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Enhanced immunosuppression by therapy-exposed glioblastoma multiforme tumor cells

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Glioblastoma multiforme (GBM) is a highly malignant brain tumor with an extremely short time to relapse following standard treatment. Since recurrent GBM is often resistant to subsequent radiotherapy and chemotherapy, immunotherapy has been proposed as an alternative treatment option. Although it is well established that GBM induces immune suppression, it is currently unclear what impact prior conventional therapy has on the ability of GBM cells to modulate the immune environment. In this study, we investigated the interaction between immune cells and glioma cells that had been exposed to chemotherapy or irradiation *in vitro*. We demonstrate that treated glioma cells are more immunosuppressive than untreated cells and form tumors at a faster rate *in vivo* in an animal model. Cultured supernatant from *in vitro*-treated primary human GBM cells were also shown to increase suppression, which was independent of accessory suppressor cells or T regulatory cell generation, and could act directly on $CD4^+$ and $CD8^+$ T cell proliferation. While a number of key immunosuppressive cytokines were overexpressed in the treated cells, including IL-10, IL-6 and GM-CSF, suppression could be alleviated in a number of treated GBM lines by inhibition of prostaglandin E2. These results reveal for the first time that conventional therapies can alter immunosuppressive pathways in GBM tumor cells, a finding with important implications for the combination of immunotherapy with standard treatment.

Tumor Immunology

Despite aggressive upfront therapy for glioblastoma multiforme (GBM), which includes surgical resection, radiotherapy and temozolomide-based chemotherapy, the median time to recurrence is only 6.9 months after standard treatment.[1] Treatment options for recurrent GBM are limited; aggressive surgical resection is often not possible due to invasion of deep structures, and retreatment with radiotherapy is limited by toxicity. Chemotherapy options include bevacizumab,[2] retreatment with temozolomide[3] and other second-line chemotherapeutic agents, but eventual tumor progression is almost universal. There is therefore a desperate need for more effective treatment options for recurrent GBM.

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Recurrent GBM tumors contain high numbers of radiation-resistant and chemotherapy-resistant cells.[4,5] Immunotherapy is a treatment option with promise in this setting because it acts in a fundamentally different way, most notably the ability to target and eliminate cells irrespective of proliferation rate. Clinical trials of therapeutic vaccination in GBM patients have provided evidence of tumor antigen-specific humoral responses, increases in tumor antigen-reactive T cells and elevated numbers of tumor-infiltrating $CD8^+$ T cells, suggesting that immunotherapy has the potential to target and eradicate GBM tumor cells.[6–8] However, although significant survival benefit has been demonstrated with immunotherapy in some other solid tumor cancers, most notably in melanoma[9–11] and prostate cancer,[12] the overall survival benefit of immunotherapy for GBM has been minimal. This lack of efficacy has been attributed to a high level of immune suppression induced by GBM tumor cells.[13,14] It is also likely that conventional treatments for GBM negatively impact upon the efficacy of immunotherapy. In this context, it is well known that radiation and chemotherapy can alter the function and survival of immune cells.[15] The widespread use of steroids such as dexamethasone in GBM patients is also likely to have a negative impact on these cells. However, another possibility to consider is that therapies like radiation and chemotherapy can actually alter, and potentially intensify, the immunosuppressive properties of GBM tumor cells themselves.

What's new?
Since recurrent glioblastoma multiforme (GBM) is often resistant to radiotherapy and chemotherapy, immunotherapy has been proposed as an alternative. Unfortunately, both GBM tumors and standard therapies can induce immune suppression. In this study, the authors asked whether these therapies might also actually enhance the ability of GBM cells to modulate the immune response. They found that treated glioma cells are indeed more immunosuppressive than untreated cells, and form tumors at a faster rate *in vivo*. This may be a critical finding for the timing of immunotherapy with conventional treatment.

In this study, we modeled chemotherapy and radiotherapy treatment of primary GBM tumors *in vitro* to investigate the ability of treated tumor cells to modulate immune responses. We demonstrate that treated cells have significantly enhanced immune suppressive activity compared to nontreated GBM cells, which is in part mediated by increases in prostaglandin E2 (PGE2). Our results have important implications for the timing of immunotherapy with conventional treatment and suggest that combinational drug therapy to remove the immune suppression induced by treated cells is likely to be required for immunotherapy to be an effective treatment option for recurrent GBM.

Material and Methods

Mice

Inbred C57BL/6 mice were purchased from The Jackson Laboratory and were bred and housed at the Malaghan Institute of Medical Research Biomedical Research Unit in Wellington, New Zealand. This project was undertaken within the provisions of the Animal Welfare Act (1999) of New Zealand and was approved by the Victoria University of Wellington Animal Ethics Committee (2009R4M). Mice were 6–10 weeks of age and matched for age and gender.

Patient samples

Tumor tissue was collected from patients with confirmed primary GBM undergoing standard care. All patient donors gave informed consent and ethical approval for tissue collection was obtained from the Central Regional Ethics Committee of New Zealand (CEN/09/06/037).

Tumor cell culture

The murine glioma cell line GL261 has been validated as an appropriate preclinical experimental model since it shares many similarities with human GBM.[16] GL261 was obtained from the DCTD Tumor Repository (National Cancer Institute, Frederick, MD) and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 20% fetal bovine serum (FBS; Sigma-Aldrich), 1% penicillin/streptomycin and 1% GlutaMax (both Invitrogen) at 37°C and 5% CO_2.

For primary GBM lines, tumor tissue was collected from GBM patients during debulking surgery. Tumor tissue was mechanically dissociated into single cell suspensions and primary GBM cultures were grown in RPMI-1640 (Invitrogen) supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% GlutaMax at 37°C and 5% CO_2.

Generation of treated glioma cells

Treated GL261 glioma cells or primary GBM cells were generated by exposure to doxorubicin or by γ-irradiation. For chemotherapy treatment, tumor cells in exponential growth were transiently exposed to a concentration of doxorubicin that kills 80% of cells (range 1.5–2 μM) or temozolomide (800 μM) or the vehicle dimethyl sulphoxide (DMSO, Sigma-Aldrich) for 16–18 hours. Surviving cells were then harvested with TrypLE (Invitrogen), replated and allowed to grow until colonies formed (usually 3–4 days). For γ-irradiation, tumor cells in exponential growth were exposed to 4 cycles of 2 Gy for four consecutive days (1 treatment per day). Surviving cells were harvested, replated after the second and forth treatments and allowed to grow until treated cells formed colonies. To produce culture supernatant, treated or control cell colonies were collected at 3–4 days and plated at 200,000 cells in 250 μl media per well of 24-well plate (BD Falcon) for 16–18 hr. Culture supernatant was collected, centrifuged and filtered to remove cells and stored at −80°C. Live cells were counted by Trypan Blue (Invitrogen) exclusion to confirm the number of cells used to produce supernatant and demonstrated that treatment-exposed cells were essentially healthy.

Tumor challenge in mice

GL261 cells were harvested with TrypLE, washed and 1×10^4 or 5×10^4 cells in 100 μl of DMEM were injected subcutaneously (s.c.) in the left flank. Mice were considered to harbor tumors when any two perpendicular diameters were both at least 2 mm.

Preparation of immune cells

Single cell suspensions were prepared from the spleens of naïve mice by passing them through a 70 μm cell strainer and subjecting them to red blood cell lysis (RBC Lysis Solution, Qiagen). For peripheral blood mononuclear cells (PBMCs), blood was collected in EDTA-coated tubes (BD Biosciences), diluted in PBS, and PBMCs collected by density gradient using Lymphoprep (Axis-Shield, Oslo, Norway). Collected PBMCs were used in assays or cryopreserved in liquid N_2 in 90% FBS and 10% DMSO. In some experiments, T cells were purified directly from donor blood with anti-CD3 magnetic beads (Miltenyi Biotec) and isolated by positive selection using an

AutoMACS Separator (Miltenyi Biotec). The T cell purity was >90%, as assessed by flow cytometry using anti-CD45 antibodies. Monocyte-derived dendritic cells (DCs) were obtained from healthy donor PBMCs by culturing cells with IL-4 (Peprotech) and GM-CSF (Leukine sargramostim, Genzyme) for 7 days, which typically yield a purity of >90% (Mo-DC Inspector Cocktail, Miltenyi Biotec).

T cell proliferation

Total splenocytes from naïve mice were cultured in 75% supernatant from doxorubicin-treated or radiation-treated GL261 cells in the presence of 2 μg/mL anti-CD3 (clone 2C11) and 2 μg/mL anti-CD28 (clone 37.51; both prepared in house). Cells were incubated for 48 hours at 37°C, 5% CO_2 before addition of 0.25 μCi thymidine (MP Biomedicals). Proliferation was measured 16 hours later by liquid scintillation counting.

PBMCs were grown with culture supernatant/media in anti-human CD3/CD28 (R&D Systems) coated 96 well plates. Purified T cells were grown with culture supernatant/media with a T Cell Expansion/Activation kit (Miltenyi Biotec). For mixed lymphocyte reactions, PBMCs (100,000 cells per well) plus DC (20,000 cells per well) were grown with culture supernatant/media. Cells were incubated for 48 hr at 37°C, 5% CO_2 before addition of 0.25 μCi thymidine. Proliferation was measured 16 hr later by liquid scintillation counting. In some experiments, PBMCs were labeled with carboxyfluorescein succinimidyl ester (CFSE) prior to use in proliferation assays. After incubation for 4 days, CFSE dilution was determined by flow cytometry. For antibody blocking experiments, prostaglandin E2 monoclonal antibody (clone 2B5, Cayman Chemical Company) or an isotype control (clone MOPC-21, BioLegend) was added to the cells during incubation.

Manipulation of supernatant

Unless otherwise stated, tumor culture supernatant was used from cryopreserved stocks. For heat-treatment prior to use, supernatant was heated at 100°C for 30 min and then snap-chilled on ice. For size fractionation experiments, GBM cells were cultured overnight in DMEM/F12 (Invitrogen) supplemented with 1% penicillin/streptomycin and 1% GlutaMax to produce serum-free culture supernatant. The supernatant was separated into two fractions using a 3,000 Da molecular weight cut-off spin column (Vivaspin 500 MWCO 3000, GE Healthcare). The remaining supernatant that had not passed through the column was reconstituted to the original volume with media. Size fractionation was confirmed by SDS-PAGE (data not shown). For 2-(2,6-dichloranilino) phenylacetic acid (diclofenac) treatment experiments, diclofenac (10uM, Sigma) or DMSO was added during the selection of treatment-exposed colonies after doxorubicin or γ-irradiation treatment. Diclofenac was not present during the 16 hr conditioning of supernatants from treatment-exposed cells.

Real-time PCR

Total RNA was extracted from treated cells using the RNeasy Plus kit (Qiagen), and cDNA was synthesized with iScript (Bio-Rad). Real-time PCR was performed with the SensiMix SYBR Low-ROX kit (Bioline) using 12.5 ng cDNA, primers *COX1*, *COX22*, *18s rRNA* (QuantiTect primer assay, Qiagen) on the ABI 7500 platform. The exponential phase of the amplification curves were used to calculate cycle threshold,[12] and each Ct was normalized to 18s rRNA (ΔCt). Efficiency of amplification of each QuantiTect primer assay was equivalent, so the ΔΔCt method was used to determine fold change from control.

Flow cytometry

Cells were stained with antibodies at 4°C for 15 min in PBS supplemented with 1% FBS, 0.01% sodium azide, and 2 mmol/L EDTA. Nonspecific binding was blocked with 2 mg/mL polyclonal human IgG (Intragam P, CSL Limited). Dead cells were excluded by staining with propidium iodide (Sigma-Aldrich). The following antibodies were used: CD3-PE or CD3-APC-H7 (clone SK7), CD8-APC (SK1), CD45-APC (H130), FoxP3-PE (259D/C7), CD4-FITC (RPA-T4) (all BD Biosciences). For FoxP3 staining, cells were incubated with a viability dye (LIVE/DEAD Fixable Blue, Molecular Probes) prior to cell surface staining. Cells were fixed/permeabilized using a FoxP3/Transcription Factor Staining Buffer Set (eBioscience) before staining with anti-FoxP3 antibody. A LSRII flow cytometer or FACSCalibur apparatus (both BD Biosciences) were used to acquire data, and FlowJo software version 9.6.1 (TreeStar Inc.) was used for analysis.

Enzyme immunoassay and cytokine analyses

PGE2 concentration was determined by colorimetric competitive enzyme immunoassay (PGE2 High Sensitivity EIA kit; Enzo). Cytokine levels in GBM supernatant were determined by cytokine bead arrays (Human Cytokine 27-plex Assay and Human Cytokine 21-plex Assay, both Bio-Rad Laboratories) and analysed on a Bio-Plex analyzer (Bio-Rad Laboratories).

Clinical survival data

Clinical survival data for the cytokines assessed in GBM lines were generated from the Repository of Molecular Brain Neoplasia Data (REMBRANDT) home page. http://rembrandt.nci.nih.gov. National Cancer Institute. 2005. Accessed 2013 May 10.

Statistical analysis

Data are represented on graphs as the mean (data point or bar) ± SEM (error bars), and n is the number of replicates within an experiment. Statistical analyses were determined

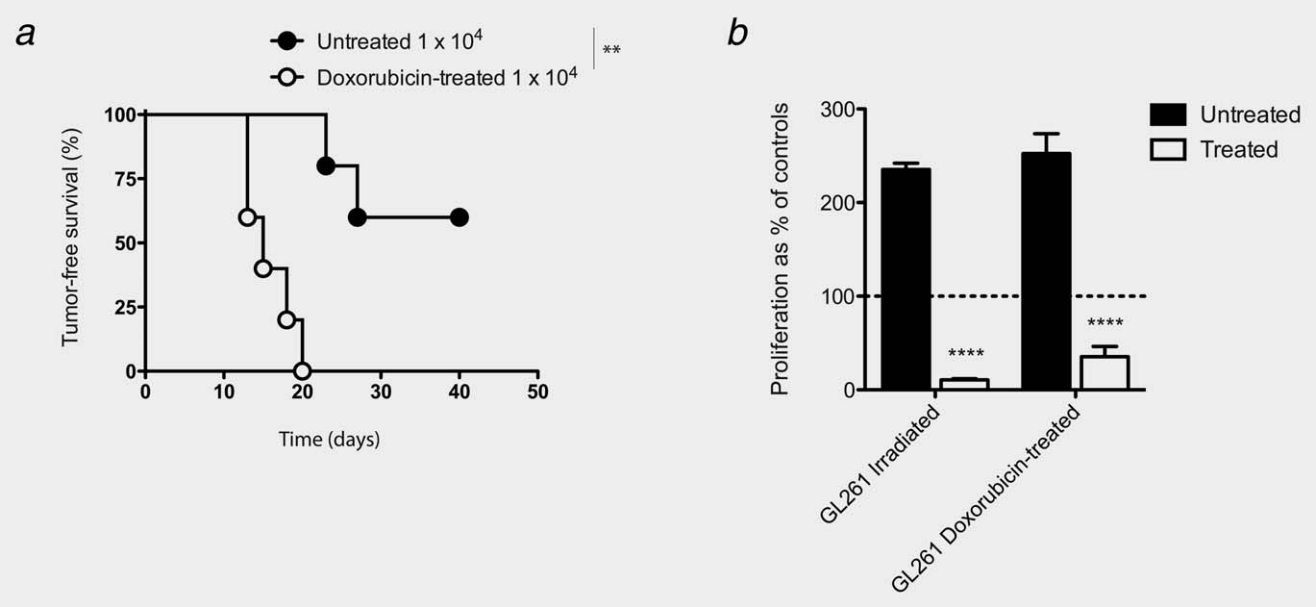

Figure 1. Treated murine glioma cells are immunosuppressive. (*a*) Doxorubicin-treated or untreated GL261 cells were implanted s.c. into C57BL/6 and monitored for tumor formation. $n = 3–5$ mice for each group (log-rank test, $^{**}p < 0.01$). (*b*) Total mouse splenocytes were cultured with 75% supernatant from treated or untreated murine GL261 cells and the ability of T cells to proliferate in response to anti-CD3/CD28 was determined by thymidine incorporation. Dotted lines represent splenocyte proliferation in the absence of supernatant. Results are displayed as mean + SEM of $n = 2–3$ for each group (unpaired *t* test, $^{****}p < 0.0001$). Results are representative of at least 2 experiments.

by the Student *t* test, log-rank test or one-way ANOVA using GraphPad Prism version 5.0.

Results

Treated murine glioma cells are immunosuppressive

The murine glioma cell line GL261, or primary cells derived from human GBM tumors, were used to investigate the immunosuppressive activity of chemotherapy-treated and radiation-treated cells by modeling treatment *in vitro*. To generate chemotherapy-exposed cells, transient exposure to doxorubicin was followed by harvest, washing and replating to select for a subset of cells that survived treatment.[17] Doxorubicin dose was titrated to select for 20% of the initial population. As controls, tumor cell suspensions were similarly exposed to the drug vehicle, DMSO. After DMSO or doxorubicin selection, cells were cultured for 3–4 days to allow for 2–3 rounds of cell division. The drug and vehicle treated cells were collected and plated separately at the same cell concentration and incubated overnight to provide a conditioned supernatant for analysis of potentially immunomodulatory secreted factors. To generate radiation-treated cells, tumor cells were subjected to four cycles of ionizing radiation (2 Gy) for four consecutive days, then replated to select for cells that survived treatment. Treated and untreated cells were then plated at the same cell concentration and cultured overnight to collect supernatants. Cell counts of the treated and control cells conducted at the end of the culture period were similar (data not shown), suggesting that the prior exposure to chemotherapy did not affect the proliferative index of the treated cells.

Initial studies were conducted with murine glioma cells. To verify that treated glioma cells could still form tumors *in vivo*, 1×10^4 doxorubicin-treated or untreated GL261 cells were implanted s.c. into C57BL/6 mice and monitored for tumor progression. Interestingly, in comparison to GL261 controls, mice that received doxorubicin-treated GL261 cells had a significantly decreased tumor-free survival time (Fig. 1*a*). As the faster tumor formation observed in mice that received treated cells could be explained by enrichment of more malignant cells, possibly reflecting alterations that render the cells more stem-like,[5,18] we investigated whether treatment could affect the ability of tumor cells to modify immune function *in vitro*. The supernatants from treatment-exposed GL261 cells were incubated with whole mouse splenocytes that had been stimulated with antibodies to CD3 and CD28 to induce T cell proliferation (Fig. 1*b*). Supernatants from doxorubicin-treated cells substantially suppressed T cell proliferation to levels significantly below those observed in the presence of media alone. Although doxorubicin alone could inhibit T cell proliferation, this only occurred at doses above 100 nM (Supporting Information Fig. 1). Given that the doxorubicin-exposed cells were extensively washed and then cultured before collection of supernatant, the T cell suppression observed was unlikely to be attributable to residual drug levels. This capacity for suppression suggests that the increased tumorgenicity of doxorubicin-treated glioma cells *in vivo* might be at least in part due to released factors that

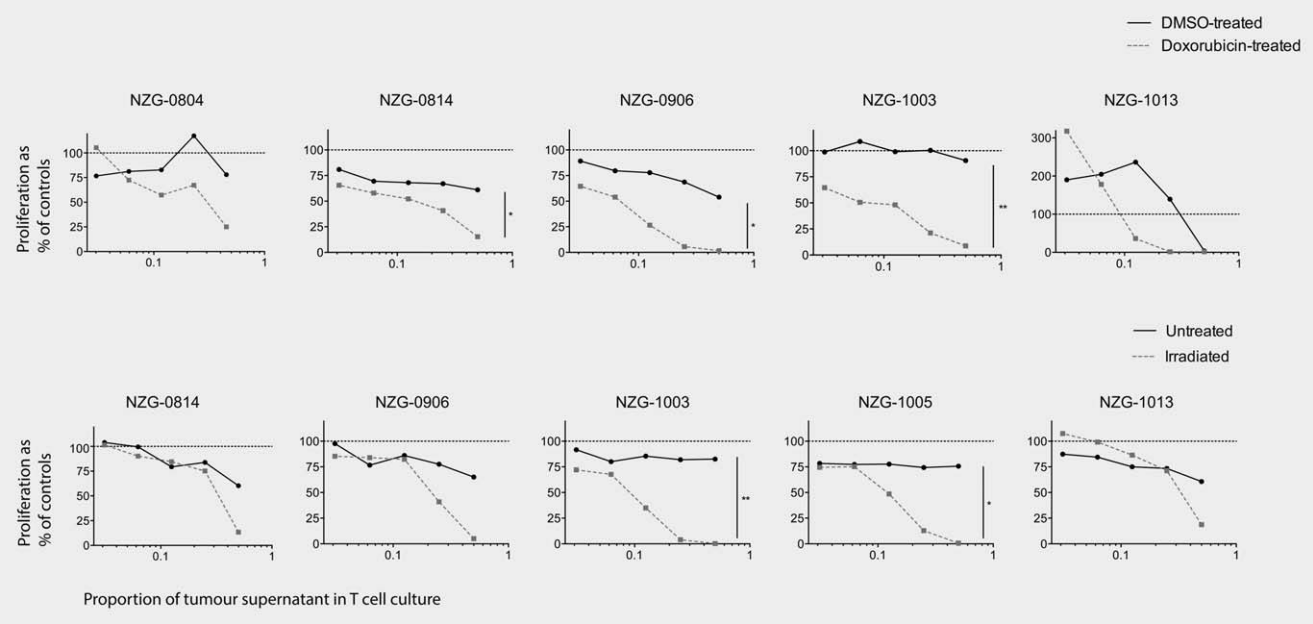

Figure 2. Therapy-exposed primary GBM cells demonstrate enhanced T cell suppression. PBMCs from healthy donors were stimulated with anti-CD3/CD28 in the presence of increasing concentrations of supernatant from doxorubicin-treated, radiation-treated or control-treated primary GBM cell lines. Proliferation was determined by thymidine incorporation. $*p < 0.05$, $**p < 0.01$ (unpaired t test).

cause evasion of immune-mediated eradication. Interestingly, supernatants from radiation-treated cells were similarly suppressive in the same T cell proliferation assays.

Conditioned media from treated primary GBM cells suppress T cell proliferation

We next determined whether therapy-exposed primary human GBM cells also have an enhanced ability to suppress the immune system. Five human primary tumor-derived GBM cell lines (the NZG series) were used to generate conditioned media as described above. Supernatants from both doxorubicin-treated and radiation-treated GBM tumor cells were incubated with PBMCs stimulated with antibodies to CD3 and CD28 (Fig. 2). Suppression of T cell proliferation was observed with supernatants from DMSO-treated or untreated cells compared to PBMC stimulated in media alone, consistent with the immunosuppressive nature of GBM. However, significantly enhanced suppression was again observed with supernatants from the treated tumor cells.

Flow cytometry was used to determine the lymphocyte populations that were suppressed by therapy-exposed GBM tumor cells. To accomplish this, CFSE-labeled PBMCs were stimulated with antibodies for CD3 and CD28, with or without conditioned media supernatant from primary GBM line NZG-0906. Levels of CFSE expression were then determined on gated $CD4^+$ and $CD8^+$ T cells at the end of the culture period, with reduced levels indicating that the cells had undergone proliferation. Supernatants from DMSO-treated or untreated primary GBM tumor cells reduced both $CD4^+$ and $CD8^+$ T cell proliferation by ~40% compared to cultures without tumor supernatant (Fig. 3*a*), indicating that this technique was more sensitive than thymidine incorporation at detecting suppressive activity in untreated cells. Importantly, proliferation was completely abrogated in T cells cultured with supernatant from either doxorubicin-treated or radiation-treated cells and inhibited both $CD4^+$ and $CD8^+$ T cell proliferation equally. This result was also confirmed in cell line NZG-1003 (data not shown). Since GBM has been shown to inhibit proliferation through the induction of T regulatory cells (Tregs), we also examined $FoxP3^+$ Tregs in the cultures. No increases in the proportion of $FoxP3^+$ Tregs were observed with supernatant from the treated GBM cells (Fig. 3*b*), suggesting that suppression is not mediated by altering the ratio of Tregs to conventional T cells.

The conditioned media supernatants from the treated GBM tumor cells were also tested in a mixed lymphocyte reaction in which PBMC were mixed with allogeneic monocyte-derived DC. Again, supernatants from both doxorubicin-treated and radiation-treated primary GBM line NZG-0906 significantly suppressed proliferative responses relative to supernatants from their respective controls. Thus the suppressive activity was observed under different modes of T cell activation and could not be overridden by the potent antigen-presenting cell function of DCs (Fig. 3*c*).

Finally, to determine whether the factors inducing the enhanced suppression could act directly on T cells, T lymphocytes were enriched from PBMCs to >90% purity before assays were conducted. Again, suppression was significantly enhanced by the supernatant from the treated cells (Fig. 3*d*). These results demonstrate that chemotherapy-treated and radiation-treated GBM cells release factors that can directly

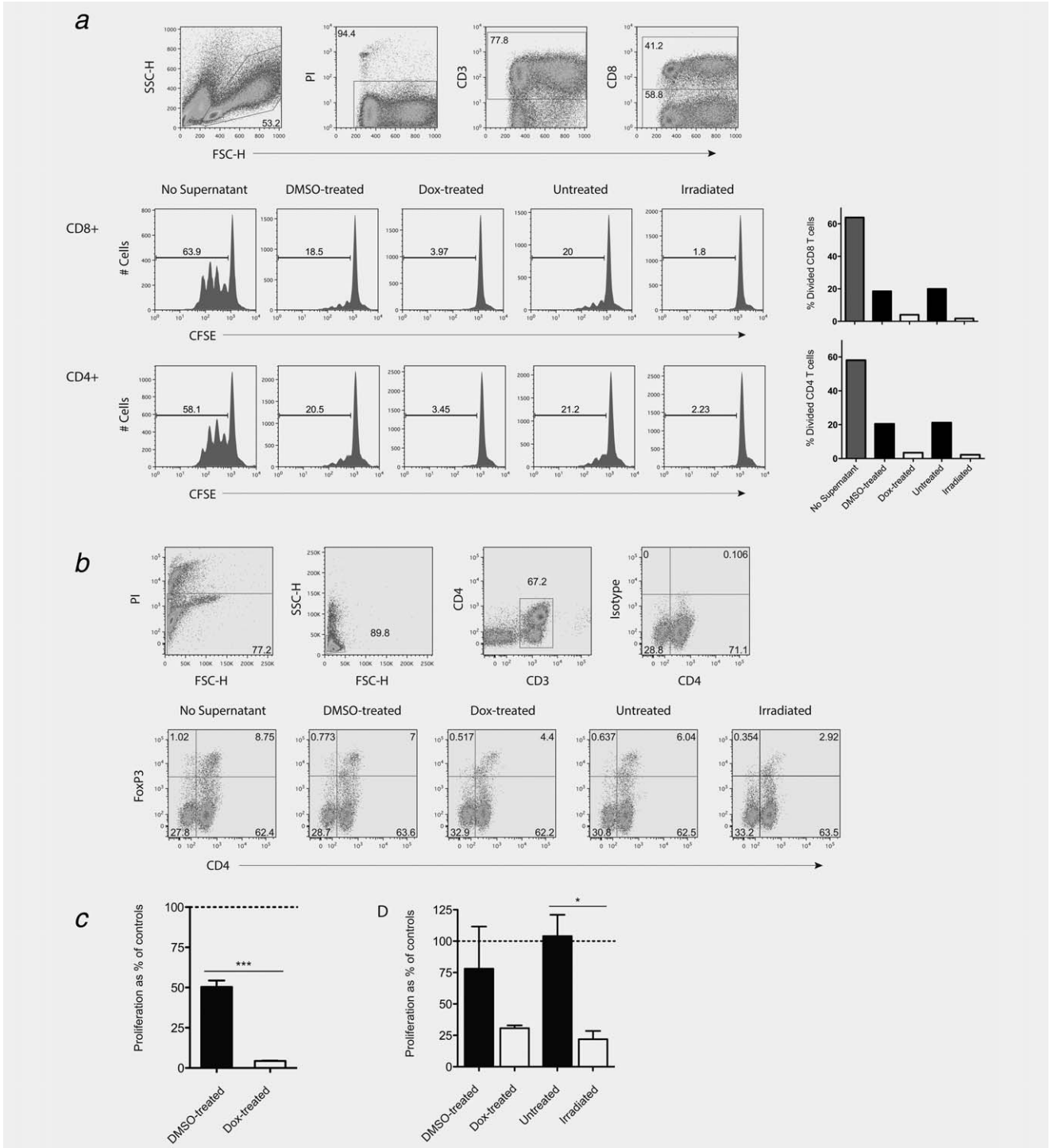

Figure 3. Treated GBM cells suppress $CD4^+$ and $CD8^+$ T cell proliferation independent of Treg induction. PBMCs were stained with CFSE and stimulated with anti-CD3/CD28 in the presence of 25% supernatant from doxorubicin-treated, radiation-treated or control-treated primary GBM cell line NZG-0906. (*a*, left) Representative flow plots show the gating strategy and CFSE dilution plots. (*a*, right) Bar graphs show the percentage of divided $CD8^+$ and $CD4^+$ T cells based on flow cytometry plots of CFSE diluted cells. (*b*) Representative flow plots show the gating strategy used to identify $CD4^+$ T cells and the proportion of lymphocytes that express FoxP3. (*c*) PBMCs were cultured with allogeneic monocyte-derived DC alone or in the presence of 25% supernatant from doxorubicin-treated or control GBM cell line NZG-0906 in a mixed lymphocyte reaction. (*d*) Purified T cells stimulated with anti-CD3/CD28 in the presence of 25% supernatant from treated or control-treated primary GBM cell line NZG-0906. Dotted lines represent T cell proliferation in the absence of supernatant. Results are representative of atleast two independent experiments. Results are displayed as mean + SEM of $n = 3$ for C, D, $*p < 0.05$, $***p < 0.001$ (unpaired *t* test).

Table 1. Cytokines detected in supernatants from treated GBM lines relative to untreated controls[1]

	Doxorubicin treatment					Irradiation treatment				Correlation
Cytokine[2]	0814	0906	1003	1013	Range[3]	0814	0906	1003	Range[3]	to survival[4]
VEGF		++++			10,227–33,165	+++	+++	++++	33,397–48,121	Negative ++++
IL-8	++++	++++	++++		1,335–15,315		++++		2,805–14,653	Negative ++++
IL-6	++	++++			3,209–14,162	++++	++++		1,949–15,868	Negative +
CXCL1	++++	++++			84–6,752	+++	++		174–7,807	Negative +++
HGF		++++		++++	243–2,250	++++	++++		323–7,606	
MIF		++++	++++	++++	370–3,510	++++	++++		1836–4,729	
G-CSF		++++	++++		17–1,921	++++	++++		123–8,418	Negative +
CXCL10		++++	++++	++++	639–4,320	+	++++	++++	599–4,320	Negative ++++
SCGF-β	++++	ND	++++		ND-1434			++++	513–4,756	
MCP-1	++	−−−−			417–1220				803–979	Negative +++
GM-CSF	−	++++			ND-742	++++	++++	ND	ND-2,696	
LIF	−	++++	−−−−	++++	166–521	++++	++++		72–3,395	Negative ++++
IL-12p70		+			316–572		+	++	574–689	Positive +
SDF-1α		++++	+++	++++	179–608	++++	++++	+++	327–688	
IFN-γ		++++	++		206–363	++	++++	++	342–385	Positive +
M-CSF		++++	+++	+++	71–198	++++	++++	+++	241–622	
RANTES		+++	++++	++++	15–411		++++	++++	27–225	Negative ++
MCP-3		++++	++++	++++	22–200	++++	++++	++++	170–313	
TRAIL	ND	++++	++++	++++	ND-163	++++	++++	++++	109–301	
MIG		++++	++	++++	16–175	++++	++++	++++	93–158	
IL-12p40	ND	ND	ND	ND	ND	++++	ND	ND	ND-171	Positive +
IL-3	ND	ND	ND	ND	ND	++++		ND	ND-128	
IL-10		+++			50–76		++++	++++	92–101	
IFN-α2		++++	++++	++++	26–92	++++	++++	++++	64–94	
SCF		++++		++++	31–36	++++	++++	++++	57–160	
IL-1Ra		++++	+		20–55	+	++++	+	31–89	
IL-9					24–43	+++	+++	+	31–65	
Eotaxin					18–54	+++	+		23–73	
IL-2Rα		++++	+++	++++	5–35	++++	++++	+++	25–49	
IL-7		++	++		23–36		+	++	35–43	
TNF-α	+	++++	+		18–23	+++	++++		18–87	
IL-13					24–33		+	++	37–45	
FGF basic		++++	++++	++++	8–30	++++	++++	ND	ND-54	Positive ++
IL-18	ND	++++	++++	++++	ND-18	++++	++++	++++	15–35	Negative +
β-NGF	++++	+++	++++	++++	10–19	++++	++++	++++	15–30	
PDGF-BB	ND	ND	ND	ND	ND	++++	++++	++++	17–22	
CTACK	ND	ND	ND	ND	ND	++++	+++	ND	ND-78	
IL-1α	ND	++++	ND	ND	ND-12	++++	++++	ND	ND-76	
IL-16	ND	ND	ND	ND	ND	++++	++++	ND	ND-30	
IL-17α	ND	ND	ND	ND	ND	+	+++	ND	ND-20	
IL-1β	ND	ND	ND	ND	ND	++++	++++	ND	ND-18	
TNF-β	ND	ND	ND	ND	ND	+++	ND	ND	ND-9	
IL-2	ND	ND	ND	ND	ND	ND	ND	ND	ND	

Table 1. Cytokines detected in supernatants from treated GBM lines relative to untreated controls (Continued)

	Doxorubicin treatment					Irradiation treatment				Correlation to survival[4]
Cytokine[2]	0814	0906	1003	1013	Range[3]	0814	0906	1003	Range[3]	
IL-4	ND	ND	ND	ND	ND	ND	ND	ND	ND	
IL-5	ND	ND	ND	ND	ND	ND	ND	ND	ND	Positive +
IL-15	ND	ND	ND	ND	ND	ND	ND	ND	ND	Negative +++
MIP-1α	ND	ND	ND	ND	ND	ND	ND	ND	ND	
MIP-1β	ND	ND	ND	ND	ND	ND	ND	ND	ND	Positive +++

[1]Cytokines measured in supernatant by bioplex technology, presented in order highest to lowest average cytokine level detected.
[2]Significance of fold-change in cytokine detected in treated versus untreated cell supernatant. Significant increases with treatment are marked: ++++ $p<0.0001$; +++ $p<0.001$; ++ $p<0.01$; + $p<0.05$. Significant reduction with treatment are marked: −−−− $p<0.0001$; − $p<0.05$. Samples below detection limit of assay are marked ND.
[3]Range of cytokine detected in treated cell supernatant in pg/ml
[4]Correlation to survival generated from queries of the NIH's REMBRANDT brain tumour database for survival and gene expression data. The expression of cytokines in glioma samples was correlated with survival using a Kaplan–Meier Survival Curve and significance is presented as cytokine upregulation correlating either positively or negatively with survival. ++++ $p<0.0001$; +++ $p<0.001$; ++ $p<0.01$; + $p<0.05$.

suppress T cell function; no other accessory cell is required for this activity.

Therapy-exposed GBM cells differentially express cytokines and chemokines associated with poor survival in glioma patients

In an attempt to define a common factor that could be responsible for mediating the increased immunosuppression observed in the different therapy-treated GBM cells, conditioned media from a number of doxorubicin-treated, radiation-treated, vehicle-treated or untreated GBM cells were analyzed for the expression of 48 different cytokines and chemokines by multiplex suspension array technology (Table 1). Many of these factors were found at increased levels in supernatants from the treated GBM cells compared to control cells. Included were factors that promote Treg function or their migration into tumors (IL-10, IL-6, IL-8, GM-CSF and HGF), or promote myeloid-derived suppressor cell (MDSC) function and/or migration (VEGF, GM-CSF, M-CSF, IL-6).[19] However, these cytokines promote immunosuppressive pathways involving accessory suppressor cells and cannot explain the immunosuppressive activity that was targeted directly at T cells in our experiments. Despite this, increased expression of these cytokines may reflect additional relevant immunosuppressive activities that could be induced by therapy-resistant tumor cells *in vivo*. In support of this, queries of the NIH's REMBRANDT brain tumor database, which is based on Affymetrix gene expression data and survival data, indicated that many of the chemokines and cytokines found at higher levels in supernatants from treated tumor cells were associated with significantly decreased survival in glioma patients (Table 1).

The increased levels of soluble IL-2 receptor α in many of the supernatants from treatment-exposed resistant cells was notable because this can have a direct inhibitory effect on T cell proliferation by depriving T cells of IL-2.[20] However, addition of IL-2 was unable to completely restore proliferation in our cultures (data not shown). Thus, although the multiplexed array analysis revealed increased expression of many potentially immunosuppressive factors as a result of prior exposure to therapy, an obvious candidate responsible for mediating the enhanced suppression of T cell function in our assays was not identified.

Treated GBM cells differentially express PGE2

To further narrow down the list of possible immunosuppressive candidates, we characterized the immunosuppressive factor based on size. To this end, conditioned media from doxorubicin-treated GBM cell line NZG-0906 was separated into <3 kDa and >3 kDa fractions before being used to assess impact on T cell proliferation (Fig. 4*a*, left). Only the <3 kDa fraction was able to suppress proliferation to the same extent as unfractionated supernatant. We also investigated the heat-stability of the suppressive factor by heating the conditioned supernatant to 100°C for 30 min. This treatment had no effect on the suppressive activity of the medium (Fig. 4*a*, right), suggesting that the suppressive factor is a heat-stable compound less than 3 kDa in size.

The small size and heat-stability of the immunosuppressive factor suggested an immune modulating metabolite. Investigations into the tryptophan metabolism pathway failed to reveal a suppressive candidate consistently increased by therapy exposure (data not shown). Further investigations were focused on PGE2 as it has been shown to exhibit considerable heat stability.[21] We investigated whether PGE2 expression was increased in conditioned media from doxorubicin-treated and radiation-treated GBM cells compared to DMSO-treated or untreated cells, respectively. This analysis revealed differential expression of PGE2 in the supernatants of some of the GBM cells lines studied; the concentration of PGE2 was significantly higher in the media of doxorubicin-resistant lines NZG-0906 and NZG-1003, and radiation-treated lines NZG-0814 and NZG-0906

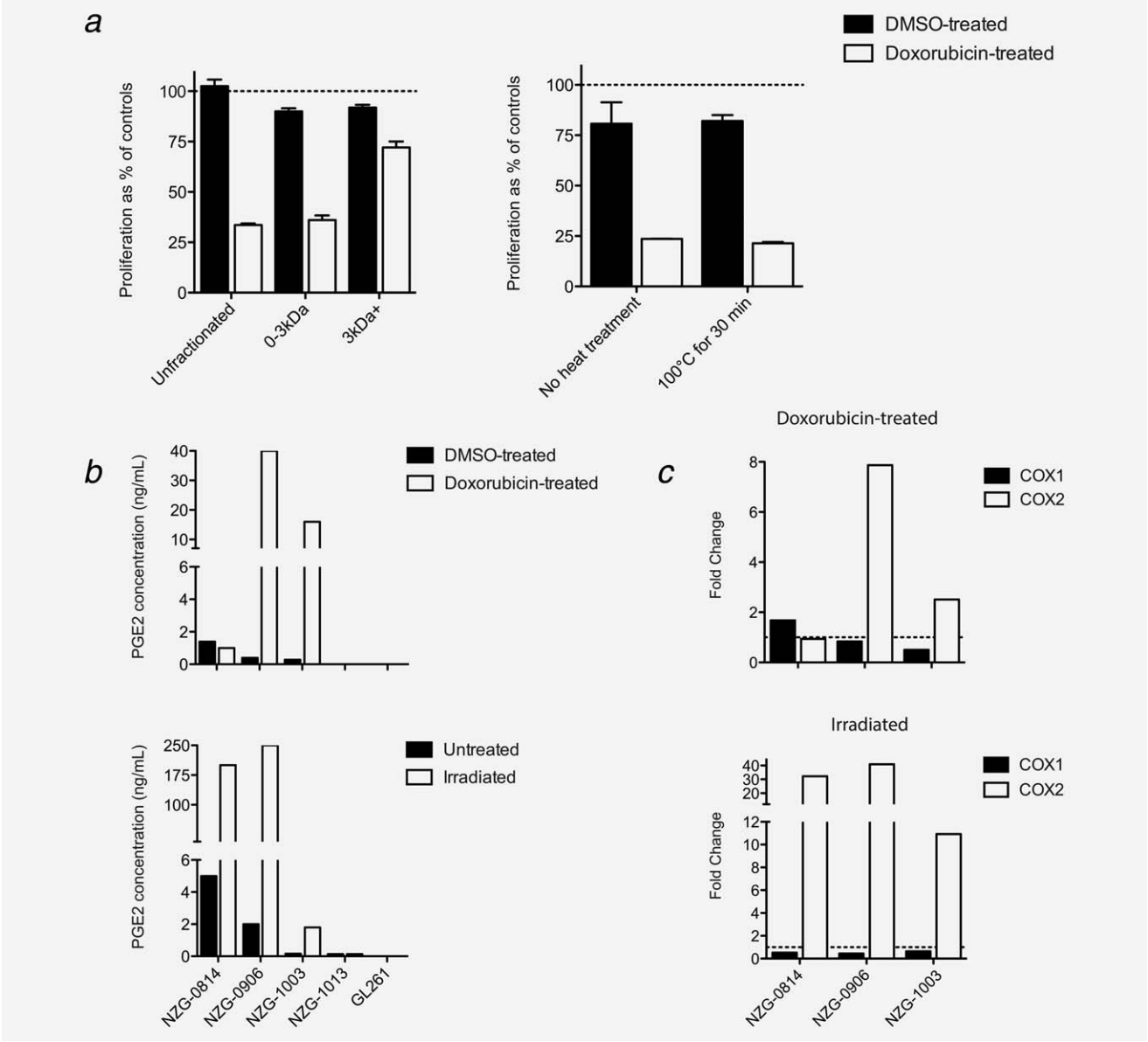

Figure 4. Therapy-exposed GBM cells differentially express cytokines and metabolites. (*a*) PBMC were stimulated with anti-CD3/CD28 in the presence of conditioned supernatant from GBM cell line NZG-0906 that had been separated into >3 kDa and <3 kDa fractions, or heat-treated. Dotted lines represent PBMC proliferation in the absence of supernatant. (*b*) The concentration of PGE2 in conditioned media from GBM cell lines and murine glioma line GL261 was measured by ELISA. (*c*) COX1 and COX2 gene expression in conditioned media from GBM cell lines was measured by quantitative RT-PCR. Fold change in gene expression is expressed relative to control-treated cells.

compared to controls, while no PGE2 was detected from GBM line NZG-1013 in control or therapy-exposed cell media (Fig. 4*b*). We also found that the increased expression of PGE2 correlated with enhanced gene expression of COX2, a key enzyme required for PGE2 biosynthesis, in both doxorubicin-treated and radiation-treated GBM cells (Fig. 4*c*). Interestingly, PGE2 was undetectable in untreated and therapy-exposed GL261 cells, demonstrating that mechanisms other than PGE2 are responsible for the immune suppression observed by treated murine GL261 glioma cells (Fig. 4*b*).

PGE2 inhibition partly reverses the immune suppression imparted by therapy-exposed GBM cells

To determine whether PGE2 was responsible for the enhanced immune suppression imparted by treated cells, we investigated the ability of a PGE2 blocking antibody, 2B5, to reverse the T cell suppression observed by doxorubicin-treated NZG-0906 GBM cells, which exhibited elevated levels of PGE2. (Fig. 5*a*). Significantly, inhibition of PGE2 completely reversed the T cell immunosuppression induced by doxorubicin-treated GBM cells, while an isotype control had no affect. Since elevated levels of PGE2 correlated with

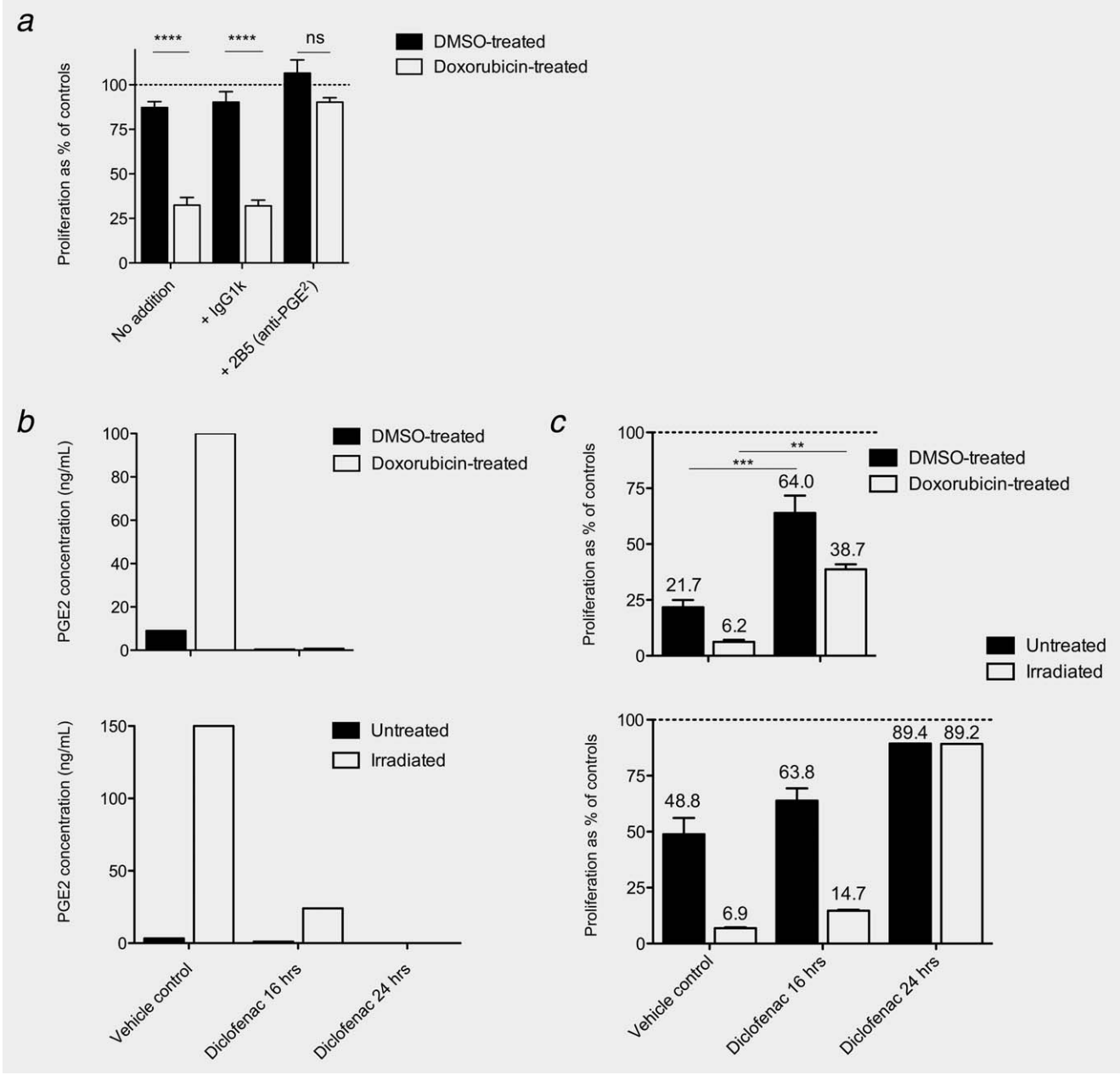

Figure 5. PGE2 blockade restores the T cell proliferation inhibited by treated GBM cells. (*a*) PBMC were stimulated with anti-CD3/CD28 in cultures containing 25% conditioned supernatant from doxorubicin-treated or control-treated GBM cell line NZG-0906, in the presence of a PGE2 blocking antibody or an isotype control. (*b*) The concentration of PGE2 in conditioned media from NZG-0906 with or without diclofenac added during the selection of treatment-exposed colonies after doxorubicin treatment. (*c*) PBMC were stimulated with anti-CD3/CD28 in cultures containing 25% conditioned supernatant from NZG-0906 that had been treated with or without diclofenac. Dotted lines represent PBMC proliferation in the absence of supernatant. Results are displayed as mean + SEM of $n = 3$ for A, C. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (two-way ANOVA, Bonferroni post-test).

COX2 upregulation, we wanted to determine whether diclofenac, an FDA approved COX2 inhibitor, could remove therapy-exposed GBM cell mediated immunosuppression. Doxorubicin-treated, radiation-treated and DMSO-treated/untreated GBM cells from line NZG-0906 were cultured in the presence of diclofenac and then conditioned media was collected and PGE2 concentration determined by ELISA (Fig. 5*b*). Exposure to diclofenac for 16 h during culture completely abrogated PGE2 release from doxorubicin-treated cells, while exposure to diclofenac for 24 h was required to abrogate PGE2 release from radiation-treated cells. Importantly, when these PGE2-depleted media were cultured with PBMCs, significantly less suppression of T cell proliferation was observed (Fig. 5*c*). Diclofenac also reversed the lower

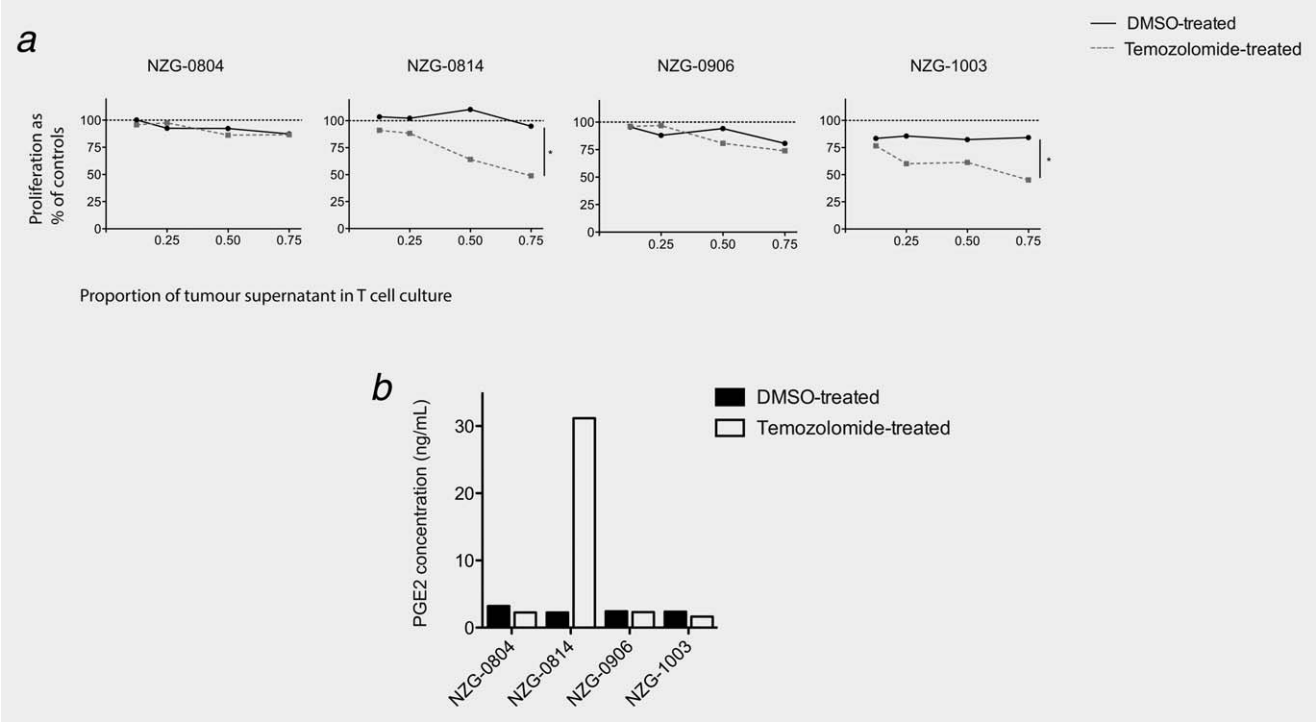

Figure 6. Temozolomide-treated cells suppress T cell proliferation and upregulate PGE2. (*a*) PBMCs from healthy donors were stimulated with anti-CD3/CD28 in the presence of increasing concentrations of supernatant from temozolomide-treated or control-treated primary GBM cell lines. Proliferation was determined by thymidine incorporation. (*b*) The concentration of PGE2 in conditioned media from GBM cell was measured by ELISA. *<0.05 (unpaired *t* test).

level suppression induced by supernatants from GBM cells that had not been exposed to chemotherapy or radiation. Diclofenac had no affect on T cell proliferation when added directly to PBMC cultures (Supporting Information Fig. 2). These results demonstrate that enhanced PGE2 release is responsible for the immune suppression mediated by exposure to therapy in some GBM tumors.

Temozolomide-exposed GBM cells can suppress T cell proliferation and upregulate PGE2

Finally, we examined whether increased immune suppression could be observed following exposure of GBM cells to temozolomide, a chemotherapeutic drug prescribed to GBM patients. Four of the human primary tumor-derived GBM cell lines were transiently exposed to temozolomide, allowed to recover, replated and cultured for 18 hr to generate conditioned media. As before, PBMCs were cultured with the conditioned media in the presence of antibodies to CD3 and CD28 to stimulate T cell proliferation (Fig 6*a*). Significant suppression of T cell proliferation was observed with supernatants from two of the temozolomide-exposed cell lines, NZG-0814 and NZG-1003, suggesting that this drug also has the potential to increase the suppressive capacity of GBM cells. Levels of PGE2 were significantly upregulated following exposure to temozolomide in NZG-0814 cells, which was the primary line that induced the most striking level of T cell suppression (Fig 6*b*).

Discussion

The modest efficacy of immunotherapeutic vaccines for the treatment of GBM has led to increasing interest in the effect that GBM has on the immune environment. It is now recognized that GBM is highly immunosuppressive, utilizing a variety of mechanisms to facilitate immune escape, most notably reduced T cell responsiveness, induction of T regulatory cells, accumulation of myeloid-derived suppressor cells, defects in dendritic cell function and release of suppressive cytokines and soluble factors.[22–26] Here, we investigated whether different forms of prior treatment, specifically exposure to chemotherapy or radiation therapy, may also influence the capacity of GBM to affect the immune environment.

We generated doxorubicin-treated and radiation-treated glioma cell lines *in vitro* and assessed their secretion of immunomodulatory factors. Using the murine glioma cell line GL261 and several primary human GBM lines, we show that cells that have survived either of these treatments can secrete factors that are highly immunosuppressive in assays of lymphocyte proliferation *in vitro*. This enhanced immunosuppression was independent of accessory suppressor cells or the induction of Tregs; rather, it was mediated by immunosuppressive factors capable of inhibiting the proliferation of purified T cells directly, affecting both $CD4^+$ and $CD8^+$ T lymphocytes. While treatment of the different GBM cell lines induced upregulation of a number of

cytokines and chemokines, many of which were associated with poor survival in glioma patients, at least one key immunosuppressive factor that could act directly on T cells was shown to be a heat-stable molecule smaller than 3 kDa in size. PGE2 fits this biochemical profile, was upregulated in some GBM cell lines exposed to doxorubicin, temozolomide or irradiation, and importantly, inhibition of PGE2 synthesis in the conditioned supernatant could significantly restore T cell proliferation. Collectively, these results demonstrate that therapies like radiation and chemotherapy can exacerbate the immunosuppressive properties of GBM tumor cells and provide a further barrier to effective immunotherapy.

The treated tumor cells used in this study were subject to transient treatment *in vitro*. For both doxorubicin and radiation therapy, this caused a dramatic change in cytokine expression, suggesting multiple pathways were affected. Doxorubicin was used in most of these assays because of its ease of use *in vitro*. Although doxorubicin has been shown to have a strong cytotoxic effect on glioma cells and is effective in animal tumor models, its use in the clinic has been restricted by its low lipid solubility and inability to cross the blood brain barrier. However, doxorubicin is still regarded as a potentially useful agent in the glioma setting and there is considerable interest in modified formulations that improve bioavailability.

It has been previously observed that GBM cancer stem cells can induce immune suppression, primarily by the expansion of Tregs.[27] We have previously observed that doxorubicin-treated cells have a cancer stem-like phenotype, with increased expression of genes associated with stem-like activity.[17] However, we did not find the same enhanced expression of these genes in the radiation-treated cells (data not shown), suggesting that the enhanced immunosuppression observed can be independent of cancer stem cell phenotype. Furthermore, our results show that this immunosuppression is not mediated by altering the ratio of Tregs to conventional T cells.

We demonstrate that treated human GBM cells have enhanced immunosuppressive functions that are mediated by PGE2 in some cancer cell lines. The ability of PGE2 to suppress immune responses is well documented; PGE2 has been shown to directly inhibit T cell proliferation by suppressing the synthesis of both IL-2 and IL-2 receptor.[28,29] This function of PGE2 may explain the inability of exogenous IL-2 to completely restore T cell proliferation in our cultures, which others have also shown to be ineffective at reversing the immune suppression imparted by PGE2.[28,29] Moreover, PGE2 has been implicated in the induction of Tregs, MDSCs and expression of indoleamine 2,3-dioxygenase (IDO), a key regulator of immune tolerance.[26,30,31] PGE2 and COX2, the rate-limiting enzyme responsible for PGE2 biosynthesis, have also both been implicated in glioma; GBM cells have been shown to overexpress PGE2 and COX2 and suppress anti-tumor immune responses.[32,33] In addition to its effect on the immune system, PGE2 and COX2 expression have also been associated with radiotherapy resistance in some cancers by promoting tumor cell progression and survival.[34] Differential expression of COX2 has been demonstrated in radiotherapy-treated and radiotherapy-sensitive GBM cells, and increased susceptibility of therapy-treated COX2-expressing cells to radiation has been observed following COX2 inhibition.[35,36] Our results suggest that increases in COX2 and PGE2 expression by GBM cells following therapy not only promotes tumor cell survival through direct mechanisms, but also by evasion of immune eradication by suppressing immune responses.

As the enhanced immune suppression induced by therapy-treated cells is likely to impede the efficacy of immunotherapy treatment for recurrent GBM, combinational therapeutic approaches are likely needed for immunotherapy to be effective. The administration of COX2 inhibitors in conjunction with temozolomide has been investigated in Phase I/II trials for the treatment of GBM with conflicting results.[37–39] Importantly, COX2 blockade in combination with immunotherapy has demonstrated increased survival benefit in preclinical murine studies of melanoma and breast cancer, and has demonstrated enhanced survival benefit in a rat glioma model compared to immunotherapy alone,[40–42] although this has yet to be confirmed in the clinic. Although we demonstrated increased T cell suppression by five different doxorubicin-treated and radiation-treated primary GBM cell lines, PGE2 expression was not consistently upregulated in all of the lines studied and was not detected in murine GL261 glioma cells. This, along with our data demonstrating differential cytokine and chemokine expression amongst treated and untreated GBM cells, suggests that therapy-exposed cells utilize more than one mechanism to induce immune suppression.

Importantly, we demonstrate that chemotherapy and radiation therapy induce drastic changes in GBM cells that influence the immune environment and ultimately, tumor survival. Therefore, a personalized approach to determine the primary suppressive mechanism, followed by combinational drug therapy to remove that immunosuppression is likely to be required for immunotherapy to be an effective treatment option for recurrent GBM.

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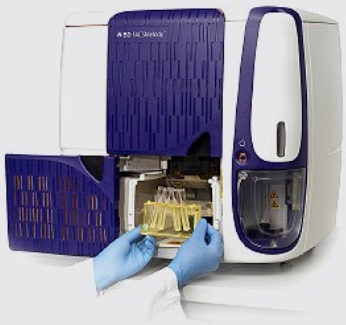

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