFL3), anti-TIGIT (1G9), anti-ICOS (C398.4A), anti-CD73 (TY/11.8), anti-IL-2 (JES6-5H4), anti-IFN-γ (XMG1.2), anti-TNFα (MP6-XT22), anti-Thy1.1 (OX-7); anti-human antibodies including, anti-CD8a (RPA-T8), anti-CD4 (RPA-T4), anti-PD-1 (EH12.2H7), anti-Tim-3 (F38-2E2), anti-CD73 (AD2), anti-CD39 (TU66), anti-IL-2 (MQ1-17H12), anti-IFN-γ (4S.B3). Invitrogen supplied: anti-mouse antibody, anti-Foxp3 (FJK-16s); anti-human antibodies, anti-Lag-3 (3DS223H), anti-TNFα (Mab11). R&D Systems supplied: anti-Hif1α (241812). Thermo Fisher supplied: CellTrace Violet Cell Proliferation Kit, anti-Tox (TXRX10), anti-Blimp-1 (5E7).

Immunoblotting analysis

Immunoblotting was performed as previously described⁷². Santa Cruz Biotechnology supplied: anti-actin (clone C4); Cell Signaling supplied: Zap70 (D1C10E), phospho-Zap70 Y319/Y352 (65E4), Akt (C67E7), phospho-Akt S473 (D9E). Immunoblots were detected via standard secondary detection and chemiluminescent exposure to film. Digitally captured films were analyzed densitometrically using ImageJ software.

Retroviral Vector Construction and T cell Transduction

Entpd1 (encoding CD39) was originally generated by and cloned into a murine stem cell virus (MSCV)-driven retroviral expression vector encoding an internal ribosome entry site (IRES)–GFP cassette, from DAA Vignali (Addgene plasmid # 52114; <http://n2t.net/addgene:52114>; RRID:Addgene_52114). For T cell transductions, T cells were activated with plate-bound anti-CD3 (5 µg/mL), soluble anti-CD28 (1 µg/mL), and IL-2 (100 U) for 24-hours. Retroviral supernatants were harvested, filtered, and supplemented with 6 µg/ml of polybrene. Prepared retrovirus was spun onto activated T cells (2,200 rpm, 2 hours, 37°C) then the cell/virus culture was rested in a tissue culture incubator for another 2 hours. Cells were then washed and cultured in fresh complete RPMI plus IL-2 (50 U) for three days to allow for expansion and expression of vector cassette. After 3 days, cells were purified via FACS by mCherry expression. CD39 expression was validated by flow cytometry and enzymatic activity verified by ENLIGHTEN ATP Assay System (Promega).

Statistics

If the data appeared to follow a normal distribution, we calculated the P values in GraphPad Prism using one-way analysis of variation (ANOVA) with Dunnett's multiple comparison test, two-way ANOVA with Sidak's multiple comparison test, unpaired Student's t-test or paired Student's t-test. For data that did not follow a normal distribution, we used the non-parametric Kruskal-Wallis and Mann-Whitney tests as indicated. The *ex vivo* suppression assays were analyzed using linear regression, treating the percent suppression as the dependent continuous variable, the type of cells as a categorical independent variable and the dilution as a continuous independent variable. Interactions were tested and included in the linear regression model if statistically significant. Values of $p < 0.05$ were considered significant and ranked as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

ACKNOWLEDGMENTS

The authors would like to thank all members of the Delgoffe lab for helpful and invigorating discussions. This work was supported by a National Institutes of Health (NIH) Director's New Innovator Award (DP2AI136598); the Hillman Fellows for Innovative Cancer Research Program; a Stand Up to Cancer–American Association for Cancer Research Innovative Research Grant (SU2C-AACR-IRG-04-16); the Alliance for Cancer Gene Therapy; the UPMC Hillman Cancer Center Skin Cancer and Head and Neck Cancer SPOREs (P50CA121973 and P50CA097190; NIH); the Mark Foundation for Cancer Research's Emerging Leader Award; a Cancer Research Institute's Lloyd J. Old STAR Award; and the Sy Holzer Endowed Immunotherapy Fund (all to G.M.D.). Trainees on this manuscript were supported by grants T32CA082084 (NIH) (to P.D.A.V., M.J.W., and K.D.), F30CA247034 (NIH) (to P.D.A.V.), F31AI149971 (NIH) (to M.J.W.), F31CA247129 (NIH) (to K.D.). This work used the UPMC Hillman Cancer Center Flow Cytometry and Animal Facilities, supported in part by grant P30CA047904 (NIH).

AUTHOR CONTRIBUTIONS

P.D.A.V. performed the majority of the experiments, analyzed data, and wrote the manuscript. K.D. performed suppression assays in the B16-ND4– and Axitinib/Metformin experiments and

contributed to writing. M.J.W. carried out initial experiments and performed assays characterizing CD39 overexpression in effector T cells. K.L. performed statistical analysis on many of the figures. N.K.M. helped characterize CD39 overexpression in effector T cells. N.E.S. characterized tumor-infiltrating T cells from B16-F10 and B16-ND4– tumors. A.V.M. supported generation of metabolic assay data in CD39 overexpression experiments. G.M.D. conceived of the study, directed the research, obtained funding, and wrote the manuscript.

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Tumor hypoxia drives suppressor function in exhausted T cells limiting antitumor immunity

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Figure 1. PD-1⁺Tim-3⁺FoxP3⁻ terminally exhausted CD8⁺ T cells predominate in cancer and adopt a regulatory signature that correlates with immunotherapy response. (A) Human CD8⁺ T cell populations from five melanoma biopsies from treatment-naïve patients. (B) CD8⁺ T cell populations from day 14 post-orthotopically implanted B16-F10 murine melanoma tumor and tumor-draining lymph nodes (dLN). (C) Percent of total CD3⁺ T cells among B16-F10 TIL and dLN with quantified fold change. (D) Total cell numbers per size matched tumor or draining lymph node. (E) Expression of CD4⁺Foxp3⁺ Treg cell-associated genes among T cell populations. (F-I) Microsuppression assays (MSA; F) of TIL from (G) B16-F10 melanoma, (H) PD-1-resistant MEER head and neck carcinoma, or (I) MC38 adenocarcinoma co-cultured with activated CTV-labeled Thy1.1⁺ responding T cells. Bivariant plots and histograms are representative of ≥3 experiments. Statistics are one-way ANOVA with Tukey's multiple comparison test (a,b,d) and linear regression (f,g,h) with **p*<0.05, ***p*<0.01, ****p*<0.001 and *****p*<0.0001.

Figure 2. CD8⁺ tTexh cells suppress T cell effector functions via CD39-mediated adenosine generation. (A) Schematic defining experimental design of (B) MSA of B16-F10 TIL from wild-type C57/BL6 or B6.1110^{-/-} mice. (C) Schematic of (D) MSA of B16-F10-derived tTexh cells co-cultured with with activated Thy1.1⁺ or B6.Bcl2^{tg} responding T cells. (E) Pearson correlation of percent tTexh-mediated suppression cell in 1:4 ratio MSA versus fold change in CD39 MFI in tTexh cells between murine tumor lines. (F) Representative bivariate plots of B16-F10-infiltrating T cell populations by expression of ectoenzymes CD39 and CD73. (G) MSA of B16-F10 TIL from Entpd1^{fl/fl} or Cd4^{Cre}Entpd1^{fl/fl} mice. (H) Expression of CD39 among transduced and untransduced T cells. (I) Western Blot analysis of transduced cells following TCR stimulation and (J) quantified band intensity normalized to t = 0 in pMSCV of phosphorylation events following of anti-CD3- and -CD28-coated microbead stimulation in transduced T cells. (K) Cytokine production of “responding” T cells in coculture experiments following anti-CD3- and -CD28-coated microbead re-stimulation. (L) Division on CTV-labeled transduced “responder” T cells from (K) over 72-hours. Western Blots, bivariate plots, and histograms are representative of ≥3 experiments. Statistics are linear regression (b,d,e,g,j), Mann-Whitney (k), and one-way ANOVA with Tukey’s multiple comparison test (l,m,n) with *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

Extended Figure 2. tTexh cells do not suppress via IL-10 secretion or cytotoxicity. (A) Day 14 B16-F10 tumor sizes from *B6. Il10*^{-/-} experiments and (B) CD8⁺ T cell infiltrate. (C) MSA of B16-F10-derived tTexh cells co-cultured with isotype IgG or neutralizing IL-10 antibody at 2.5 or 5.0 μ g/mL. (D) Cell viability of responding T cells from Fig. 2c-d. (E) *Ex vivo* TIL and associated draining lymph node (dLN). (F) MSA of B16-F10-derived tTexh cells from wild-type C57/BL6 or *B6. Bcl2*^{tg} mice. Bivariant plots and histograms are representative of ≥ 3 experiments. Statistics are student t-test (a,b), linear regression (c,f), Mann-Whitney (d) and one-way ANOVA with Tukey's multiple comparison test (e) with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

Extended Figure 3. Texh suppress ex vivo through CD39-mediated extracellular ATP depletion and adenosine production. (A) Percent CD8⁺ TIL from B16-F10 expressing CD39. (B) Percent CD8⁺ T cells expressing CD39 in various tissues in a B16-F10 tumor-bearing C57/BL6 mice. (C) MSA at 1:2 ratio of B16-F10-derived tTexh cells co-cultured with CTV-labeled responding T cells with either live or Mytomycin C-fixed T cell-depleted splenocytes, or anti-CD3/anti-CD28 bound microbeads. (D-E) MSA of B16-F10 TIL, CD8⁺ tTexh (D) or CD4⁺ Treg (E) cells, co-cultured with with activated CTV-labeled Thy1.1⁺ or *B6.Nt5e*^{-/-} responding T cells. (F-G) MSA of B16-F10-derived CD8⁺ tTexh (F) or CD4⁺ Treg (G) cells co-cultured with DMSO vehicle or A2AR/A2BR small-molecule inhibitor AB928 at 3 μ g/mL. (H-I) CD8⁺ TIL from B16-F10 tumors from *Entpd1*^{fl/fl} and *Cd4*^{Cre}*Entpd1*^{fl/fl} mice. (J) Tumor sizes at day 14 of growth *in vivo*. Bivariant plots and histograms are representative of ≥ 3 experiments. Statistics are one-way ANOVA with Tukey's multiple comparison test (a), linear regression (b,c,d,e), and student t-test (g,i) with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

Extended Figure 4. Enforced CD39 expression on CD8⁺ T cells limits TCR signaling and effector functions.

(A) Schematic of CD8⁺ T cell transduction with pMSCV-encoded CD39. (B) Extracellular ATP consumption by CD39⁺mCherry⁺ T cells versus empty vector controls. (C) Extracellular acidification rate (ECAR) in in-Seahorse activation of rested CD8⁺ T cells stably transduced with pMSCV or pMSCV-*Entpd1*. (D) Delta-maximal ECAR quantified by basal ECAR – maximal ECAR; minutes till maximal ECAR per sample well. (E) Associated oxygen consumption rate (OCR) from (C) with (F) quantified basal OCR and delta-minimal OCR. (G) Intracellular calcium flux in stably transduced rested CD8⁺ T cell following activation with anti-CD3- and -CD28-coated microbeads in the presence of calcium indicators, Fluo-4 and Fura Red, and in a calcium-buffered solution. (H-I) Cytokine production of transduced T cells following anti-CD3- and -CD28-coated microbead stimulation (H) or PMA and Ionomycin stimulation (I). Western Blots and bivariate plots are representative of ≥ 3 experiments. Statistics are one-way ANOVA with Tukey's multiple comparison test (b,c,e,g), student t-test (d,f), and Mann-Whitney (h) with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

Figure 3. Tumor-infiltrating CD8⁺ T cell exposed to hypoxia differentiate to a suppressor CD39^{hi} tTexh cell fate. (A) Histogram overlay displaying hypoxia exposure in CD8⁺ dLN and TIL from B16-F10 tumors. (B) PD-1 and Tim-3 staining in hypoxyprobe positive and negative samples and quantification. (C) CD39 staining in populations in (B). (D) Hypoxia staining and (E) CD39 expression in CD8⁺ T cell populations from wild-type or Ndufs4-deficient B16-F10 tumor or associated draining lymph node. (F) Schematic and (G) MSA of B16-F10 or B16^{ND4}- TIL from C57/BL6 mice. (H) Schematic and (I) MSA of B16-F10 TIL following two treatments of vehicle or Axitinib. Histograms are representative of ≥ 3 experiments. Statistics are student t-test (b,c,d,e) and linear regression (g,i) with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

Extended Figure 5. Hypoxia exposure and continuous TCR stimulation drives CD8⁺ T cells to a suppressor tTexh cell fate. (A) Percent of CD8⁺ T cells expressing CD39 following 72-hour stimulation at atmospheric or tumor hypoxic conditions (1.5% O₂). (B) Validation of inhibitory receptor expression and (C) CD39 expression in Continuous Activation under Hypoxia (CSH) assay cells. In brief, naïve T cells are either acutely (24 hours, then rested for 5 days) or continuously (6 days) stimulated with anti-CD3 and -CD28-coated microbeads in either atmospheric or tumor-hypoxic (1.5% O₂) conditions, then isolated and co-cultured with activated CTV-labeled Thy1.1⁺ T cells. (D) MSA of CD8⁺ T cells following *in vitro* CSH assay. (E) Hypoxia staining and (F) CD39 expression in CD8⁺ T cell populations from day 14 B16-F10 or MC38 or associated draining lymph node. (G) Validation of mitochondrial respiration knockdown (via OCR measurement) in B16^{ND4-} tumor cells versus parental B16-F10. (H) Bivariant plots of CD8⁺ TIL from parental B16-F10 or B16^{ND4-} tumors. (I) MSA of B16-F10 or B16^{ND4-}-infiltrating CD4⁺Foxp3⁺ Treg cells from C57/BL6 mice. Bivariant plots and histograms are representative of ≥ 3 experiments. Statistics are two-way ANOVA with Sidak's multiple comparison test (a), one-way ANOVA with Tukey's multiple comparison test (c), linear regression (d,i), and student t-test (e,d,h) with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

Extended Figure 6. Reversal of intratumoral hypoxia rescues suppressor tTexh cell fate. (A) Tumor sizes pre- and post- treatment with Axitinib or metformin versus vehicle. (B) Hypoxia staining, (C) expression of coinhibitory receptors, PD-1 and Tim-3, and (D) CD39 expression in CD8⁺ T cell populations from tumor or draining lymph node from mice treated with Axitinib or metformin versus vehicle. (E) MSA of B16-F10 TIL following two treatments of vehicle or metformin. (F) MSA as before but with CD4⁺Foxp3⁺ Treg cells from tumors treated with Axitinib or metformin versus vehicle. Bivariate plots and histograms are representative of ≥3 experiments. Statistics are one-way ANOVA with Tukey's multiple comparison test (b,c,d) and linear regression (e,f) with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

Figure 4. tTexh cell-restricted deletion of CD39 invigorates immune response in aggressive tumor model. (A) Schematic of (B) tumor growth over time in *Entpd1^{fl/fl}* or *E8i^{Cre-ERT2}Entpd1^{fl/fl}* mice bearing B16-F10. Tamoxifen (TAM) treatment began at day 5 post-tumor inoculation continuing daily for three days then thrice weekly thereafter; immunotherapy (IMT) treatment began on day 8 and was administered thrice weekly. (C) Survival of mice from (B). (E-F) Flow analysis of CD8⁺ TIL at day 8 of tumor growth, day 3 of TAM treatment. (G) Reactivation of day 8 CD8⁺ TIL by PMA and Ionomycin. Bivariate plots and histograms are representative of ≥ 3 experiments. Tumor growth data are pooled data from ≥ 3 experiments; the solid black line represents the average. Statistics are Log-Rank test (c) and student t-test (d,e,f,g) with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.