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IMMUNOGENIC OXIDATIVE THERAPY-BASED NEXT-GENERATION DENDRITIC CELL VACCINES AGAINST HIGH-GRADE GLIOMA

Lien VANDENBERK

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List of abbreviations

5-ALA	5-aminolevulinic acid
APC(s)	antigen presenting cell(s)
ATP	adenosine triphosphate
BBB	blood brain barrier
CAR	chimeric antigen receptor
CD	cluster of differentiation
CMV	cytomegalovirus
CNS	central nervous system
CNTR	control
COX	cyclo-oxygenase
CRT	calreticulin
CSF	colony stimulating factor
CTL(s)	cytotoxic T lymphocyte(s)
CTLA-4	cytotoxic T lymphocyte-associated protein 4
DAMP(s)	damage-associated molecular pattern(s)
DC(s)	dendritic cell(s)
Ecto	surface exposed
EGFRvIII	epidermal growth factor receptor variant III
eIF2α	eukariotic inititiation transcription factor 2-alpha
ER	endoplasmatic reticulum
Exo	secreted
FasL	Fas ligand
FDA	food and drug administration
F/T or FT	freeze-thawing or freeze-thaw or freeze-thawed
GBM	glioblastoma multiforme
GITR	glucocorticoid-induced TNFR family related gene

GM	GentleMACS®
GM-CSF	granulocyte/macrophage colony-stimulating factor
HGG	high-grade glioma
HHP	high hydrostatic pressure
HMGB	high-mobility group box
HSP(s)	heat shock protein(s)
Hyp-PDT	hypericin-based photodynamic therapy
ICD	immunogenic cell death
IDO	indoleamine 2, 3-dioxygenase
IFN	interferon
IL	interleukin
i.p.	intraperitoneal
IR	irradiation or irradiated
KO	knockout
L-Hist	L-Histidine
LN	lymph node
LPS	lipopolysaccharide
mAb	monoclonal antibody
MDSC(s)	myeloid-derived suppressor cell(s)
MGMT	O^6-methylguanine methyltransferase
MHC	major histocompatibility complex
MyD88	myeloid differentiation primary response gene 88
NAC	N-acteylcysteine
NK	natural killer
OAMP(s)	oxidation-associated molecular pattern(s)
OS	overall survival
PD-1	programmed cell death protein 1

PD-L1	programmed death-ligand 1
PERK	PKR-like ER kinase
PFS	progression-free survival
PGE_2	prostaglandin E2
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PTEN	phosphatase and tensin homolog
SPLs	splenocytes
TAA(s)	tumor-associated antigen(s)
TAM(s)	tumor-associated macropage(s)
TGF	transforming growth factor
Th1 or Th17	T helper type-1 polarized cells or T helper IL17A-secreting cells
TIL(s)	tumor-infiltrating lymphocyte(s)
TLR	toll-like receptor
TMZ	temozolomide
TP53	tumor protein p53
Treg(s)	regulatory T cell(s)
TSA(s)	tumor-specific antigen(s)
TTF(s)	tumor treating field(s)
TUDCA	Tauroursodeoxycholic acid
UPR	unfolded protein response
UV	ultraviolet
Vacc	vaccine
VEGF	vascular endothelial growth factor
WHO	world health organization

1. GENERAL INTRODUCTION

"There is no medicine like hope, no incentive so great, and no tonic so powerful as expectation of something tomorrow."

- Orison Swett Marden

1.1 High-grade glioma: a devastating disease in dire need of therapeutic advancement

1.1.1 Classification, epidemiology and risk factors

High-grade glioma (HGG) is a terrible disease with a universally dismal prognosis in children and adults. Gliomas in general are the most common primary intracranial tumor, representing 81% of malignant brain tumors. The term glioma refers to a group of central nervous system (CNS) tumors that occur primarily in the brain. Gliomas are either astrocytic (most frequent), oligodendrocytic or mixed oligoastrocytic, depending on the glial cell type from which they originate (1, 2). The World Health Organization (WHO) classifies gliomas into different grades (from I to IV) based on several histopathological criteria such as presence or absence of necrosis, endothelial proliferation, extend of cellular and nuclear polymorphism and frequency of mitotic activity (1). Grade III (anaplastic astrocytoma, anaplastic oligodendroglioma and anaplastic oligoastrocytoma) and grade IV (glioblastoma multiforme/ GBM) represent the most aggressive HGGs. Of these, GBM is not only the most frequent but also the most malignant tumor type (1). GBM can either occur as a primary *de novo* lesion (in 90% of the cases) or it can progress from a low grade astrocytic tumor (secondary GBM). In comparison to anaplastic astrocytoma, GBM shows more microvascular proliferation and necrosis. GBM tumors rarely metastasize outside the brain and are most frequently located in the white matter of the frontal or temporal lobes. Patients often present with progressive neurological deficit, headache and to a lesser extent with seizures, local edema and increased intracranial pressure.

The overall age-adjusted incidence rates for glioma range from 4.67 to 5.73 per 100,000 persons per year (2). Although this is a relatively smaller number as compared to certain other cancer types, the years of life lost from brain tumors are the highest amongst all cancer types. HGG is often diagnosed at a young age and results in short term fatal outcomes (3). Both anaplastic astrocytoma and GBM increase in incidence with age, peaking in the 75-84 age groups (2). Gliomas are more common in men than in women and it seems that glioma incidence rates do not increase over time (2).

With regards to glioma risk factors, they can be subdivided into environmental and genetic factors. To date, the only proven environmental risk factor for glioma is ionizing radiation (4).

On the other hand, epidemiologic studies of large and diverse groups of cases and controls consistently suggest that allergic conditions (asthma, hay fever, eczema and food allergies) can reduce glioma risk (2). Other exogenous causes like smoking, non-ionizing radiation (cellular phone use), high dose chemotherapy and occupational chemical exposures have also been linked to glioma, but with inconclusive results (4, 5). When it comes to genetic risk factors, several monogenic Mendelian disorders (e.g. Li-Fraumeni syndrome, Lynch syndrome, Neurofibromatosis 1 and 2) are associated with increased risk of GBM and glioma in general (2). However, these disorders can only account for a small proportion of adult glioma incidence. To characterize other genetic contributors to adult glioma, five genome-wide association studies were performed. These studies have identified eight independently significant germline DNA single nucleotide polymorphism associations located in seven genes (i.e., TERT, RTEL1, EGFR, TP53, CDKN2B, PHLDB1 and CCDC26) (6). Although no single molecular factor or signature has been determined to drive gliomagenesis, these genetic mutations can provide an excellent basis to unravel the main signaling pathways underlying this disease.

In spite of multidisciplinary treatment, the median prognosis of GBM patients is limited to 14.6 months, with less than 10% of patients surviving 5 years post diagnosis (7). Relapse is universal and upon relapse, prognosis is even worse. Relapsed GBM patients have a median survival expectancy of only 9 months and almost all patients succumb within 18 months (8). The treatment failure is attributed to the diffuse infiltration of therapy-resistant tumor cells into healthy tumor tissue. This results in recurrent tumor growth which, in 95% of the cases, develops very close to the resection cavity.

1.1.2 Standard of care and major breakthroughs

Despite therapeutic advances, HGG remains an incurable disease with no change in the standard of care for already a decade. This standard of care is based on a protocol first reported by Stupp *et al.* in 2005 (7). It involves maximal safe surgical resection followed by postoperative radiotherapy with concomitant temozolimide (TMZ) chemotherapy and continued use of adjuvant TMZ for 6 to 12 months. As compared to patients treated with radiotherapy alone, this treatment protocol could prolong the median overall survival (OS) of over 500 newly diagnosed GBM patients by 2.5 months (from 12.1 to 14.6 months) and could improve the 2-year and 5-year survival rates (7, 9). TMZ is an oral alkylating agent that adds a methylgroup on the O^6 position of guanine in the DNA. As a consequence, this methylated guanine residue can no longer basepair with thymine during the following DNA replication cycle, resulting in the G2/M arrest and subsequent autophagy followed by apoptosis (10).

The most decisive prognostic factors for GBM are age at the moment of diagnosis, Karnofsky performance status and the degree of tumor resection (2). In view of the latter, fluorescence-guided resection with 5-aminolevulinic acid (5-ALA) is introduced since 2006, resulting in a

higher rate of complete resection and an improved 6-month progression free survival (PFS) in patients diagnosed with HGG (11).

Another important milestone in HGG therapy is the discovery that patients with a methylated O^6-methylguanine-DNA methyltransferase (MGMT) promoter have better responses to chemotherapy with alkylating agents like temozolomide (12). MGMT is a DNA repair enzyme that can remove alkyl groups from the DNA, enabling the prevention of DNA mismatch during replication and transcription. Methylation of the genetic promoter of this enzyme inhibits its activity. Hence, MGMT promoter methylation is considered a very valuable predictive biomarker for therapy response to TMZ that can be tested on a routine basis for therapeutic decision making.

Given the recent advances in molecular diagnostics, alternative tumor classification methods (other than the WHO histological classification) are proposed based on molecular abnormalities and signaling pathways that play a role in glioma. These molecular subtypes show distinct prognosis and therapy responses. While there is significant correlation between traditional pathologic groupings and the newer molecular subtypes, this overlap is incomplete. Three core pathways have been proven to be targeted in GBM patients: retinoblastoma signaling, tumor protein 53 (TP53) signaling and receptor tyrosine kinase signaling (2). Subsequent to the characterization of the main signaling pathways, a GBM categorization based on gene expression profiles was proposed containing the following subtypes: classical, neural, proneural and mesenchymal (13). The most frequent classical subtype harbors hallmark epidermal growth factor receptor (EGFR) alterations and deletion of cyclin dependent kinase inhibitor 2A. Recently, the proneural subtype of GBM (characterized by isocitrate dehydrogenase mutations and hypermethylation across the genome) was shown to be the only subgroup with improved survival (2). Hence, molecular classification opens the door for personalized treatment of HGG by transforming diagnosis, dictating survival and elucidating subgroups that may respond better to specific therapies.

Unfortunately, targeted molecular therapies have not been really successful thus far with the exemption of the antiangiogenic agent bevacizumab (a neutralized monoclonal antibody against VEGF, a key regulator of angiogenesis) (14). In 2009, bevacizumab received approval from the Food and Drug administration (FDA) for recurrent GBM based on an uncontrolled phase II trial demonstrating improved PFS at 6 months (15).

Another therapy that was recently approved by the FDA for recurrent GBM is Nova-TFF (14). This treatment consists of tumor treating fields (TTFs) that are low amplitude alternating electric fields that can disturb cell division and tumor development.

1.2 Cancer immunoediting: from immune surveillance to immune escape

Despite the heterogeneity of different cancer types, they all appear to share six hallmarks: angiogenic capacity, unlimited replication, resistance to apoptosis, autonomous growth, unresponsiveness to inhibitory growth signals and the ability to metastasize (16). In the last decade, it was proposed that the ability of tumors to evade immunological pressure can be considered an extra characteristic of tumorigenesis (16, 17). The rationale for this constatation lies within the immunoediting hypothesis originally proposed by Dunn *et al*. (17)

The idea that the immune system can control tumor growth without therapeutic intervention (also termed immunosurveillance) is being discussed since the 1950s. Three main findings have meanwhile confirmed its existence (17). First, the incidence of chemically-induced, spontaneous and genetically engineered tumors is increased in immune compromised mice. Also in immunosuppressed or immunocompromised humans, both virus-induced and non-virus-induced tumors develop at higher incidences. Second, the presence of tumor-infiltrating lymphocytes (TILs) can correlate with improved survival in cancer patients. Finally, owing to the discovery of tumor-specific antigens, it became clear that cancer patients can develop tumor-antigen specific immune responses.

However, the interplay between the cancer and the immune system was not completely resolved with this concept of immunosurveillance. How would immunosurveillance then alow cancers to develop in immunologically intact individuals? The immunoediting paradigm gives a plausible explanation to this question (Figure 1). In this concept the tumor progresses through three different phases during its interplay with the immune system: elimination, equilibrium and escape (17). In the first phase, the innate and adaptive immune system work together to recognize and destroy the malignant cells before they become a clinically apparent entity. However, cancer cells that manage to survive elimination are subjected to continuous immune selection pressure. This causes the tumor cells to genetically adapt, giving rise to new variants with increased resistance to immune attack. This equilibrium phase is considered a latent period in which those tumor cells that are not eliminated in the elimination phase persist in a dynamic interaction with immunity (18). Finally, in the escape phase, the edited tumor cells can outstrip the immune pressure to constrain tumor growth and become clinically detectible. A broad spectrum of tumor escape mechanisms have been discovered that can be grouped into two categories: tumor cell intrinsic and tumor cell extrinsic mechanisms. The most relevant mechanisms for immune escape in the context of HGG will be discussed in chapter 1.4.

A key feature of cancer immune editing is that tumors express tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs) that can be targeted by T cells, leading to their destruction and/or selection of tumor escape clones. Indeed the importance of T cells, in particular $CD8^+$ T cells in cancer immune editing has long been recognized, with large

annotated data sets often demonstrating favorable prognosis in patients with strong $CD8^+$ T cell infiltrates. In addition to $CD8^+$ T cells, other adaptive and innate immune cells and parameters eventually orchestrate the antitumor immune response. The term immune contexture defines this complex immunological composition of the tumor microenvironment in which the location, density and functional orientation of infiltrating hematopoietic cells correlates with the clinical outcome of patients. Although this human immune contexture can only be evaluated at the stage of tumor escape (when the tumor is clinically apparent), it can predict the ability of the natural immune reaction or therapies to drive the tumor into equilibrium or to even destroy the tumor.

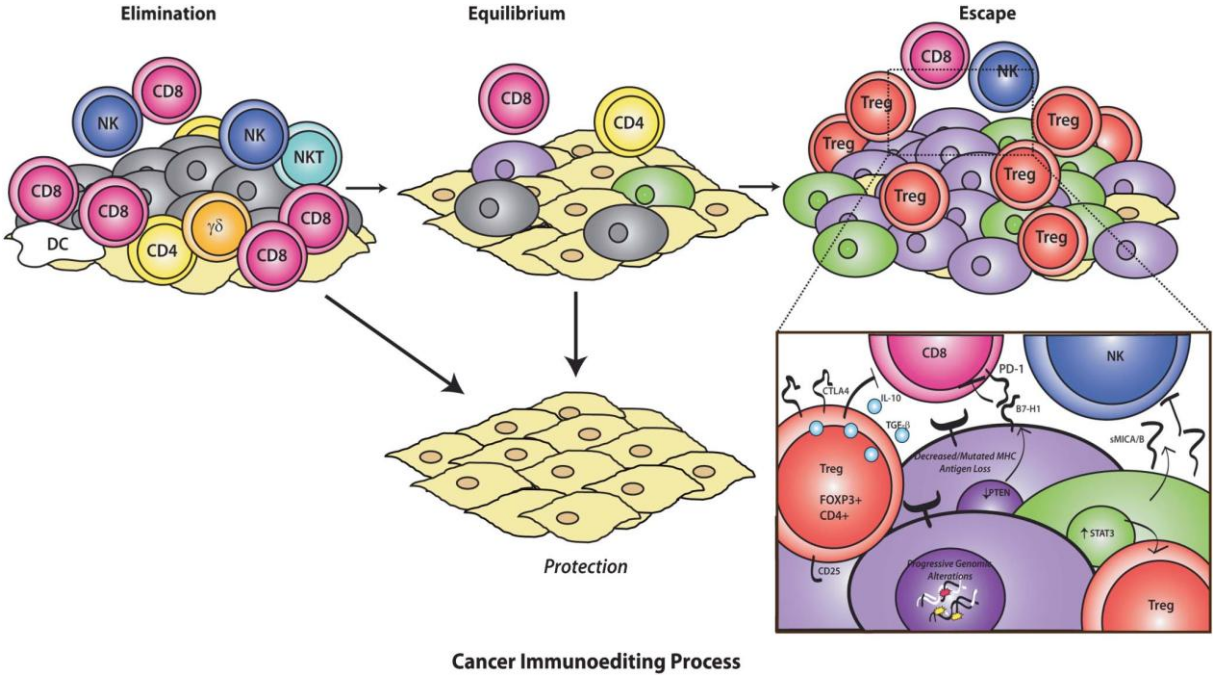

Figure 1: An overview of the cancer immunoediting process in high-grade glioma. *Cancer immunoediting encompasses three main phases, i.e. elimination, equilibrium and escape. Tumors can be eliminated in either the elemination or equilibrium phase. However, due to immune selection pressure and genemic instability, tumors may ultimately escape immune protection and become clinically apparent in the escape phase. The molecular basis of several immune escape mechanisms is discussed in chapter 1.4 of this work. NK, natural killer; NKT, NK T cell; PTEN, phosphate and tensin homolog; Treg, regulatory T cell. (Modified adapted from Dunn et al. (19)).*

1.3 Immune contexture in HGG

1.3.1 The CNS as an immunologically specialized site

The brain has long been envisaged as an immunoprivileged site due to the presence of the tight blood-brain barrier (BBB) and the absence of a conventional lymphatic drainage system. Later experiments have invalidated this dogma by revealing that the CNS is

immunocompetent and can exhibit dynamic interactions with the peripheral immune system. Although the cellular biology of the BBB remains poorly understood, it is now a certainty that this barrier is deteriorated in the context of malignant glioma (20). In addition, it is now well recognized that intracerebral antigens and T cells can access the cervical lymph nodes via drainage through Virchow-Robin spaces (20). Moreover, dendritic cells (DCs) injected into brain tumors have been shown to migrate to the cervical lymph nodes in a mouse glioma model (19, 21). In order to manifest antiglioma cellular immunity, glioma antigens have to be presented in major histocompatibility complex (MHC) molecules (either within the brain itself or in the peripheral lymphoid structures) to naïve precursor T cells and B cells. Within the brain, DCs and microglia (the brain's resident macrophages) are the main antigen-presenting cell (APC) types, although B lymphocytes and perivascular macrophages may also exert this function (20). DCs and microglia are both able to cross-present intracerebral antigens in MHC-I context to naïve $CD8^+$ T cells *in vivo*. A last argument in favor of the immunocompetent state of the CNS is the ability of antibodies and activated T cells, but not naïve T cells, to bypass or to penetrate the BBB to interact with their target antigens (19). Once arrived in the brain, these antigen-specific T cells are capable of proliferating and acquiring effector function.

1.3.2 The presence of glioma-infiltrating lymphocytes and their prognostic relevance

In order to mount effective antitumor immunity, activated lymphocytes like activated antigen-specific $CD4^+$ T helper (Th) cells, $CD8^+$ cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells must traffic into the glioma microenvironment. Several chemokines (in particular CXCL12) and adhesion molecules (integrins α4β1 and αEβ7) expressed by T cells can attribute to their CNS homing (19). Both $CD4^+$ and $CD8^+$ T cells make up 4 to 40% of tumor-infiltrating immune cells in glioma (22). Moreover, it has been shown that TILs are able to lyse autologous tumor cells (19). It is increasingly being reported that increased number of TILs may correlate with improved survival in many cancer types (23). In the context of glioma, tumor-infiltrating $CD8^+$ T cells are considered a good prognostic indicator (24-28). With regards to the infiltration of general $CD4^+$ lymphocytes, the results are less straightforward. While three studies report a longer survival in patients with an enriched $CD4^+$ T cell infiltrate (28-31), two studies contradict these results (26, 31). In line with these data, Yu *et al.* have demonstrated a higher $CD8^+/CD4^+$ T cell ratio in less aggressive brain tumors (Yu 2003). These conflicting results for the $CD4^+$ subset might be attributed to the fact that the $CD4^+$ population also comprises immunosuppressive regulatory T cells (Tregs) and different Th subsets. The significance of Treg infiltration in HGG will be discussed in chapter 1.4.2.3. While the positive prognostic value of the Th1 subset has already been demonstrated in the context of DC vaccinated GBM patients (32), the prognostic significance of the Th2 subset is still not elucidated. Nevertheless, as further elaborated in chapter 1.4.2.1, the cytokine profiles of glioma infiltrating lymphocytes are generally skewed towards Th2-

biased humoral immunity, suggesting a negative effect of Th2 infiltration on prognosis (19). The role of Th17 cells, a Th subset involved in antimicrobial inflammation and autoimmunity, remains controversial in most tumor types. In the setting of glioma, a recent study by Cui and colleagues revealed a better OS and PFS in GBM patients with high IL-17 (the main Th17 cytokine) expression levels (33). Furthermore, this study identified IL-17 expression as an independent prognostic indicator in GBM patients. This result is reminiscent of that in the GL261 mouse model in which intracranially implanted tumors grew equally well in wildtype and IL-17 deficient mice (34). Along the same lines, Hu *et al.* showed no difference in IL-17 expression between human low-grade and high-grade gliomas.

NK cells consist of cytotoxic effector lymphocytes that play an important role in anti-tumor immune responses through apoptotic killing of tumor cells and the secretion of IFN-γ. A high density of NK cells was shown not to correlate with clinical outcome in non-small lung cancer, while it is associated with improved survival in early stages of breast cancer (23). Moreover, recent reports have underlined that tumor-infiltrating NK cells have an anergic phenotype, characterized by downregulation of activating receptors (e.g. NKp30, NKp46, NKp80) and upregulated expression of co-inhibitory receptors (23). These data suggest that NK cells may have a protective role in early stages of tumor development, while exerting negligible or even immunosuppressive effects in clinically apparent tumors. When it comes to NK cells infiltrating in brain tumors, their suppressive role has been demonstrated both *in vitro* and *in vivo* (35, 36). These cells have been shown to infiltrate in HGGs as $CD56^{dim}CD3^{-}CD16^{-}$ cells (25). Nevertheless, the tumor infiltration by NK cells remains low (approximately 2% of all tumor-infiltrating immune cells) and their functionality is often harmed by tumor-derived factors (25). As an example, tumor-derived TGF-β downregulates the expression of the NKG2D activating receptor on NK cells isolated from GBM patients (37).

The role of B cells in gliomas remains enigmatic. Although these cells have been shown to infiltrate in HGGs, they only represent a tiny fraction (0.03 – 0.7% of all tumor-infiltrating immune cells) (25). As documented by Candolfi *et al.*, B cells can function as APCs for T cells in a murine GBM model, suggesting their favorable prognostic impact (38). In contrast, several reports suggest a protumoral role of B cells relating to their ability to negatively regulate macrophage function through IL-10 production (37).

1.4 Immune escape mechanisms at play in HGG

The observation that most patients who develop HGGs are not immunocompromised underscores the concept of immune escape. Indeed, there is a profound local immune suppression in the glioma microenvironment that can provoke systemic depression of cellular immunity, even in a more rigorous fashion than observed in other solid tumors. A myriad of immunoevasive cues are at play in HGG (Table 1). These can be grouped in tumor intrinsic and tumor extrinsic mechanisms.

Table 1: Escape mechanisms involved in glioma immunoediting

Intrinsic mechanisms establishing invisibility		**Therapeutic strategies**
- Escape of adaptive T cell recognition	Defective MHC class I antigen presentation/processing, antigen loss (39)	Induction of immunogenic cell death, DC vaccination (40)
	Aberrant T cell costimulation (decreased B7-H1 and increased PD-L1 expression) (19, 41)	Adjuvants (e.g. CD40 agonist, anti-CD137), PD-1/PD-L1 blockade (42)
- Evasion of innate NK cell recognition	Increased expression of HLA-E and HLA-G (19, 41, 43)	NKG2A blocking antibodies (44)
	Release of soluble NKG2D ligands (19)	
Extrinsic mechanisms impairing immune cell function		**Therapeutic strategies**
- Lymphocyte dysfunction and Th2 skew	T cell anergy and lymphopenia (19, 45, 46)	Adoptive T cell therapy, checkpoint blockade (e.g. anti-CTLA-4, anti-PD-1/PD-L1) (42, 47-51)
	Th2 skew in TILs and blood (52-54)	Adoptive T cell therapy, DC vaccination (40, 47, 50, 51)
- Immunosuppressive factors	Immunosuppressive enzymes (e.g. IDO, COX-2) (20, 44)	IDO and COX-2 inhibitors (19, 55)
	Immunosuppressive molecules (TGF-β, IL-10, PGE_2, Galectin-1, VEGF) (19, 55)	Anti-VEGF therapy, TGF-β receptor 1 inhibitors/ TGF-β blocking antibodies, COX-2 inhibition (55)
- Recruitment of immune suppressive populations	Tregs (56-61)	Anti-CD25 antibodies, cyclophosphamide, low dose TMZ, IDO inhibitors (20, 55, 62)
	MDSCs (63, 64)	COX-2 inihibition, CCL2 neutralization, all-trans retinoic acid (20, 55, 65)
	TAMs/Microglia (20, 66)	CSF-1 receptor inhibition, CCL2 neutralization, STAT3 inhibition (20, 55, 65)

1.4.1 HGG intrinsic mechanisms

HGG intrinsic mechanisms encompass intrinsic changes within the glioma cell that impair their recognition by the immune system (Table 1).

1.4.1.1 Escape of adaptive T cell recognition

To establish invisibility, glioma cells can alter their MHC class I antigen processing and presentation capacity, thereby impairing CTL lysis. Facoetti *et al.* have demonstrated the loss of HLA class I antigens in about 50% of GBM patients (39). Moreover, this selective antigen

loss correlated positively with tumor grade. On the other hand, the expression of HLA-DR, a major MHC class II antigen increased with increasing tumor grades, hinting towards a skewing of the immune response towards Th cells rather than CTLs (19, 20). Likewise, GBM tumor cells have been shown to downregulate key molecules in the antigen processing machinery like LMP2 and TAP1 or they can even completely abolish the expression of certain antigens as was shown for epidermal growth factor receptor variant III (EGFRvIII) (19, 20, 48). The EGFRvIII is an example of a tumor-specific antigen (TSA) that is expressed in 30 to 40% of GBM patients, but its expression is absent on nontransformed cells (67). In addition, GBM cells that exhibit increased levels of MHC relative to nontransformed cells, show impaired expression of costimulation signals belonging to the B7 family (44). Programmed Death Ligand-1 (PD-L1) is a member of this B7 family, but exerts primarily inhibitory functions when expressed on tumor cells. Upon binding to its receptor PD-1, expressed on antigen-specific T cells, PD-L1 can infer anergy and even apoptosis of activated T cells (68). Already a decade ago, Wintrelle *et al.* documented the constitutive expression of PD-L1 in glioma cell lines and tumor specimens (68). Few years later, the loss of phosphatase and tensin homolog (PTEN), a suppressor gene playing a role in gliomagenesis, was shown to enhance the expression of PD-L1 on glioma cells (41). Of note, upon stimulation of PTEN deficient glioma cells with IFN-γ, immune evasion was even further potentiated by increased expression of PD-L1 and increased T-cell apoptosis (69). PD-L1 expression is positively correlated with tumor grade in the context of glioma (70) and co-culturing of alloreactive T cells with glioma cells expressing PD-L1 significantly depressed the production of proinflammatory cytokines like IL-2 and IFN-γ (68).

1.4.1.2 Evasion of innate NK cell recognition

Besides evading recognition by T cells, glioma cells have also developed smart strategies to disrupt the activity and recognition by NK cells. NK cells are innate effector lymphocytes that play a significant role in antitumor immune responses through e.g. the apoptotic killing of tumor cells and priming of Th1 responses (37). The functional status of NK cells largely depends on the delicate balance between the signaling via activating receptors or inhibitory receptors expressed on this innate cell type. NKG2A is an important inhibitory receptor expressed on NK cells and $CD8^+$ T cells. Its ligand, the non-classical MHC class I molecule HLA-E is overexpressed in primary GBM cell cultures, rendering them resistant to NK cell and CTL cytotoxicity (43). NKG2D on the other hand is an activating receptor expressed on NK cells, $CD8^+$ T cells and γδ T cells (19). Its ligands, MICA/B and ULBP2 are expressed in glioma specimens and their expression correlates inversely with WHO tumor grade (19). Moreover, glioma cells can evade NKG2D-dependent cytotoxicity by producing soluble forms of these ligands or by expressing the non-classical MHC class I molecule HLA-E (19).

1.4.2 HGG extrinsic mechanisms

HGG extrinsic mechanisms include the impairment of immune cells induced by the glioma cells (Table 1).

1.4.2.1 Lymphocyte dysfunction and a Th2 cytokine skew

A first extrinsic mechanism is the lymphocyte dysfunction observed in HGG patients. Several studies have outlined quantitative and functional deficits, specifically in the $CD4^+$ T cell subset (19). Additionally, T cells isolated from glioma patients show decreased responsiveness to IL-2 signaling and defects in early T cell receptor signaling events (45, 46). Another critical observation encompasses the predominant expression of Th2 cytokines (e.g. IL-4 and IL-10) in glioma specimens, cell lines and glioma TILs (53, 54). In line with this notion, HGG patients show increased serum levels of IL-10 (a general immunosuppressive cytokine related to Th2 responses) and decreased levels of IL-12 (a pro-inflammatory cytokine related to Th1 responses) (52). Although the mechanism for this polarization towards a protumoral Th2 profile in gliomas is not elucidated yet, glioma-derived IL-10 may play a curcial role here. IL-10 is known to contribute to the priming of Th2 responses and to interfere with Th1 differentiation. Primary glioma cultures produce high levels of IL-10 and IL-10 expression is higher in HGGs as compared to LGGs (44, 53).

1.4.2.2 Secretion and expression of immunosuppressive factors

Besides the secretion of IL-10, glioma cells secrete or express a myriad of immunsuppressive factors like STAT3, TGF-β, PD-L1, PGE_2, indoleamin 2, 3-dioxygenase (IDO), galectin-1 and Fas ligand (FasL) (20, 44). Several of these factors are implicated in the induction/expansion of Tregs (e.g. TGF-β, PGE_2, IDO), while others contribute to anergy induction in T cells (e.g. PD-L1, STAT3) or the induction of apoptosis (e.g. PD-L1, FasL and Galectin-1). Most of these factors have been extensively studied in experimental glioma mouse models, with promising drugs interfering with these pathways now being tested in clinical trials. For a more detailed overview of these immunoregulatory pathways, we refer to two recent reviews by Ahn *et al.* and Dunn *et al*. (19, 55).

1.4.2.3 Recruitment of immunosuppressive leukocyte populations

$CD4^+FoxP3^+$ Tregs, our natural guardians against autoimmunity, play a key role in glioma-induced immune depression. Here, Tregs have the highest predilection for tumors of the astrocytic lineage and specifically in the HGGs such as GBM (58, 60). Several studies have meanwhile documented a worse prognosis in glioma patients with high Treg levels (26, 57, 59, 61). Importantly, the frequency of tumor-infiltrating Tregs in GBM patients is higher than the blood frequency of Tregs (56, 57). This suggests that the systemic immune status does not accurately represent the ongoing immune reaction in the brain. Treg accumulation in the

blood of GBM patients has been shown to correlate with the impairment of T cell proliferation (71). The majority of Tregs in the context of gliomas are thymus-derived, natural Tregs (55). They are suggested to be recruited to the tumor site by the CC chemokines ligands (CCL) CCL2 and CCL22 that are abundantly produced by glioma cells (20, 60). Interaction of these ligands with their receptors, CCR2 and CCR4 respectively, highly expressed on glioma-infiltrating Tregs, facilitates their recruitment to the tumor site. Although the majority of glioma-infiltrating Tregs are thymus-derived, induced Tregs may also contribute to glioma progression. Indeed, IL-10 and TGF-β, both of which are produced in significant amount by gliomas *in vivo*, have been shown to induce the induction of Tregs from conventional T cells *in vitro (20)*. Tregs in glioma express a plethora of immunoregulatory molecules like CD25, glucocorticoid-induced TNFR family related gene (GITR), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and CD62L (55). The crucial role of Tregs in bolstering antiglioma immunity is further illustrated by Treg depletion studies in experimental glioma models. Several of these studies have demonstrated better tumor control in Treg depleted glioma-bearing mice (62).

When it comes to immunosuppressive glioma-infiltrating myeloid cells, their role in promoting tumor growth and invasion has gained much attention in recent years. Here, we can distinguish two main populations in glioma: the myeloid-derived suppressor cells (MDSCs) and the tumor-associated macrophages (TAMs)/microglia. Both cell types are known to promote tumor development via T cell suppression-dependent and independent mechanisms.

MDSCs represent a population of highly suppressive immature myeloid cells that can concentrate in the tumor, secondary lymphoid structures and blood of cancer patients in response to tumor-derived factors (e.g. VEGF, TGF-β, IL-6, IL-10, PGE_2 and CCL2) (20). In mice, MDSC are phenotypically characterized by the co-expression of CD11b and Gr1 (72). Further stratification into monocytic and granulocytic MDSCs is based on the expression of Ly6C and Ly6G, respectively. Human MDSC subsets are identified by the expression of CD11b and CD33 and by lack of expression of HLA-DR (72). Accordingly, human MDSCs can be subclassified into monocytic and granulocytic MDSC, based on the expression of CD14 and CD15, respectively. MDSCs can suppress T cell immunity through divergent mechanisms, including the production of nitric oxide and ROS, L-arginine and cysteine deprivation and the expansion of Tregs (20). In glioma context, Gielen *et al.* recently documented increased levels of monocytic and granulocytic MDSCs in the blood of glioma patients, while the glioma tissue showed a predominant infiltration of granulocytic MDSCs (63). Moreover, circulating MDSCs isolated from GBM patients have been shown to suppress IFN-γ production by autologous T cells (64).

In the setting of glioma, macrophages and microglia are the predominant tumor-infiltrating immune cells. They can comprise up to 30% of the glioma mass (20). Depending on

environmental signals, they can embody pro-inflammatory/antitumoral (M1) or alternatively activated immunosuppressive/protumoral (M2) phenotypes. In glioma patients, macrophage infiltration tends to increase with increasing tumor grades, suggesting their contribution to tumor progression (66). Interestingly, glioma infiltrating TAMs and microglia predominantly show M2 characteristics (e.g. reduced expression of co-stimulatory signals, secretion of M2 cytokines such as IL-4, IL-10 and TGF-β, and the upregulation of CD163 and CD204), particularly in late stage disease progression (55). TAMs are recruited and sustained in the glioma microenvironment via CCL2, colony-stimulating factor-1 (CSF-1) and stromal cell-derived factor-1 (SDF-1/CXCL12) amongst others (55). Gliomas can induce TAMs and microglia to upregulate the expression of PD-L1, FasL and STAT3, and to significantly downregulate the expression of pro-inflammatory cytokines and MHC molecules (20).

Given the substantial contribution of TAMs and MDSCs to glioma-mediated immune suppression, strategies targeting these myeloid populations are being exploited intensively. Depletion of the general $CD11b^+$ myeloid subset in GL261 glioma-bearing mice delays tumor progression (65). This finding highlights the tumor-promoting role of tumor-infiltrating myeloid cells in glioma. Along the same lines, the more specific depletion of MDSCs (either via COX-2 inhibition, Gr-1-mediated depletion or CCL2 neutralization) and TAMs (CFS-1 receptor blocking) has been shown to confer survival advantage in various murine glioma models (20, 55).

Over the last decade, oncologists have recognized that simply debulking the tumors via a combination of surgery and radio- and chemotherapy is not sufficient to eradicate therapy-resistant, heterogeneous and diffusely infiltrating glioma cells. Given the malevolent immunosuppressive and immunoevasive capabilities of HGGs described above, the need to tackle the glioma-induced immunosuppression and/or to actively stimulate antiglioma immune responses arose.

1.5 Immunotherapy to tilt the balance in favor of anti-glioma immunity

As opposed to standard cytotoxic therapies, immunotherapy is highly specific for eradicating cancer cells with low toxicity to surrounding normal brain tissue. Moreover, several immunotherapeutic strategies have been shown to evoke immunological memory. This memory response is considered extremely beneficial in the context of HGG, given the inevitable tumor relapse following standard-of care therapy.

Box 1 summarizes the main characteristics of the two most extensively studied immunocompetent murine brain tumor models for testing of immunotherapeutic approaches.

Box 1: Characteristics of the two most widely applied immunocompetent brain tumor models for testing of immunotherapeutic approaches		
	CT2A	**GL261**
Host	C57BL/6 mice	C57BL/6 mice
Induction	20-Methylcholantrene	3-Methylcholantrene
Ease of transplantation	Very good	Very good
Tumorigenesis	100%	100%
Histologic resemblance	High-grade astrocytoma	Ependymoblastoma
GBM characteristics	Highly proliferative and invasive, pseudopalisading necrosis, intratumoral heterogeneity, angiogenesis, *in vivo* migratory patterns	Pleomorphism, pseudopalisading necrosis, angiogenesis, invasiveness
Grade of stemness	High	Moderate
Immunogenicity	Unknown	Low baseline expression of MHC-I, but not MHC-II; Increased MHC-I and II expression upon stimulation with IFN-γ
TAAs	Unknown	HMP/AN2, EphA2, GARC-1
Oncogenic mutations	PTEN	PTEN, K-ras, P53
MGMT promotor methylation	Unknown	Yes

1.5.1 Checkpoint blockade to unleash the brakes of the immune system

Inhibitory checkpoint mediators like CTLA-4 and PD-1 function as brakes to attenuate normal T cell responses, respectively during the priming and effector phases of T cell activation (47). However, cancers exploit this mechanism to depress the antitumor immune response. Therapeutic blockade of CTLA-4 and PD-1 or its ligands PD-L1 and PD-L2 have demonstrated exciting antitumor benefit in several cancer types. FDA approval of ipilimumab, a monoclonal antibody targeting CTLA-4, for metastatic melanoma, including patients with brain metastasis, has paved the way for testing checkpoint blockade in the setting of HGG (73). CTLA-4 blockade led to 80% long-term survival in mice bearing SMA-560 brain tumors and PD-1 blockade could significantly improve overall survival of intracranial GL261 tumors when combined with radiation therapy (47). Very recently, two mAbs targeting PD-1 receptor signaling (nivolumab and pembrolizumab) have joined the list of FDA-approved check-point blockers (respectively for the treatment of metastatic squamous non-small cell lung cancer and relapsed/refractory melanoma patients). Several clinical trials are currently evaluating ipilimumab and PD-1/PD-L1 checkpoint inhibitors in recurrent and newly diagnosed HGG patients (NCT02529072, NCT02311920 and NCT02017717) (47).

1.5.2 Passive immunization with adoptive T-cell therapy

Adoptive T cell transfer involves the infusion of T cells with high avidity for tumor antigens. Originally this encompassed the administration of T cells isolated from draining lymph nodes from GBM patients or *ex vivo* expanded autologous T cells cocultured with tumor cells (47). A more contemporary application of this approach is the use of expanded autologous cytomegalovirus (CMV)-specific T cells from CMV-seropositive GBM patients (74). Indeed, recent studies assigning a potential association between human CMV and glioma, suggest a potential pool of tumor antigens of viral origin (51). Recent progress in this field has been made by applying genetically modified T cells engineered to express a chimeric antigen receptor (CAR) with high reactivity against TAAs (50). Due to their direct antigen-binding capacity, these so called CAR T cells can bypass MHC restriction. Preclinical experiments in glioma models have documented promising results with CAR T cells engineered to react with IL13R2α, Her2 and EphA2 (47). Moreover, clinical trials with CARs targeting EGFRvIII and Her2 are running for GBM patients. Major drawbacks of this type of immune therapy are related to the costs and the labor intensiveness of the *ex vivo* culturing, as well as the possibility of eliciting cytokine storms.

1.5.3 Active vaccination therapy

As opposed to passive immunization, active immunotherapy (also referred to as tumor vaccination) attempts to stimulate the host's intrinsic immune response against the malignancy. Several vaccination strategies have been exploited in the setting of HGGs. Peptide vaccines are the simplest application of this strategy. They comprise the administration of synthetic peptides derived from TAAs or TSAs. The majority of peptide vaccines currently under evaluation for glioma target the HLA-A2 haplotype (e.g. Her2, gp100, IL13R2α), this way limiting the application of this therapy to HLA-$A2^+$ tumors (19). In contrast, the TSA EGFRvIII is a highly promising target for peptide vaccination. Sampson *et al.* developed a promising EGFRvIII vaccine that could prolong the median OS of newly diagnosed GBM patients in comparison to a matched control group (48). This strategy was proven to be safe (even in adjuvant setting after radiochemotherapy) and both humoral and cellular immune responses were documented. However, upon recurrence after vaccination, 80% of the GBM tumors were EGFRvIII negative, suggestive of tumor immune editing by induction of tumor antigen escape (48). To overcome this antigen escape, several groups are exploring the use of multipeptide vaccines and autologous tumor cell vaccines. Indeed, data provided by Neller *et al.* suggest a better response rate in immunotherapies using whole tumor cell approaches than therapies targeting single antigens (75). A recent modification of the multipeptide approach involves vaccination with tumor antigens bound to 96 kD heat shock proteins (HSP96) (47). HSPs are natural adjuvants that can facilitate the presentation of peptides to circulating APCs such as DCs. Early clinical trials in recurrent GBM patients have

shown that this therapy can induce immunogenicity with potential survival benefit (47). Whole tumor cells approaches usually apply autologous tumor cells that can be irradiated, combined with cytokine-producing fibroblasts or transduced with Newcastle disease virus (76). However, due to the difficulties associated with culturing inactivated autologous tumor cell cultures and the impact on the immunogenicity of the tumor cells, this form of active immunotherapy is the least widely applied.

In general, the success of peptide and whole tumor cells vaccines is considered limited. This can be attributed to the lack of immune adjuvants, resulting in poor immunization and even tolerance induction (75). Moreover, *in vivo* circulating APCs often show low MHC expression and frequently lack costimulatory signals in glioma-bearing individuals (20). DC vaccines can bypass these difficulties. DCs are positioned at the crucial interface between the innate and adaptive immune system as APCs capable of inducing antigen-specific $CD4^+$ and $CD8^+$ T cell responses. Therefore, they are the most frequently used cellular adjuvant in clinical trials. Since the publication of the first DC vaccination trial in melanoma patients in 1995, the promise of DC immunotherapy is underlined by numerous clinical trials, frequently showing survival benefit in comparison to non-DC control groups (40, 77). The FDA approval of Sipileucel (Provenge), an autologous DC-enriched vaccine for hormone-resistant metastatic prostate cancer, in 2010 is considered as a milestone and proof-of-principle in this field (78).

DCs reside in an immature state in most organs and tissues, specifically at sites where pathogen entry is very likely. This immature state allows them to sample and process antigens efficiently. Upon exposure to the antigens in the presence of proper danger signals (e.g. inflammation, pathogens), DCs upregulate their expression of adhesion, MHC and costimulatory molecules (like CD86, CD80 and CD40) and show enhanced expression of CCR7. This latter chemokine receptor drives their migration to the draining lymph nodes where they secrete pro-inflammatory cytokines and present the captured exogenous antigens in either MHC I (a process known as cross-presentation) or MHC II context to $CD8^+$ CTL cells and $CD4^+$ Th cells, respectively. Both cell types are required for the priming and persistence of cellular antitumor immunity. Inappropriate stimulation of DCs (e.g. in the absence of proper danger signaling or costimulation) may render them tolerant, resulting in the elimination or suppression of T cells. In view of this, *ex vivo* generated and antigen loaded DC vaccines that are activated and primed in controlled conditions, are ideal candidates to stimulate potent tumor-specific immunity.

1.6 DC-based immunotherapy for HGG: clinical status and prognostic markers

DC vaccination constitutes the administration of autologous DCs enriched or cultured from a patient's blood and loaded with selective TAAs/TSAs, or whole tumor cell preparations. To date, over 20 DC clinical trials were performed in HGG patients, involving over 500 patients (77). Despite the notion that these early phase clinical trials differ in multiple vaccine parameters (e.g. site and frequency of injection, nature of the DCs, choice of antigen), DC vaccination in HGG patients is considered feasible as well as safe. More specifically, this therapy has never elicited autoimmune reactions nor demonstrated evidence of autoimmune encephalitis. This is in sharp contrast with the use of monoclonal antibodies or cytokine therapies. Ipilumumab has for instance been shown to induce immune-related adverse events in 60% of treated melanoma patients (73).

DC vaccines have been shown to elicit antigen-specific cellular and humoral immune responses in HGG patients and several reports describe an association between DC vaccine-induced immune responses and patient outcome (79, 80). Moreover, a recent meta-analysis demonstrated high IFN-γ in the peripheral blood of DC vaccinated HGG patients (77). This study also revealed that DC vaccination can significantly prolong the 1-, 1.5-, 2-, 3-, 4- and 5-year OS and PFS in newly diagnosed and recurrent HGG patients as compared to a non-DC control group. Moreover, the objective response rate (either complete or partial response according to the WHO or RECIST criteria) in DC vaccinated HGG patients (15.6%) is higher than that observed in melanoma, prostate cancer and renal cell carcinoma patients, three other tumor types that are most frequently targeted with DC-based immunotherapy (40). Together these data indicate the susceptibility of brain tumors, despite their somehow shielded location, to this efficacious treatment. However, in order to evaluate if DC vaccination can confer a meaningful survival advantage to HGG patients, the results of ongoing large phase III randomized controlled (multicenter) clinical trials will have to be awaited (NCT01280552 and NCT00045968).

Our increased understanding of intrinsic patient biomarkers (clinical, immunologic or molecular factors) that can associate with survival in DC vaccine settings may significantly improve the clinical results. Among clinical factors, younger patients and patients with lower tumor burden are more likely to respond to DC-based immunotherapy (81). In line with this notion, a recursive partitioning analysis model proposed by our group has identified age, pathology and performance status as predictors of outcome in DC vaccinated recurrent HGG patients (82). When it comes to molecular biomarkers, Prins *et al.* have recently shown that GBM patients that have a mesenchymal gene signature (but not patients with a proneural signature), show increased levels of $CD8^+$ and $CD3^+$ tumor-infiltrating lymphocytes, associated with improved survival following DC vaccination (83). Due to significant variability in methodology and the notion that a single immune parameter will most likely not

correlate with clinical outcome in all patients, clustering of several immune parameters may represent a promising strategy. In this regard, the immunoscore that was originally developed for colon cancer might be a promising tool to identify patients that will most likely benefit from DC vaccination. This scoring system derived from the immune contexture is a clinically useful prognostic marker based on the enumeration of $CD3^+$ and $CD8^+$ T cells in the tumor microenvironment (84). In the setting of glioma, the presence of pre-existing tumor antigen-specific immune responses has been shown to correlate with response in several DC vaccine studies (79, 81). In line with this notion, Fadul and colleagues have demonstrated that GBM patients with increased postvaccination frequencies of peripheral IFN-γ^+CD4^+ T cells and IFN-γ^+CD8^+ T cells respond better to DC therapy (32). In contrast, a decreased frequency of systemic Tregs following DC vaccination has been shown to associate with improved survival in GBM patients (85).

1.7 The impact of DC biology on the efficacy of DC vaccines

Despite the notion that promising objective responses and tumor-specific T cell responses have been observed in DC vaccinated HGG patients (clearly providing proof-of-principle for DC-based immunotherapy), the clinical success of this treatment is still considered suboptimal. This poor clinical efficacy can in part be attributed to the severe tumor-induced immune suppression and the selection of patients with advanced disease status and poor survival prognostics (40). In addition, there is a consensus in the field that step-by-step optimization and standardization of the production process of DC vaccines, in order to obtain Th1-driven antitumor immune responses, might enhance clinical efficacy (40). The next section addresses some recent DC vaccine adaptations that impact DC biology. Combining these novel insights might bring us closer to an ideal DC vaccine product that can trigger potent CTL- and Th1-driven antitumor immunity (Figure 2).

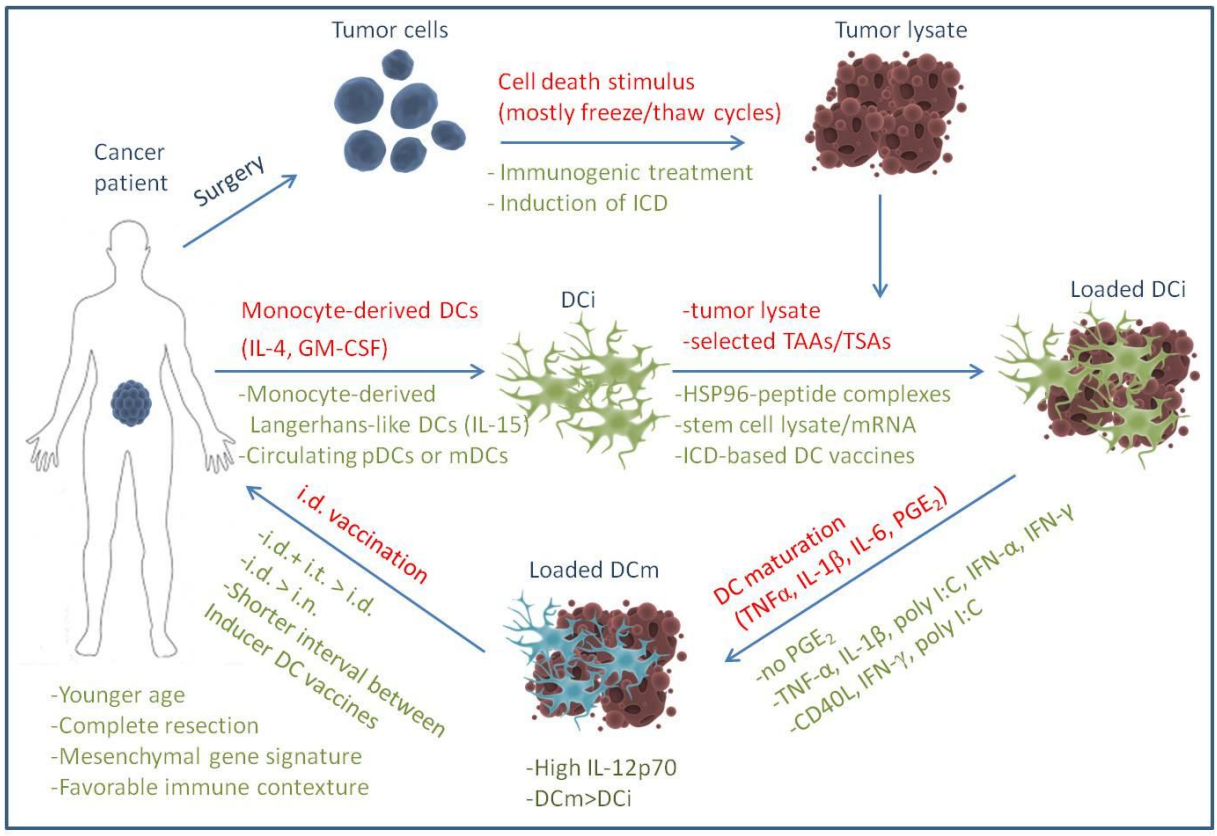

Figure 2. DC vaccine protocol adaptations to maximize therapeutic potential. *DC-based immunotherapy seeks to exploit the maximal intrinsic capacity of DCs to stimulate antigen-specific CTLs and NK cells. In recent years, several standardized steps in the production process of DC vaccines (as indicated in red) have been subjected to changes (as indicated in green) to obtain a more potent DC preparation. Besides DC vaccine parameters, intrinsic patient variables may also affect the therapeutic effectiveness of DC vaccines. DCi, immature DC; DCm, mature DC; ICD, immunogenic cell death; i.d., intradermal; i.n., intranodal; i.t., intratumoral; mDCs, myeloid DCs; pDC, plasmacytoid DC; TSAs, tumor-specific antigens: TAAs, tumor-associated antigens.*

Given the labor-intensive *ex vivo* culturing protocol of monocyte-derived DCs and inspired by the results of the Provenge study, several groups are currently exploiting the use of blood-isolated naturally circulating DCs (86-88). In this context, De Vries *et al.* evaluated the use of antigen-loaded purified plasmacytoid DCs for intranodal injection in melanoma patients (89). This strategy was feasible and induced only very mild side effects. In addition, the overall survival of vaccinated patients was greatly enhanced as compared to historical control patients. However, it still remains to be determined whether this strategy is more efficacious than monocyte-derived DC vaccine approaches (88). In contrast, experiments in the preclinical GL261 HGG model have recently demonstrated that vaccination with tumor antigen-loaded myeloid DCs resulted in a more robust Th1 response and a stronger survival benefit as compared to mice vaccinated with their plasmacytoid counterparts (90).

In view of their strong potential to stimulate CTL responses, several groups are currently exploring the use of Langerhans cell-like DCs as sources for DC vaccines (91-93). These so-called IL-15 DCs can be derived from $CD14^+$ monocytes by culturing them with IL-15 (instead of the standard IL-4). Recently it has been shown that in comparison to IL-4 DCs, these cells have an increased capacity to stimulate antitumor NK cell cytotoxicity in a contact- and IL-15-dependent manner (94). NK cells are increasingly being recognized as crucial contributors to antitumor immunity in DC vaccination setups (95, 96). Three clinical trials are currently evaluating these Langerhans cell-type DCs in melanoma patients (NCT00700167, NCT 01456104 and NCT01189383).

Targeting cancer stem cells is another promising development, particularly in the setting of glioma (47). Glioma stem cells can foster tumor growth, radio- and chemotherapy-resistance and local immunosuppression in the tumor microenvironment (47, 97). On the other hand, glioma stem cells may express higher levels of tumor-associated antigens and MHC complex molecules as compared to non-stem cells (98, 99). A preclinical study in a rodent orthotopic GBM model has shown that DC vaccines loaded with neurospheres enriched in cancer stem cells could induce more immunoreactivity and survival benefit as compared to DCs loaded with GL261 cells grown under standard conditions (100). However, as the *in vitro* production of neurospheres can be considered a rather artificial technique to enrich for glioma stem cells, studies that compare DC vaccination efficacy in two glioma models with varying grades of stemmness (e.g., the GL261 model versus the CT2A model) will be much more informative. Currently there are four clinical trials ongoing in HGG patients to evaluate this approach (NCT00890032, NCT00846456, NCT01171469 and NCT01567202).

Regarding the DC maturation status of the vaccine product, a phase I/II clinical trial in metastatic melanoma patients has confirmed the superiority of mature antigen-loaded DCs to elicit immunological responses as compared to their immature counterparts (101). This finding was further substantiated in patients diagnosed with prostate cancer and recurrent HGG (102, 103). Hence, DCs need to express potent costimulatory molecules and lymph node homing receptors in order to generate a strong T cell response. In view of this finding, the route of administration is another vaccine parameter that can influence the homing of the injected DCs to the lymph nodes. In the context of prostate cancer and renal cell carcinoma it has been shown that vaccination routes with access to the draining lymph nodes (intradermal/intranodal/intralymphatic/subcutaneous) resulted in better clinical response rates in comparison to intravenous injection (102). In melanoma patients, a direct comparison between intradermal vaccination and intranodal vaccination concluded that although more DCs reached the lymph nodes after intranodal vaccination, the melanoma-specific T cells induced by intradermal vaccination were more functional (104). Furthermore, the frequency of vaccination can also influence the vaccine's immunogenicity. Our group has shown in a

cohort-comparison trial involving relapsed HGG patients that shortening the interval between the four inducer DC vaccines improved the PFS curves (81, 105).

Another variable that has been systematically studied is the cytokine cocktail that is applied to mature the DCs. The current gold standard cocktail for DC maturation contains TNF-α, IL-1β, IL-6 and PGE_2 (106, 107). Although this cocktail can upregulate DC maturation markers and the lymph node homing receptor CCR7, it failed to evoke IL-12 production by DCs (106, 107). Nevertheless, IL-12 is a critical Th1 driving cytokine and DC-derived IL-12 has been shown to associate with improved survival in DC vaccinated HGG and melanoma patients (108, 109). Recently a novel cytokine cocktail including TNF-α, IL-1β, poly-I:C, IFN-α and IFN-γ was introduced (110, 111). The type 1-polarized DCs obtained with this cocktail produced high levels of IL-12 and could induce strong tumor-antigen specific CTL responses through enhanced induction of CXCL10 (108). In addition, CD40 ligand (CD40L) stimulation of DCs has been used to mature DCs in clinical trials (109, 112). Binding of CD40 on DCs to CD40L on $CD4^+$ Th cells licenses DCs and enables them to prime $CD8^+$ effector T cells.

A final major determinant of the vaccine immunogenicity is the choice of antigen to load the DCs. Two main approaches can be applied: loading with selected TAAs or TSAs and loading with whole tumor cell preparations (113). The former strategy enables easier immune monitoring, has a lower theoretical risk of inducing auto-immunity and can provide "off-the-shelf" availability of the antigenic cargo. Whole tumor cell-based DC vaccines on the other hand are not HLA-type dependent, have a reduced risk of inducing immune-escape variants and can elicit immunity against multiple tumor antigens. Meta-analytical data provided by Neller *et al.* have demonstrated enhanced clinical efficacy of cancer vaccines that employed whole tumor cells as antigens in comparison to the use of molecularly defined antigens (75). This finding was recently also substantiated in DC vaccinated HGG patients, although this study was not set-up to compare survival parameters (80).

With regard to whole tumor cell approaches, one factor requiring more attention is the immunogenicity of the dying or dead cancer cells used to load the DCs. The majority of clinical trials that apply autologous whole tumor lysate to load DC vaccines report the straightforward use of multiple freeze-thaw cycles to induce primary necrosis of cancer cells (102, 114). In addition, this technique can easily meet the rigid clinical requirements like the absolute avitalization of cancer cells. However, freeze-thaw induced necrosis is considered non-immunogenic and has even been shown to inhibit toll-like receptor (TLR)-induced maturation and function of DCs (115). To this end, many research groups have focused on tackling this roadblock by applying immunogenic treatment modalities to induce cell death in the context of DC vaccines (115-119). These treatments can potentiate antitumor immunity by inducing immune responses against tumor neo-antigens and/or by selectively increasing the exposure/release of particular damage-associated molecular patterns (DAMPs) which can

trigger the innate immune system (116, 118, 119). The emergence of the concept of immunogenic cell death (ICD) might even further increase the immunogenicity of DC vaccines. Cancer cells undergoing ICD have been shown to exhibit excellent immunostimulatory capacity owing to the spatiotemporally-defined emission of a series of critical DAMPs acting as potent danger signals (120, 121). These danger signals are able to activate innate immune cells like DCs. Thus far, three DAMPs have been attributed a pivotal role in the immunogenic potential of nearly all ICD inducers: the surface-exposed "eat me" signal calreticulin (ecto-CRT), the "find me" signal ATP and the TLR ligand high-mobility group box 1 (HMGB1) (121). Moreover, these ICD-experiencing cancer cells have been shown in various mouse models to act as very potent Th1-driving anticancer vaccines, even in the absence of any cellular or molecular adjuvants (121, 122). The ability to reject tumors in syngeneic mice after vaccination with cancer cells (of the same type) undergoing ICD is a crucial hallmark of ICD, in addition to the molecular DAMP signature (121). The concept of ICD, as well as its associated DAMPs and its promising application in DC-based immunotherapy are further elaborated in later sections of this work. In the following section, we will focus on non-ICD dependent enhancers of immunogenicity and their application in DC vaccines.

1.8 Immunogenic treatment modalities to establish a more productive DC-cancer cell interphase

Table 2 lists some frequently applied treatment methods to enhance the immunogenic potential of the tumor cell cargo for DC vaccines in an ICD-independent manner (i.e. these treatments do not meet the molecular and/or cellular requirements of ICD). The induction of particular DAMPs in the dying cancer cells positively impacts DC biology (Table 2). Table 3 summarizes the preclinical and clinical studies that investigated the *in vivo* potential of DC vaccines loaded with immunogenically killed tumor cells. Figure 3 schematically represents the application and the putative modes of action of these immunogenic enhancers in the setting of DC vaccines.

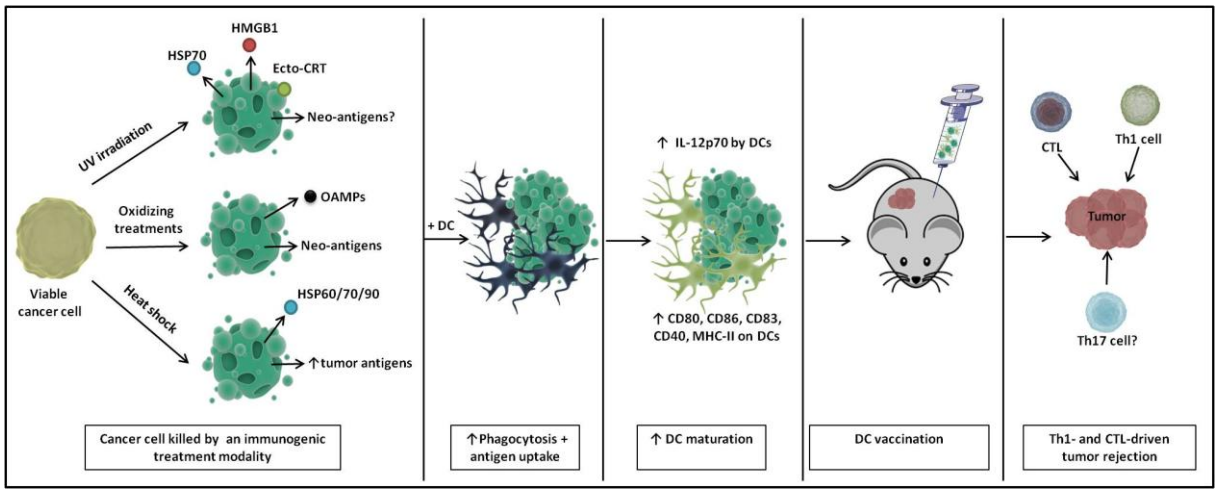

Figure 3. A schematic representation of immunogenic DC vaccines. *Cancer cells show enhanced immunogenicity upon treatment with UV irradiation, oxidizing treatments and heat shock characterized by the release of particular danger signals and the (increased) production of tumor (neo-)antigens. Upon loading onto DCs, they undergo enhanced phagocytosis and antigen uptake and show phenotypic and partial functional maturation. Upon in vivo immunization, these DC vaccines elicit Th1- and cytotoxic T lymphocyte (CTL)-driven tumor rejection.*

Table 2. A list of prominent enhancers of immunogenicity and ICD inducers applied in DC vaccine setups and their associations with DAMPs and DC biology

Treatment modality	Associated DAMPs	Effect on DC biology
Immunogenic treatment modalities		
UV irradiation	Pre-apoptotic ecto-CRT (123); post-apoptotic passive release of HSP70 and HMGB1 (124); mutation-induced neo-antigens (125)	Efficient engulfment; phenotypic maturation; increased IL-12 secretion; stimulate the polarization of T cells towards CTLs (119, 124, 126, 127)
Oxidation-inducing modalities (HOCl treatment)	OAMPs (reactive protein carbonyls, peroxidized phospholipids, oxidized low-density lipoprotein) (116, 128-130); carbonylated protein products presented as neo-antigens (130, 131)	Efficient antigen uptake and presentation; induction of IL-12; increased *in vivo* induction of tumor-reactive T cells (116)
Heat shock	Passive release of HSPs like HSP60/70/90 (118, 132); passive release of HMGB1 (133); increased expression of tumor-specific antigens (134)	Upregulation of DC maturation markers (CD40, CD80 and CD86) and induction of IL-12 (132); enhanced priming of CTL responses (118, 134)
Inducers of immunogenic cell death		
Radiotherapy	Pre-apoptotic exposure of ecto-CRT (123, 124, 135); early/mid-apoptotic exposure of ecto-HSP70 (136); post-apoptotic passive release of HMGB1 (133, 135); mutation-induced neo-antigens (125)	Efficient phagocytosis and enhanced phenotypic maturation (137); increased infiltration in the tumor environment (138, 139); enhanced stimulation of antigen-specific CTL responses (140)
Shikonin	Early/mid-apoptotic induction of ecto-HSP70, ecto-CRT and ecto-GRP78 (an inducer of pro-tumorigenic effects) (141)	Increased phenotypic ($CD40^{high}$, $CD80^{high}$, $CD86^{high}$) and functional maturation ($IL\text{-}12p70^{high}$, $TGF\text{-}\beta^{high}$, $IL\text{-}6^{high}$, $IL\text{-}23^{low}$) but only in combination with LPS; increased capacity to induce Th1 and Th17 differentiation (141)
High-hydrostatic pressure	Early/mid-apoptotic exposure of ecto-HSP70, ecto-HSP90, ecto-CRT; pre-apoptotic ATP release; post-apoptotic passive release of HMGB1, HSP70/90 and CRT (142)	Efficient phagocytosis; enhanced phenotypic and functional maturation; induction of antigen-specific T cells without inducing Tregs (142)
Oncolytic viruses	CVB3 and oncolytic adenovirus: (early-apoptotic) exposure of ecto-CRT; (early/mid-apoptotic) secretion of ATP and (post-apoptotic) release of HMGB1 (143, 144) NDV: early/mid-necroptotic exposure of ecto-CRT and post-necroptotic release of HMGB1 (145)	Enhanced expression of CD80/CD86 (144, 146, 147) and CCR7 (144); more efficient priming of tumor-specific $CD8^+$ CTL responses (143, 146, 147) and Th1 responses (143); increased accumulation in tumor microenvironment (143, 144)
Hypericin-based PDT	Pre-apoptotic ecto-CRT, ecto-HSP70 and secreted ATP; late apoptotic passive release of HSP70/90, CRT and HMGB1; accumulation of OAMPs like protein carbonyls (148-150)	Enhanced phagocytosis; phenotypic maturation ($CD80^{high}$ $CD86^{high}$ $CD83^{high}$ $MHC\text{-}II^{high}$) and immunogenic functional stimulation (NO^{high} $IL\text{-}10^{absent}$ $IL\text{-}6^{high}$ $IL\text{-}1\beta^{high}$ $IL\text{-}12p70^{medium}$); clonal expansion of human IFN-γ producing $CD4^+$ and $CD8^+$ T cells (150, 153, 154)
Photofrin-based PDT	Early/mid-apoptotic exposure of CRT, HSP60/70, ceramide and S1P; post-apoptotic release of HMGB1 (151, 152)	Increased phenotypic maturation ($CD86^{high}$, $MHC\text{-}II^{high}$) and enhanced IL-12 production (155); increased infiltration in tumor draining lymph nodes after peritumoral vaccination (156)

Abbreviations: CRT, Calreticulin; CTL, Cytotoxic T lymphocyte; CVB3, Coxsacievirus B3; DAMPs, Damage-associated molecular patterns; HMGB1, High mobility group box-1 protein; HSP, Heat shock protein; ICD, Immunogenic cell death; IFN, Interferon; LPS, lipopolysaccharide; NDV, Newcastle disease virus; NO, nitric oxide; OAMPs, oxidation-associated molecular patterns; PDT, photodynamic therapy; TGF, transforming growth factor; Treg, regulatory T cell

Table 3. A list of preclinical tumor models and clinical studies for evaluation of the in vivo potency of DC vaccines loaded with immunogenically killed tumor cells

Treatment modality	**Preclinical experience in DC vaccine settings**	**Clinical experience in DC vaccine settings**
Immunogenic treatment modalities		
UV irradiation	B16 melanoma in C57BL/6 – curative immunizations (119); ID8-ova ovarian carcinoma model in C57BL/6 mice – weekly curative immunizations (116)	Only in combination with γ-irradiation and heat shock in B-cell lymphoma patients (157)
Oxidation-inducing modalities (HOCl)	ID8-ova ovarian carcinoma model in C57BL/6 mice – weekly curative immunizations (116)	Freeze-thaw cycles in combination with high-dose irradiation are often reported in clinical trials involving high-grade glioma and melanoma patients (32, 81, 82, 114, 158-163) HOCl: Pilot study in 5 recurrent ovarian cancer patients demonstrated potent T cell responses against tumor antigens, decreased circulating Treg levels and serum IL-10 levels and two patients experienced durable PFS responses of ≥ 24 months (116)
Heat shock	PANCO2 pancreatic cancer model in C57BL/6 mice – curative vaccinations (118); in combination with 30 Gy irradiation in B16-ova model in C57BL/6 mice – prophylactic vaccinations (115)	Non-randomized trial in newly diagnosed glioblastoma patients (164): significantly improved tumor control rates and survival rates in DC vaccine group than in control group; increased proportions of peripheral $CD4^+$ and $CD8^+$ T cells post vaccination compared to control group; In combination with other cell killing modalities in B-cell lymphoma and melanoma patients (157, 165)
Inducers of immunogenic cell death		
Radiotherapy	B16 melanoma in C57BL/6 - prophylactic immunization model with critical involvement of $CD4^+$ and $CD8^+$ T cells (117, 137); E.G7 (SCCVII) in C57BL/6 - curative vaccination model (140)	Radiotherapy as a single intervention: multiple clinical trials in melanoma patients (114) and two clinical trials in high-grade glioma patients (166, 167). This study by Cho and colleagues reported a survival advantage of more than 15 months in the vaccinated glioblastoma patients in comparison to the control group (receiving conventional treatment); Radiotherapy as part of an ICD-inducing cell death protocol in B-cell lymphoma patients (157)
Shikonin	B16 melanoma in C57BL/6 - curative immunization model with strong induction of CTL responses (141)	Not available
High-hydrostatic pressure	Preclinical experiments are currently ongoing (168)	Multiple clinical trials are initiated involving prostate and ovarian cancer patients (168)
Oncolytic viruses	Not applied as ICD-based DC vaccines yet; curative combination of intratumoral oncolytic virus	Case report of breast cancer patient treated with combination of local hyperthermia, intravenously administered NDV and

	treatment and peripheral DC vaccination in B16 melanoma (C57BL/6) (169) and in subcutaneous CMT64 or KNL205 tumors (in C57BL/6 mice and DBA/2 DREG mice, respectively) (170)	intradermal DC vaccines loaded with NDV-oncolysate (171)
Hypericin-based PDT	Hypericin-PDT based DC vaccines in the ID8 ovarian carcinoma model (Coosemans and Baert *et al.*, unpublished results)	Not available
Photofrin-based PDT	*In vivo* photofrin-PDT treatment in combination with curative DC vaccination in C-26 colon carcinoma (BALB/c) (172); curative vaccinations with DCs charged with PDT-induced tumor lysate in EMT6, Renca and 4T1 non-orthotopic tumor models (BALB/c), induction of CTL and Th1 responses	Not available

Abbreviations: CRT, Calreticulin; CTL, cytotoxic T lymphocyte; ICD, Immunogenic cell death; NDV, Newcastle disease virus; PDT, photodynamic therapy; PFS, progression free survival

1.8.1 Ultraviolet irradiation

Ultraviolet (UV) light is considered an electromagnetic non-ionizing radiation with a wavelength between 100 and 400 nm. Its immunogenic potential was discovered in 1991 when Begovic *et al.* demonstrated that vaccination of immunocompetent mice (but not immunodeficient nude mice) with UV-irradiated cancer cells could induce resistance to subsequent rechallenge with live tumor cells (123, 173). This antitumor effect was crucially mediated by NK cells and $CD8^+$ T cells. UV-treated cancer cells are efficiently engulfed by DCs, leading to their phenotypic maturation and increased IL-12 production (119, 124, 126) (Table 2). Moreover, these matured DCs in turn stimulated the polarization of T cells towards IFN-γ producing $CD8^+$ T cells (124, 126). Of note, human DCs that had ingested UV-irradiated apoptotic tumor cells were shown to be more effective in generating $CD8^+$ CTLs than DCs pulsed with freeze-thaw lysates (127). In addition, immunization with DCs loaded with UV-treated tumor cells could elicit effective antitumor therapeutic efficacy in a B16 mouse melanoma model (119) (Table 3). The induction of specific DAMPs like ecto-CRT and the release of HSP70 and HMGB1 determines the immunogenicity of UV light (123, 124, 133) (Table 2). Moreover, as UV light is known to affect mainly DNA, mutation-induced tumor neo-antigens might also contribute to increased host antitumor immune responses (174). Vaccination with UV-induced tumor neo-antigens might be particularly useful in UV-induced tumors (e.g. cutaneous and uveal melanoma) that might share the *ex vivo* UV-induced tumor neo-antigens. Besides, it has previously been shown that immunization of tumor-bearing mice with mutated melanoma-derived self-antigens can elicit efficient cross-reactive $CD8^+$ T cell responses against multiple non-mutated epitopes of the tumor protein and against

the melanoma cells (175). This led to the rejection of established poorly immunogenic B16 melanoma tumors (175).

To the best of our knowledge, there are no reports of clinical trials that used UV irradiation as a single treatment for obtaining an antigen source to pulse DC vaccines (Table 3). This is probably related to the notion that UV light as a single treatment is not able to induce high levels of cancer cell death, an absolute requirement for clinical translation of DC vaccines.

1.8.2 Oxidation-inducing modalities

In recent years, an increased number of data were published concerning the ability of oxidative stress to induce oxidation-associated molecular patterns (OAMPs) like reactive protein carbonyls and peroxidized phospholipids which can act as DAMPs (128, 129) (Table 2). Protein carbonylation, an indicator of irreversible protein oxidation, has for instance been shown to improve cancer cell immunogenicity and to facilitate the formation of immunogenic neo-antigens (130, 131).

Through a series of elegant *ex vivo* and *in vivo* mouse experiments, Coukos *et al.* recently selected hypochlorous acid (HOCl)-based oxidation (to induce primary necrosis of tumor cells) as the method of choice (as compared to UV irradiation and freeze-thaw cycles) for preparing whole tumor lysate-loaded DC vaccines in the ID8 ovarian cancer model (116) (Table 3). In a small pilot study containing five recurrent ovarian cancer patients, these autologous DCs loaded with HOCl-oxidized autologous tumor lysate could produce high levels of IL-12, elicited strong antigen-specific T cell responses and reduced the levels of circulating Tregs and serum IL-10 (116). Moreover, two patients experienced durable progression free survival intervals of more than 24 months after vaccination (Table 3).

One prototypical enhancer of oxidation-based immunogenicity is radiotherapy (121, 123). In certain tumor types like HGG and melanoma, clinical trials that apply autologous whole tumor lysate to load DC vaccines report the random use of freeze-thaw cycles or a combination of freeze-thaw cycles and subsequent high-dose γ-ray or x-ray irradiation (32, 81, 82, 114, 158-163) (Table 3). Preclinical and clinical data comparing these two easily applicable treatment modalities are unfortunately lacking. In the light of the oxidation-based immunogenicity that is associated with radiotherapy, we however envisaged that x-ray irradiation might enhance the immunogenic potential of freeze-thaw necrotic tumor lysate. We investigated this nagging research question in the setting of HGG in chapter 3 of this work.

1.8.3 Heat shock treatment

Heat shock is a term that is applied when a cell is subjected to a temperature that is higher than that of the ideal body temperature of the organism from which the cell is derived. Heat

shock can induce apoptosis (41 to 43°C) or necrosis (> 43°C) depending on the temperature that is applied (176). The immunogenicity of heat shock treated cancer cells largely resides within their ability to produce HSPs like HSP60, HSP70 and HSP90 (118, 132) (Table 2). These HSPs can function as chaperones for tumor antigens, facilitating their cross-presentation (118). Moreover, after recognition by their receptors (CD91, TLR2/4), these HSPs can instigate the attraction of neutrophils and monocytes and the activation of NK cells and DCs (177). These events are crucial for the initiation of tumor-specific immune responses. Independent of the induction of HSPs, heat shock treatment has also been shown to upregulate the transcription of TAAs (134).

Co-incubation of heat-stressed apoptotic cancer cells with immature DCs resulted in the upregulation of DC maturation markers (CD40, CD80 and CD86) and higher IL-12 levels (132) (Table 2). Interestingly, splenocytes from mice immunized with heat-stressed apoptotic cancer cells got polarized towards a Th1 cytokine profile. Furthermore, DCs loaded with heat shock stressed melanoma cells can efficiently cross-prime tumor-antigen specific CTLs both *in vitro* and *in vivo* (134). Of note, direct comparison of heat shock treated tumor lysate with freeze-thaw tumor lysate in a DC vaccine setup demonstrated a stronger tumor regression in favor of heat shock lysate in a mouse model for pancreatic cancer (Table 3). Again, this was associated with a stronger priming of tumor-specific CTL responses (118).

DCs loaded with heat shocked cancer cells have already been successfully applied in clinical practice in high-grade glioma patients (Table 3). Jie *et al.* recently published an open labeled non-randomized clinical trial in which 12 newly diagnosed GBM patients received conventional therapy and 13 patients received additional DC vaccines loaded with heat shock treated autologous GBM cells (164). The vaccinated patients had a significantly improved OS and PFS. Interestingly, the proportions of peripheral $CD4^+$ T cells, $CD8^+$ T cells and NK cells were significantly elevated following DC vaccination in comparison to the control group. Moreover, increased levels of IFN-γ, IL-2 and IL-12 were measured in the sera of DC vaccinated patients.

All together, these data suggest that an immunogenic treatment of cancer cells can positively impact the potency of DCs interacting with them (Figure 2). In the light of this finding, the relatively new concept of ICD of cancer cells can be considered a promising strategy for loading DC-based anticancer vaccines, potentially giving rise to a next generation of potent Th1-driving DC vaccines (177-179) (Figure 3).

1.9 ICD: ignited by ROS-based ER stress and executed by DAMPs

ICD is a peculiar apoptotic demise that can trigger potent adaptive antitumor responses. It is accompanied by the emission of a series of immunostimulatory DAMPs in a specific spatiotemporal pattern (Figure 4) (121, 178). This type of cell death is considered stressor-

dependent, as only selected agents have been shown to induce it (178, 179). Since the initial screening study in 2007 (this study identified mitoxantrone, the anthracyclines doxorubicin and idarubicin and ionizing radiation as efficient ICD inducers), the list of ICD inducers has been growing considerably, further highlighting their emerging therapeutic potential (123, 179).

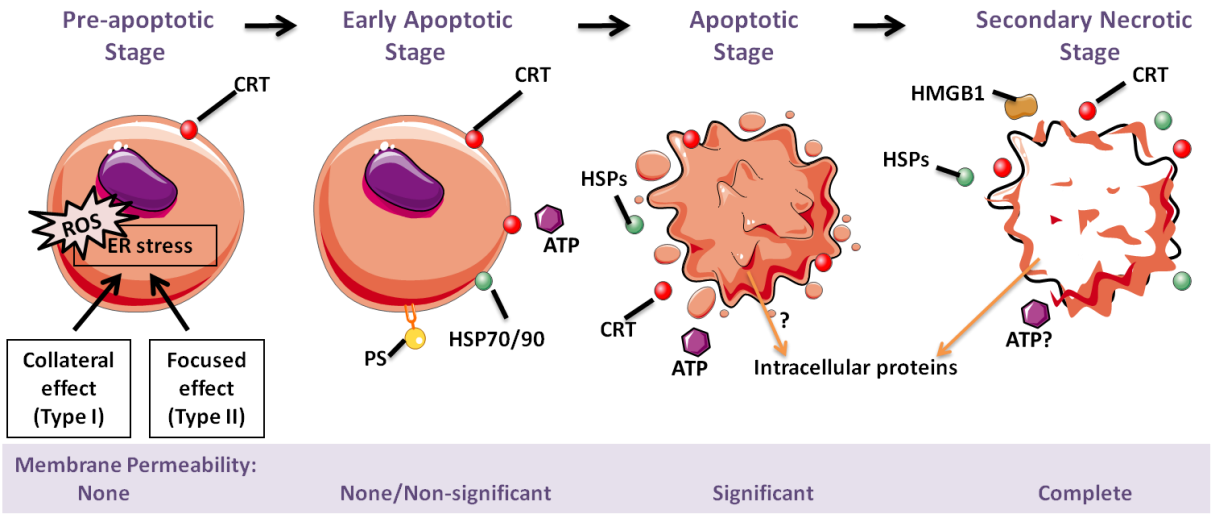

Figure 4: Overview of the generalized DAMP profile elicited by most ICD inducers. *While type I ICD inducers elicit ROS-based ER stress as an off-target effect, type II ICD-inducers instigate ER stress-mediated danger signaling and apoptosis through the selective induction of ER stress. Following ROS-based ER stress, CRT gets surface exposed in the pre-apopttic stage, followed by the externalization of phosphatidylserine (PS), the exposure of HSPs and the active secretion of ATP in the early apoptotic phase. Subsequently, the apoptotic process allows membrane permeabilization, entailing the passive release of HMGB1 and chaperones like CRT and HSPs (Modified adapted from (180)).*

1.9.1 ROS-based ER stress

ICD inducers all share a common feature, being the ability to induce endoplasmatic reticulum (ER) stress accompanied by the production of ROS (Figure 4) (177). More specifically, their effects (e.g. ecto-CRT exposure and the induction of apoptosis) are abrogated in the presence of anti-oxidants (181). Moreover, the observation that ROS inducers (like cisplatin) need to be combined with ER stressors (like tunicamycin) to induce ICD further emphasizes the importance of ROS-based ER stress in ICD (182). However, most ICD inducers affect the ER as an off-target effect (177). More precisely, they trigger cell death via non-ER associated targets (mostly the DNA replication/repair machinery as applicable to anthracyclines, oxaliplatin, radiotherapy and UVC irradiation), while danger signaling is triggered via the collateral induction of ER stress. These ICD inducers are categorized as type I ICD inducers. On the other hand, type II ICD inducers selectively target the ER to induce pro-death

signaling and ICD-associated danger signaling (160). To date, mainly five type II inducers have been characterized; Hypericin-based photodynamic therapy (Hyp-PDT), three oncolytic virus strains (Newcastle disease viruses, adenoviruses and coxsackievirus B3) and Pt^{II} N-heterocyclic carbene complexes (179). The collateral nature of the ER stress that is associated with type I ICD inducers ensures that the ICD-associated danger signaling here is milder and more complex (177).

The ROS-based ER stress is crucial for activating the danger signaling pathways that lead to the intracellular trafficking and the subsequent surface expression/emission of DAMPs (Figure 4). ER stress holds an imbalance between protein-folding load and capacity as a consequence of pathophysiological conditions (e.g. hypoxia, ROS production, ER-Ca^{2+}-depletion and hypoglycemia) that can disturb proper protein folding. The ER then attempts to re-establish homeostasis by activating the unfolded protein response (UPR). This complex signaling cascade can originate from 3 ER-sessile proteins i.e. PERK, IRE1α and ATF6. However, if the ER stress is too strong, this UPR response shifts to a pro-death pathway (instead of a pro-survival pathway), generally resulting in mitochondrial apoptosis.

1.9.2 DAMPs

In homeostatic conditions, DAMPs reside within the cell where they exhibit non-immunological functions. However, they tend to get exposed or secreted by damaged and dying cells in a spatiotemporally-defined manner (Figure 4), enabling them to bind pattern-recognition receptors like Toll-like receptors (TLRs). To date, three DAMPs have been attributed a pivotal role in ICD i.e. CRT, ATP and HMGB1 (121). Other DAMPs like surface-exposed or released HSPs (notably HSP70 and HSP90) have also been shown to contribute to the immunogenic capacity of ICD inducers (120, 121). The binding of these DAMPs to their respective immune receptors (CD91 for HSPs/CRT, P2RX7/P2RY2 for ATP and TLR2/4 for HMGB1/HSP70/HSP90) leads to the recruitment and/or activation of innate immune cells and facilitates the uptake of tumor antigens by antigen presenting cells and their cross-presentation to T cells eventually leading to IL-17 and IFN-γ dependent tumor eradiation (122).

1.9.2.1 Surface-exposed CRT (ecto-CRT): an established ‘eat-me’ signal

CRT is an ER protein that serves multiple functions here like chaperone activity, assistance in MHC assembly and regulation of Ca^{2+} homeostasis. The cell surface exposure of CRT during apoptosis binds to and activates LDL-receptor-related protein (LRP, also termed CD91) on professional phagocytes (183). This interaction allows the activation of Rac-1 in the phagocytes, enabling efficient phagocytosis. Ground-breaking research by Obeid *et al.* has identified a key role for ecto-CRT in the overall immunogenicity of dying cancer cells (123). Here, they showed that the exposure of CRT on cancer cells undergoing ICD in response to

several chemotherapeutics can facilitate their phagocytosis by DCs. Hence, ecto-CRT exposure is considered an 'eat me' signal for professional phagocytes. Later it was shown that anti-CRT antibodies could also impair the engulfment of Hyp-PDT treated cancer cells by murine and human DCs (150).

CRT gets exposed in the pre-apoptotic phase of ICD, preceding apoptosis-associated exposure of phosphatidylserine on the outer leaflet of the plasma membrane and mitochondrial depolarization. The molecular mechanisms underlying this ICD hallmark have meanwhile been dissected for certain chemotherapeutics and Hyp-PDT, revealing some interesting discrepancies (177). Chemotherapy-induced ICD is highly multifactorial, involving PERK-dependent posphorylation of eIF2α (the ER stress module), the activation of caspase-8 and the subsequent cleavage of BCAP31 leading to the activation of the pro-apoptotic Bcl-2 family members BAX and BAK proteins (the apoptotic module) and the proximal and PI3K-regulated distal secretory pathway enabling its docking and binding to CD91 (exocytosis module) (121). The CRT translocation pathway induced by the type II ICD inducer Hyp-PDT is much simpler as it is dispensable of caspase-8 and eIF2α (150). In addition, in the setting of Hyp-PDT, ecto-CRT did not colocalize with ERp57 in lipid rafts, as is demonstrated for chemotherapy-induced ICD (150).

1.9.2.2 ATP release: more than a 'find me' signal

Extracellularly released ATP can modulate different cellular functions such as death, survival, proliferation, adhesion and differentiation. ATP released by apoptotic cells is an established 'find me' signal that facilitates the recruitment and differentiation of monocytes and macrophages via its interaction with the purinergic P2Y2 receptor (184). Chemotherapy-induced ICD has been shown to induce early-apoptotic ATP secretion (during the phase of phosphatidyl exposure), which was vital for the generation of an effective antitumor immune response (185). In addition to its chemoattractant function, extracellular ATP stimulates P2RX7 receptors on DCs, this way activating the NLRP3 inflammasome-mediated maturation and consequent release of IL-1β (186). This cytokine stimulates the polarization of IFN-γ producing $CD8^+$ CTLs and IL-17 production by Th17 cells.

The mechanisms involved in ATP release depend on the apoptotic stage and the cell death stimulus involved. Pre-apoptotic secretion of ATP by Hyp-PDT treated cancer cells was autophagy independent and was mediated by the PERK-regulated proximal secretory pathway and PI3K-dependent exocytosis with no contribution of BAX or BAK (150). On the other hand, early apoptotic secretion as elicited by chemotherapy-induced ICD was positively regulated by autophagy-related genes (177).

1.9.2.3 Passively released HMGB1: a highly context-dependent DAMP

HMGB1 is a nuclear chromatin-binding protein that can support the transcription of various genes like p53 and NF-κB. It can be passively released, however, during primary necrosis and immunogenic apoptosis (during secondary necrosis). Once secreted it can stimulate the production of pro-inflammatory cytokines (e.g. TNFα, IL-1β, IL-6 and IL-8) from innate immune cells, by binding to various immune receptors like TLR2, TLR4 and RAGE (177).

Importantly, extracellular HMGB1 is crucial for the immunogenicity of chemotherapy- and radiotherapy-induced ICD (187, 188). More specifically, the tumor-rejecting effect of chemotherapy treated CT26 murine colon carcinoma cells undergoing ICD was abrogated in the presence of HMGB1-specific antibodies, hindering the binding of HMGB1 to TLR4 (187). In accordance with these data, the immunogenicity of tumor cells exposed to anthracyclines is lost in mice presenting with genetic defects in TLR4 or its downstream mediator MyD88 (myeloid differentiation primary response gene 88) (187, 188). The TLR4/MyD88 pathway instigated by HMGB1 inhibits the fusion between lysosomes and phagosomes, this way encouraging antigen processing (187, 188).

Nonetheless, HMGB1 has also been shown to have a protumorigenic role in several murine models and binding of HMGB1 to TIM3 on tumor-infiltrating DCs has been shown to abolish nucleic-acid mediated antitumor immunity (189). To make things even more complex, the activity of extracellular HMGB1 is highly dependent on the redox state in the cell such that full oxidation of HMGB1 results in its inactivation (177). Although the tumor microenvironment *in vivo* tends to be highly pro-oxidative, *in vitro* this can be highly variable depending on the cancer cell type and it progression stage.

1.10 ICD-based DC vaccines

Although the list of ICD inducers is constantly growing, only few of these immunogenic modalities have been tested in the light of generating an immunogenic tumor cell cargo to load DC vaccines (Tables 2 and 3). Figure 5 schematically represents the preparation of ICD-based DC vaccines and their putative modes of action.

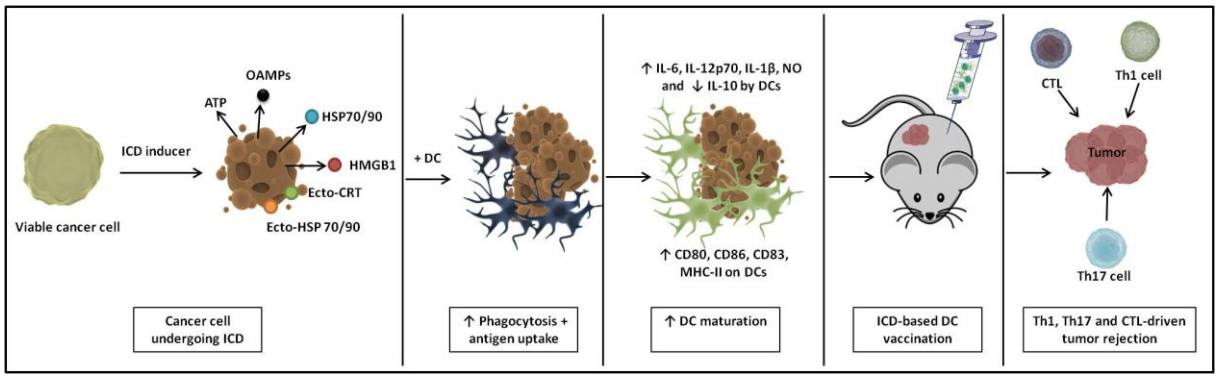

Figure 5. A schematic representation of ICD-based DC vaccines. *ICD causes cancer cells to emit a spatiotemporally defined pattern of danger signals. Upon loading of ICD-undergoing cancer cells onto DCs, they induce extensive phagocytosis and antigen uptake by DCs. Loaded DCs show enhanced phenotypic and functional maturation and immunization with these ICD-based DC vaccines instigates Th1-, Th17-, and cytotoxic T lymphocyte (CTL)-driven antitumor immunity in vivo.*

1.10.1 Radiotherapy

Ionizing x-ray or γ-ray irradiation exerts its anticancer effect predominantly via its capacity to induce DNA double-strand breaks leading to intrinsic cancer cell apoptosis (190). The idea that radiotherapy could also impact the immune system was derived from the observation that radiotherapy could induce T-cell mediated delay of tumor growth in a non-irradiated lesion (191). This abscopal (ab-scopus, away from the target) effect of radiotherapy was later explained by the ICD-inducing capacity of radiotherapy (192). Although this type I ICD inducer is known to induce ROS, its ER stress-inducing capability remains largely unexplored (177). The DAMPs that are induced following radiotherapy treatment of cancer cells include the exposure of ecto-CRT (123, 124, 135) and ecto-HSP70 (136) and the release of HMGB1 (133, 135) (Table 2). Irradiated B16 melanoma cells have been shown to be efficiently phagocytosed by DCs and to induce phenotypic DC maturation (117, 137). In addition, human DCs pulsed with irradiated tumor cells could efficiently stimulate antigen-specific CTL responses (140) (Table 2). Furthermore, mice immunized with DCs loaded with irradiated cancer cells could efficiently suppress tumor growth following inoculation with live syngeneic tumor cells in multiple preclinical cancer models (117, 140). In this setting, splenocytes from vaccinated animals could efficiently prime $CD4^+$ and $CD8^+$ T cells and exerted antigen-specific cytolytic activity (117) (Table 3).

DC vaccines exposed to irradiated cancer cells have been successfully implemented in clinical practice in melanoma and HGG patients (114, 166, 167) (Table 3). Cho and colleagues have shown that the implementation of DC vaccines loaded with irradiated autologous tumor cells in the conventional treatment regimen of newly diagnosed GBM patients could significantly prolong the median overall survival (by more than 15 months) as compared to a control group

receiving solely conventional treatment (167). Interestingly, Di Nicola *et al.* reported that vaccination with DCs loaded with dying autologous tumor cells after exposure to a cell death protocol consisting of heat shock, γ-ray and UVC ray could elicit clinical responses in 6 out of 18 relapsed B-cell lymphoma patients (193). Later they showed the impaired ability of the neoplastic cells used to vaccinate non-responders to undergo ICD upon exposure to the cell death protocol (157). Importantly, they revealed a positive association between the extent of CRT and HSP90 surface expression in the DC antigenic cargo and the clinical and immunological responses achieved (157).

1.10.2 Shikonin

The phytochemical shikonin, a major component of Chinese herbal medicine, is known to inhibit proteasome activity. It serves multiple biological roles and can be applied as an antibacterial, antiviral, anti-inflammatory and anticancer treatment. The latter application has been shown to yield responsiveness in late-stage lung cancer patients (194). Apoptotic cell death elicited by this type I ICD inducer can be inhibited by anti-oxidants, suggesting a role of shikonin-induced ROS (195). The ICD that is induced in shikonin-treated cancer cells is characterized by the early induction of HSP70, HSP90, GRP78 and HMGB1 (141) (Table 2). Importantly, shikonin treatment could significantly improve the survival of mice bearing P388 leukemia and this antitumor effect of shikonin was less pronounced in immunodeficient mice (195). Moreover, the tumor lysate from shikonin-treated B16 cells could enhance phenotypic and functional DC maturation and differentiation of Th1 and Th17 cells, two important features of ICD-associated antitumor immunity (141) (Table 2). Additionally, curative vaccination of B16 melanoma-inoculated mice with shikonin-lysate loaded DCs could delay tumor growth (141). This was associated with increased cytolytic activity of splenocytes on target tumor cells (Table 3). Although shikonin is administered to breast cancer patients for observational purposes (NCT01287468), clinical experience evaluating shikonin-lysate loaded DC vaccines is unfortunately still lacking (Table 3).

1.10.3 High-hydrostatic pressure

High-hydrostatic pressure (HHP) is an established method to sterilize pharmaceuticals, human transplants and food. HHP between 100 and 250 megapascal has been shown to induce apoptosis of murine and human (cancer) cells (196). While DNA damage does not seem to be induced by HHP < 1000 megapascal, HHP can disrupt membranes and denature proteins (197). Increased ROS production was detected in HHP-treated cancer cell lines and ER stress was evidenced by the rapid phosphorylation of eIF2α (142).

The anticancer activity of HHP was already demonstrated more than 4 decades ago in bladder cancer patients (168). Later, preclinical experiments demonstrated *in vivo* immunogenicity of HHP-treated cancer cells in the B16 melanoma model and the 3LL-D122 lung metastasis

model (168). Subsequently, it was shown that HHP-treated mammalian cancer cell lines undergoing apoptosis can release HSP70 and HMGB1, while retaining their immunogenicity *in vivo* (198). Very recently, Spisek and colleagues have shown the ability of HHP to induce prototypical ICD in human prostate and ovarian cancer cell lines and in acute leukemia cells (142). HHP treatment induced the rapid expression of ecto-HSP70, ecto-HSP90 and ecto-CRT and the release of HMGB1 and ATP (Table 2). Interestingly, HHP-treated cancer cells were rapidly phagocytosed by DCs and induced the upregulation of CD83, CD86 and HLA-DR and the release of pro-inflammatory cytokines (Table 2). This led to the stimulation of high numbers of tumor-specific T cells without inducing Tregs. Hence, all ICD-associated molecular criteria are fulfilled for HHP. This group is currently testing the *in vivo* immunogenicity of HHP killed tumor cells in prophylactic and curative murine vaccination settings (Table 3). Moreover they have initiated multiple clinical trials to evaluate the potential of DC vaccines loaded with HHP-treated cancer cells in ovarian and prostate cancer patients (168).

1.10.4 Oncolytic viruses

Oncolytic viruses are self-replicating, tumor selective virus strains that can directly lyse tumor cells. Over the past few years, a new oncolytic paradigm has risen; entailing that, rather than utilizing oncolytic viruses solely for direct tumor eradication, the cell death they induce should be accompanied by the elicitation of antitumor immune responses to maximize their therapeutic efficacy (199). One way in which these oncolytic viruses can fulfill this oncolytic paradigm is by inducing ICD (199).

Thus far, three oncolytic virus strains can meet the molecular requirements of ICD; Coxsackievirus B3 (CVB3), oncolytic adenovirus and Newcastle disease virus (NDV) (table 2) (179). Infection of tumor cells with these viruses causes the production of viral envelop proteins that induce ER stress by overloading the ER. Hence, all three virus strains can be considered type II ICD inducers (179). While CVB3 and oncolytic adenoviruses induce the surface expression of CRT, followed by the release of ATP and the passive release of HMGB1 in apoptotic tumor cells (in non-small cell lung carcinoma and adenocarcinoma cells, respectively) (143, 144), NDV induces necroptosis accompanied by the surface exposure of ATP and the post-necroptotic release of HMGB1 in GL261 glioma cells, with no contribution of ATP (Table 2)(145). In addition, NDV-infected GL261 cells upregulated the expression of the PMEL17 tumor antigen (145).

Intratumoral administration of CVB3 in nude mice resulted in the marked infiltration of NK cells, macrophages, granulocytes and mature DCs into the tumor tissue (Table 2) (144). Tumor-infiltrating DCs expressed significantly higher levels of costimulatory molecules CD80 and CD86, as well as the lymph node homing receptor CCR7 (144). CD40-ligand encoding oncolytic adenoviruses have also been shown to facilitate the recruitment of DCs to

the tumor tissue, this way entailing efficient Th1 and $CD8^+$ CTL responses (Table 2) (143). Measles virus is another oncolytic virus that requires further investigation. Although extensive analysis of *in vitro* ICD determinants is lacking for this virus (only the release of HMGB1 has been documented), DCs exposed *in vitro* to measles-virus treated melanoma cells upregulated CD80 and CD86 expression levels (Table 2) (146). This resulted in the efficient priming of melanoma-specific cell killing by IFN-γ producing $CD8^+$ T cells. Moreover, measles virus-infected melanoma cells constituted more effective tumor lysates (also termed oncolysates) than uninfected melanoma cell lysates as an antigen source for loading of DCs in terms of priming these melanoma-specific CTL responses (146). The DC stimulatory capacity of NDV-derived oncolysates has already been demonstrated more than a decade ago by the group of Schirrmacher (147). Here, DCs derived from breast cancer patients pulsed with NDV oncolysates showed increased expression of costimulatory molecules in comparison to DCs loaded with tumor lysate from non-infected breast carcinoma cells (Table 2). In addition, these oncolysate-loaded DCs were more effective in stimulating bone-marrow-derived reactive memory T cells *in vitro* (147).

Oncolytic viruses hold great potential for application in ICD-based DC vaccines given their potential to elicit several ICD-related DAMPs. Furthermore, these viruses might directly affect DC maturation and activation through interaction with pathogen recognition receptors on the DCs. This way, biological oncolysates may render the use of an artificial maturation cocktail otiose. Unfortunately, there are no preclinical *in vivo* data available yet to evince the efficacy of DC vaccines loaded with immunogenic oncolysates (Table 3). Nevertheless, several studies have documented the beneficial effect of intratumoral application of oncolytic viruses in combination with tumor-directed systemic DC vaccinations (169, 170). Very recently, Schirrmacher *et al.* disclosed a case report of a breast cancer patient with liver metastasis that was treated with local hyperthermia, intravenously administered NDV and subcutaneous vaccination with DCs loaded with NDV-infected breast cancer cells (oncolysate) (171) (Table 3). This combination therapy led to long-lasting tumor-specific memory T cell responses and stable disease for more than 66 months in this particular patient (171). The use of autologous DCs loaded with NDV-mediated oncolysate is licensed by the Paul Ehrlich Institute to the Immunologic-Oncologic Centre Cologne (IOZK) since May 2015.

Of note, in October 2015, the FDA approved the first oncolytic virus, Imlygic (a genetically modified live oncolytic herpes virus) for the treatment of melanoma lesions in the skin and lymph nodes. This FDA approval should facilitate the approval of other oncolytic viruses as well as the application of oncolysates in DC vaccine settings.

1.10.5 Photodynamic therapy

Photodynamic therapy (PDT) is an established, minimally invasive physico-chemical anticancer treatment modality. It has a two-step mode of action involving the uptake of a photosensitizer by the tumor tissue, followed by its activation by light of a specific wavelength. The absorption of photons with this specific wavelength will cause the photosensitizers to excite and a part of them will fall back to a more stable triplet state. These triplet states can undergo type I or II reactions, in the presence of oxygen, to return to their ground state, leading to the production of ROS (preferably singlet oxygen) (200-202). In type I reactions, the excited triplet state photosensitizer reacts with the neighbouring biomolecules (e.g. proteins or lipids) to form radical species that can react with molecular oxygen to form hydrogen, hydrogen radicals and superoxide (200). Type II reactions on the other hand, consist of the triplet state directly reacting with molecular oxygen thereby leading to the generation of $^1O_2$ (200).

One attractive feature of PDT is that the ROS-based oxidative stress (due to the short half-life and limited diffusion range of ROS species) originates in the particular subcellular location where the photosensitizer tends to accumulate, ultimately leading to the destruction of the tumor cell (203). PDT-based antitumor effects are multifactorial and depend on its abilities to damage the tumor vasculature, directly kill tumor cells, exert cytotoxic effects towards tumor-infiltrating immune cells and recruit and activate immune cells that can instigate adaptive antitumor immune responses (202).

Increasing preclinical information is available regarding the impact of PDT on the immune system. Recent studies have demonstrated that PDT can effectively generate several DAMPs. HSP70, the best studied DAMP associated with PDT, is exposed on the surface of cancer cells treated with photofrin-PDT, 5-ALA-PDT and Foscan-PDT (151, 204, 205). Of note, the uptake of tumor antigens and DC maturation induced by 5-ALA-PDT treated GBM spheroids were inhibited when HSP70 was blocked (204). Later it was reported that photofrin-PDT also promotes the early/mid-apoptotic surface expression of CRT and the post-apoptotic release of HMGB1 (152) (Table 2). Very recently, the DAMPs profile induced by Rose Bengal Acetate (RBAc)-based PDT was unraveled. RBAc photosensitized apoptotic/autophagic Hela cells were found to expose and/or release ATP, HSP70/90, HMGB1 and CRT (206). In terms of its immunogenicity, hypericin can be considered the best studied photosensitizer. Few years ago, Hyp-PDT became the first PDT modality capable of inducing prototypical ICD in cancer cells (Figure 6) (120, 149, 150, 177). Hypericin localizes predominantly in the ER and upon irradiation it causes photo-oxidative ER stress, making Hyp-PDT the only known PDT modality able to induce ICD through focused ROS-based ER stress (Type II ICD inducer), eventually culminating in mitochondrial apoptosis (150, 207). In the pre-apoptotic stage it induces the active emission of three crucial ICD-associated DAMPs i.e. ecto-CRT, ecto-

HSP70 and secreted ATP, followed by the passive release of HSP70 and HMGB1 (149, 150) (Table 2 and Figure 6).

The immunogenic features of Hyp-PDT-treated cancer cells have also been confirmed by *ex vivo* and *in vivo* experiments (Tables 2 and 3 and Figue 6). Hyp-PDT-treated cancer cells form a productive interface with DCs in terms of phagocytosis (CRT-dependent) and maturation (150) (Table 2). More specifically, the interacting DCs exhibit functional stimulation (NO^{high}, $IL\text{-}10^{absent}$, $IL\text{-}6^{high}$, $IL\text{-}1\beta^{high}$ and $IL\text{-}12p70^{median}$) and phenotypic maturation ($CD80^{high}$, $CD83^{high}$, $CD86^{high}$ and $MHC\text{-}II^{high}$) (150, 153). Moreover, these immunogenic and fully mature DCs induce the clonal expansion of human IFN-γ producing $CD4^+$ and $CD8^+$ T cells (153, 154). Consequently, this *in vitro* antitumor immunity induced by Hyp-PDT-induced ICD led to the efficient rejection of murine tumors *in vivo* in the absence of any adjuvants (both in prophylactic and curative vaccination models) – a key signature of antitumor immunity and ICD (150, 208). Following Hyp-PDT, photofrin-based PDT became the second PDT modality that is capable to fulfill this critical *in vivo* requirement for ICD characterization. Here, peritumoral curative immunization with benzoporphyrin-based-PDT treated squamous cell carcinoma cells constituted a potent anticancer vaccine (as opposed to x-ray irradiated or freeze-thawed cells) in this poorly immunogenic model (156).

Importantly, inoculation of mature DCs in PDT-treated tumors resulted in the cytolytic activation of T cells and NK cells, leading to effective tumor eradication (172). Moreover, DC vaccines loaded with porphyrin PDT-induced tumor lysates have been shown to cure fully established solid non-orthotopic tumors. This was associated with enhanced CTL responses and Th1 immunity (209) (Table 3). Hyp-PDT-based DC vaccines have not been studied yet in preclinical tumor models. To this end, and considering the very attractive ICD-related features of hypericin-PDT (as elaborated chapter 1.11), we investigated the potential of Hyp-PDT-based DC vaccines in preclinical orthotopic HGG models (chapter 4).

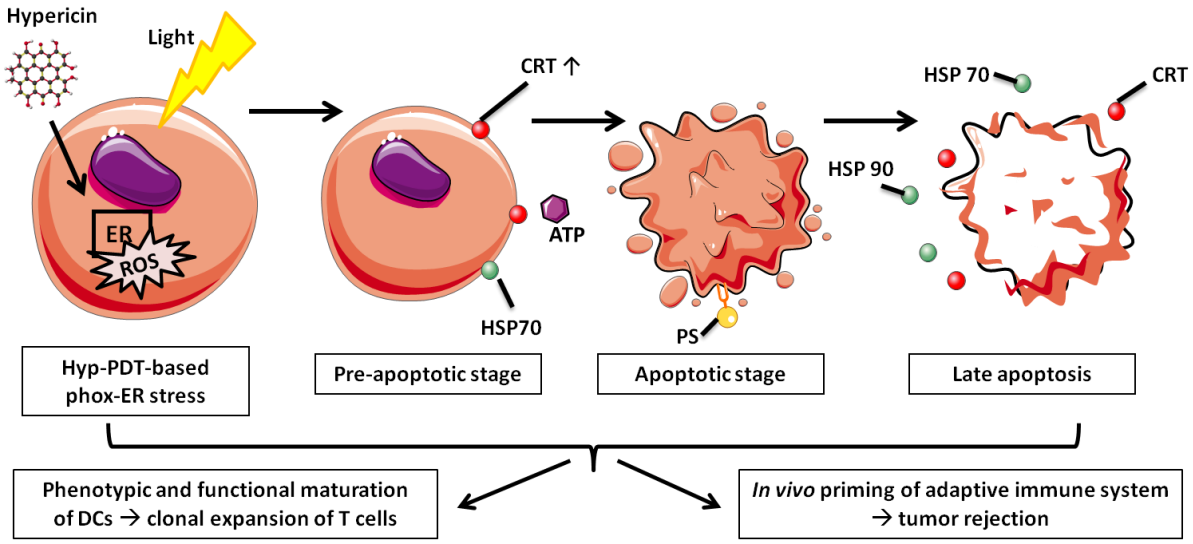

Figure 6: Photo-oxidative (Phox) ER stress induced by Hyp-PDT elicits prototypical ICD in cancer cells. *In contrast to other ICD inducers (like doxorubicin and mitoxantrone), Hyp-PDT induces the pre-apoptotic exposure of ecto-CRT (at higher levels as compared to chemotherapeutic ICD inducers) and ecto-HSP70, accompanied by the active secretion of ATP in the absence of plasma membrane permeabilization and prior to the externalization of phosphatidylserine (PS). This is followed by the passive release of HSP70, HSP90 and CRT in the late apoptotic phase. Overall, Hyp-PDT stressed dying/dead cancer cells cause phenotypic and function maturation of DCs and priming of the adaptive immune system in vivo upon prophylactic vaccination (Modified adapted from (180)).*

1.11 Hyp-PDT-based ICD in the spotlight

1.11.1 Hyp-PDT: an evolutionary conserved elicitor of host-defense mechanisms?

Hypericin is a naturally occurring photosensitizer that is exploited by plants of the genus Hypericum to achieve light-mediated killing of pest (203, 210). Hence, Hyp-PDT might constitute evolutionary conserved defense mechanisms against non-self molecules.

As discussed previously, hypericin preferentially localizes in the ER, and upon irradiation with an appropriate wavelength (around 595 nm) it causes predominantly singlet oxygen species (210). These species can interact with proteins and lipids, thereby eliciting protein carbonyl derivatives or lipid hydroperoxides that can pass on this photo-oxidative damage to other biological targets. In particular, Hyp-PDT instigates ER dilatation and damage to SERCA (a sarcoplasmic reticulum Ca^{2+} ATPase), causing the exit of Ca^{2+} from the ER into the cytosol (211). This triggers the rapid activation of the UPR response in which PERK plays a central role, both in activating the danger signaling pathways responsible for the exposure/release of DAMPs (as discussed previously) as well as in triggering apoptosis (150, 153, 203). In view of the latter, three pro-death signaling pathways were uncovered,

ultimately provoking BAX/BAK mediated mitochrondrial apoptosis accompanied by mitochondrial depolarization, release of cytochrome c and activation of caspase-3. In the first pathway, the PERK-eIF2-ATF branch robustly induces CHOP (a pro-apoptotic transcription factor) (212). In case of the second pathway, PERK induces the expression of Noxa (a pro-apoptotic BH3-only protein), independent of CHOP signaling (212). In the last pathway, PERK functions as a component of the mitochondria-associated membranes to physically connect the ER to the mitochondria, allowing the ROS originated in the ER to transfer to the contacting mitochondria (207).

1.11.2 Hyp-PDT as an ideal ICD inducer for application in DC vaccines?

Recently, a list of properties of an ideal ICD inducer was proposed (177). The following of these requirements should also be met in the setting of ICD-based DC vaccines; the capability to induce efficient cell death, the ability to induce strong antitumor immunity, being non-susceptible to drug-efflux channels, the capacity to induce severe focused ER stress, the ability to overcome loss of function mutations that can diminish danger signaling during cancer micro-evolution, the capacity to down regulate tumor-based transcription of pro-inflammatory genes, having negligible inhibitory effects on the infiltrating antitumorigenic immune populations, being able to exert inhibitory effects on tumor-infiltrating protumorigenic immune cell. In contrast to other ICD inducers, Hyp-PDT is able to measure up to all of these requirements, except the last one. Indeed, the influence of Hyp-PDT on the tumor influx of immunosuppressive immune cells like Tregs is not investigated yet. Moreover, the group of Agostinis has managed to put Hyp-PDT in the spotlight when it comes to advantages associated with ICD and ER stress biology. More specifically, the number (ATP, ecto-CRT and ecto-HSP70) and relative amount of pre-apoptotically induced DAMPs associated with Hyp-PDT is higher than that reported for chemotherapeutics (mitoxantrone or doxorubicin) (203). Also the speed of their emission is unmatched. Moreover, as elaborated in previous sections, the Hyp-PDT induced danger signaling pathways involve reduced numbers of molecular components as compared to chemotherapy-induced ICD (150, 203). This makes Hyp-PDT treated cancer cells less susceptible to tumor cell evasion owing to the ability of tumor cells to undergo loss of function mutations for several signaling molecules involved in ICD-associated danger signaling (as is demonstrated for caspase-8 and BAX, amongst others) (177, 203). These advantages of Hyp-PDT are predominantly related to its ability to induce on target photo-oxidative ER stress, able to trigger most potent danger signaling. Other advantages include its favorable safety profile (in contrast to the strong cardiotoxicity that is associated with mitoxantrone and doxorubicin) and its straightforward use as a single-agent ICD inducer. Additionally, unlike HHP that requires expensive and sophisticated HHP equipment, Hyp-PDT only needs hypericin and visible light in the orange region. Furthermore, Hyp-PDT is an FDA approved treatment and its *in vivo*

application in bladder carcinoma and HGG patients, amongst others, has been proven to be safe (178, 213).

2. AIMS AND OBJECTIVES

"The scientist is not a person who gives the right answers; he's one who asks the right questions."

- Claude Lévi-Strauss

Over the last decade our research group and others, have demonstrated that DC-based immunotherapy can be considered a promising treatment strategy in the fight against HGG. Nevertheless, the clinical benefit associated with this tumor-specific therapy is still considered suboptimal. This can in part be attributed to the strong HGG-induced immunosuppression and the selection of advanced disease patients. In addition, potentiating the immunogenicity of DC vaccines by making step-by-step protocol optimizations might also bring us closer to an effective CTL- and Th1-driving vaccine product. In this regard, the immunogenicity of the tumor lysate that is used for loading DC vaccines might constitute a crucial influencing factor.

The general aim of this project was to increase the immunogenicity of the whole tumor cell preparations that are used to load DC vaccines, in the setting of preclinical HGG. Given the residual toxicity that might be associated with chemical inducers of cancer cell death, we focused here on two promising physico-chemical treatment modalities: x-ray irradiation and Hyp-PDT. These two cell killing modalities share the capability to generate high levels of ROS that can induce immunogenic OAMPs. While the former induces ROS species throughout the complete cell, the latter generates ROS specifically in the ER, this way inducing prototypical ICD, associated with the exposure/release of high levels of DAMPs.

To study our hypothesis, we used the orthotopic murine GL261 HGG model, which is the most abundantly used immunocompetent mouse model to study the potency of immunotherapeutic strategies against intracranial HGG.

With this background, we wished to achieve the following general aims in the two projects:

I. To test the *in vivo* potency of the DC vaccine preparations in a prophylactic vaccination setup and to study the brain immune-contexture within this setup;
II. To identify/develop the most clinically relevant version of the vaccine that can still trigger potent protective immunity against HGG;
III. To identify the molecular and cellular determinants contributing to the vaccine's *in vivo* immunogenicity;
IV. To study the efficacy of the DC vaccine preparations in a curative, more clinically relevant setup and to study the brain immune-contexture associated with this setup;

3. RESULTS – PART I

"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less."

- Marie Curie

Irradiation of necrotic cancer cells, employed for pulsing dendritic cells (DCs), potentiates DC vaccine-induced antitumor immunity against high-grade glioma

OAMPs like carbonylated proteins and peroxidized phospholipids have emerged as important molecular determinants of cancer immunogenicity, in line with the family of DAMPs. More specifically, protein carbonylation has been shown to improve cancer cell immunogenicity by facilitating efficient Ag processing by DCs and by inducing the formation of tumor-specific carbonylated neo-antigens which are less susceptible to central tolerance. Most clinical reports involving DC vaccinated HGG patients report the *ad random* use of freeze-thaw cycles (to induce necrosis of tumor cells) or the combination of freeze-thaw cycles and high-dose x-ray irradiation to obtain a whole tumor lysate for loading onto the DCs. There is, however, no consensus yet on which of the two methodologies is preferred. As radiotherapy is known to generate ROS that can induce OAMPs, we hypothesized that x-ray irradiation might potentiate the immunogenicity of freeze-thawed tumor lysates. Given that radiotherapy on its own is a known inducer of ICD, we also investigated the immunogenicity associated with this single intervention in this DC vaccine setting. Furthermore, we studied the brain immune-contexture of vaccinated mice to identify the pivotal players of the innate and adaptive immune system that are contributing to the *in vivo* immunogenicity of the vaccines.

With this background in mind, we wished to accomplish the following aims in the murine GL261 HGG model:

I. To compare the *in vivo* efficacy of DC vaccines loaded with freeze-thawed GL261 lysate (FT-DC vaccine), irradiated freeze-thawed GL261 lysate (FT+IR-DC vaccine), freeze-thawed irradiated GL261 lysate (IR+FT-DC vaccine) and irradiated GL261 cells (IR-DC vaccine) in a prophylactic DC vaccination setup;
II. To compare the brain immune-contexture of mice prophylactically immunized with FT-DC vaccines and FT+IR-DC vaccines;
III. To unravel the molecular mechanisms behind the potentially increased immunogenicity (as studied in aim I) of the FT+IR-DC vaccines;
IV. To compare the brain immune-contexture induced by FT-DC vaccines and FT+IR-DC vaccines in a clinically more relevant curative treatment setup;

The research paper describing the results associated with this section and the corresponding supplementary information is presented on the following pages.

Irradiation of necrotic cancer cells, employed for pulsing dendritic cells (DCs), potentiates DC vaccine-induced antitumor immunity against high-grade glioma

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The authors don't have any conflict-of-interest or financial disclosure statements to declare.

Key words: High-grade glioma; dendritic cell vaccination; whole tumor lysate; protein carbonylation; antitumor immunity; oxidation-associated molecular patterns (OAMPs)

Abbreviations: CD, cluster of differentiation; CTL, cytotoxic T lymphocyte; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; FT, freeze-thaw; GBM, glioblastoma multiforme; GM-CSF, granulocyte/macrophage colony-stimulating factor; HGG, high-grade glioma; ICD, immunogenic cell death; IFN, interferon; IL, interleukin; i.p., intraperitoneal; IR, irradiation; L-Hist, L-Histidine; LPS, lipopolysaccharide; MDSC, myeloid-derived suppressor cell; mAb, monoclonal antibody; MHC, major histocompatibility complex; NAC, N-acetylcysteine; OAMP, oxidation-associated molecular pattern; ROS, reactive oxygen species; TAM, tumor-associated macrophage; TGF, transforming growth factor; Tregs, regulatory T cells; WHO, world health organization;

Abstract

Dendritic cell (DC)-based immunotherapy has yielded promising results against high-grade glioma (HGG). However, the efficacy of DC vaccines is abated by HGG-induced immunosuppression and lack of attention towards the immunogenicity of the tumor lysate/cells used for pulsing DCs. A literature analysis of DC vaccination clinical trials in HGG patients delineated the following two most predominantly applied methods for tumor lysate preparation: freeze-thaw (FT)-induced necrosis or FT-necrosis followed by X-ray irradiation. However, from the available clinical evidence, it is unclear which of both methodologies has superior immunogenic potential. Using an orthotopic HGG murine model (GL261-C57BL/6), we observed that prophylactic vaccination with DCs pulsed with irradiated FT-necrotic cells (compared to FT-necrotic cells only) prolonged overall survival by increasing tumor rejection in glioma-challenged mice. This was associated, both in prophylactic and curative vaccination setups, with an increase in brain-infiltrating Th1 cells and cytotoxic T lymphocytes, paralleled by a reduced accumulation of regulatory T cells, tumor-associated macrophages and myeloid-derived suppressor cells. Further analysis showed that irradiation treatment of FT-necrotic cells considerably increased the levels of carbonylated proteins - a surrogate-marker of oxidation-associated molecular patterns (OAMPs). Through further application of antioxidants and hydrogen peroxide, we found a striking correlation between the amount of lysate-associated protein carbonylation/OAMPs and DC vaccine-mediated tumor rejection capacity thereby suggesting for the first time a role for protein carbonylation/OAMPs in at least partially mediating antitumor immunity. Together, these data strongly

advocate the use of protein oxidation-inducing modalities like irradiation for increasing the immunogenicity of tumor lysate/cells used for pulsing DC vaccines.

Introduction

Gliomas are a group of central nervous system tumors of glial cell origin, accounting for at least 50% of all primary intrinsic brain tumors. Amongst gliomas, glioblastoma multiforme (GBM) is the most aggressive and most frequently occurring primary brain tumor in adults. According to the World Health Organization (WHO) it is classified as a grade IV tumor.[1] Together with WHO grade III anaplastic astrocytoma, anaplastic oligodendroglioma and anaplastic oligoastrocytoma, GBM are categorized as high-grade gliomas (HGG). Despite multidisciplinary treatment consisting of surgical resection and radiochemotherapy, median overall survival of GBM patients is restricted to approximately 14.6 months.[2] Hence, there is an urgent need for novel, effective treatment strategies with minimal off-target effects against normal brain parenchyma.[3] In this regard, dendritic cell (DC)-based immunotherapy can be an attractive option, because of its relative tumor-specific nature and its ability to elicit immune memory responses crucial for controlling or eliminating residual cancer cells.[4, 5] A recent meta-analysis revealed that DC-based immunotherapy in HGG patients markedly prolonged the overall and the progression-free survival compared with a non-DC group.[6] However, there is a consensus that DC vaccines have not yet reached their full potential. This is in part due to the selection of advanced disease stage patients and the presence of tumor-induced immunosuppressive mechanisms.[7, 8] In addition, the lack of optimization and standardization of the different steps in the production process of DC vaccines, especially those pertaining to the overall immunogenicity of the tumor cells/lysate itself, also contributes notably to the limited DC vaccine efficacy.[9]

HGG-induced immunosuppression is associated with a profound infiltration of anti-inflammatory and pro-tumorigenic immune cells like T regulatory cells (Tregs), myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs).[10-14] Therefore, an immunotherapeutic strategy that activates antitumor immunity and simultaneously tackles the infiltration of these immunosuppressive immune cell populations is highly desirable.[4, 15]

DCs pulsed with whole tumor lysate tend to have superior efficacy over DCs pulsed with selected cancer-associated antigen peptide(s).[16] Moreover, the tumor specificity of the antitumor immune response elicited by whole tumor cell-based DC vaccination has been demonstrated in the preclinical GL261 HGG model.[17] Interestingly, multiple preclinical studies in different cancer models have convincingly demonstrated that the methodology utilized for tumor lysate preparation can influence its immunogenicity and in turn the overall efficacy of antitumor immunity elicited by tumor lysate pulsed DCs.[18-21] There is, however, no consensus yet on the methodology of whole tumor lysate preparation; most clinical reports involving DC vaccinated HGG patients describe the use of multiple freeze-thaw (FT) cycles to induce necrosis of tumor cells (i.e. FT-necrosis) or a combination of FT-necrosis and subsequent high-dose X-ray irradiation. The latter preparation methodology offers interesting, but as-yet-untested avenues in light of the fact that radiotherapy is a known enhancer of oxidation-based immunogenicity.[15, 22] More specifically, radiotherapy is known to generate reactive oxygen species (ROS)[23] that have the ability to induce oxidation-associated molecular patterns (OAMPs) like carbonylated proteins.[24-29] This is important because protein carbonylation, a surrogate indicator of irreversible protein oxidation, has been shown to improve cancer cell immunogenicity and to facilitate the formation of neo-immunogenic antigens.[26] In addition, OAMPs like protein carbonylation can facilitate efficient antigen processing by DCs.[25]

The primary goal of this study was to directly compare the *in vivo* immunogenicity of DCs pulsed with either FT-necrotic cells or X-ray irradiated FT-necrotic cells, in the context of HGG. Moreover we explored the contribution of protein carbonylation-based OAMPs in this setting. To address these questions, we utilized the well-established, immunocompetent, orthotopic GL261 mouse HGG model. This model has been abundantly used to evaluate the potency of anti-HGG immunotherapies.[30]

Results

Clinical evidence generated from DC vaccination trials in HGG patients hints towards improved efficacy of irradiated FT-necrotic lysate

Since 2000, over thirty phase I/II studies of DC-based immunotherapy for HGG have been published in which over 500 patients were involved.[31] To this end, we decided to do a literature-based meta-analysis to ascertain the methodologies of tumor lysate preparation used and the associated patient responses. We found that 19 trials reported the use of whole tumor lysate as an antigen source for loading DCs (Table I). The method of preparing this lysate however randomly (i.e. without any specified reason or rationale) involved either FT-necrotic

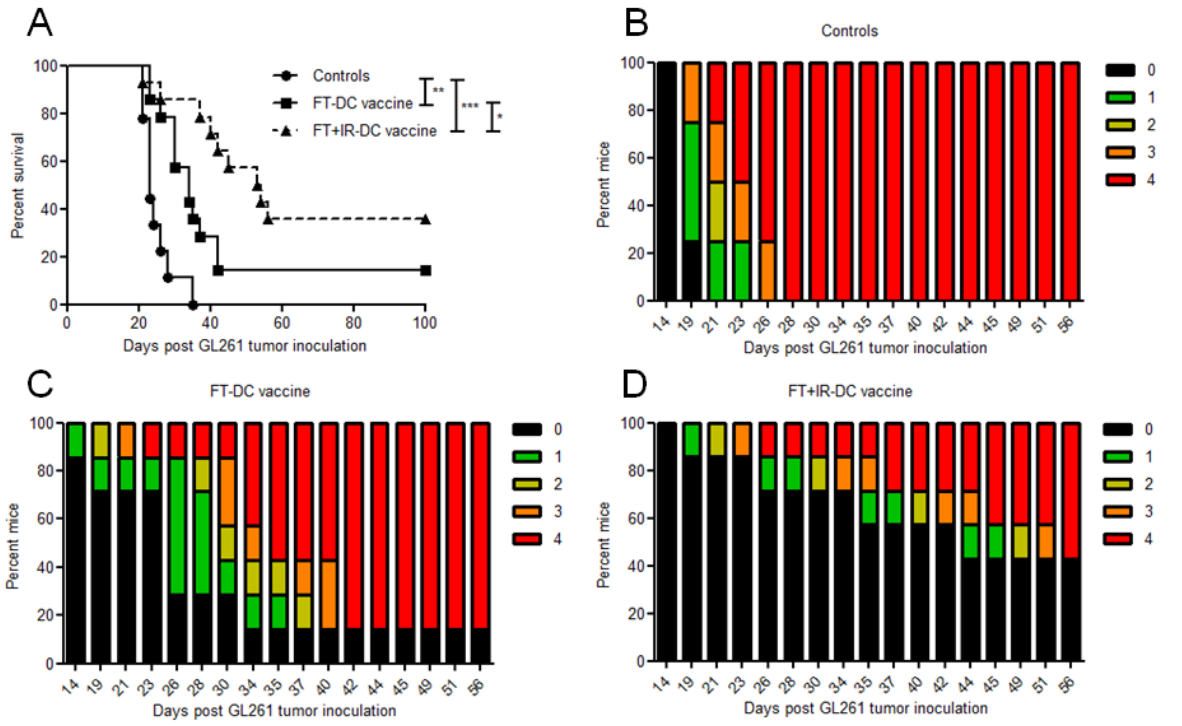

Figure 1. Irradiation of necrotic tumor lysate prolongs DC-vaccine induced survival of glioma-bearing mice. (A) Kaplan-Meier graph of two independent experiments depicting survival of mice immunized with the FT-DC vaccine (■, n = 14), the FT+IR-DC vaccine (▲, dashed line, n = 14) and untreated control mice (●, n = 9). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$ (Log-rank test). (B-D) The tumor-induced neurological deficit of one representative experiment is displayed graphically over time by color-coding symptom severity for (B) control mice (n = 4), (C) FT-DC vaccine treated mice (n = 7) and (D) FT+IR-DC vaccine immunized mice (n = 7): grade 0 (black), healthy mice; grade 1 (green), slight unilateral paralysis; grade 2 (yellow), moderate unilateral paralysis and/or beginning hunchback; grade 3 (orange), severe unilateral or bilateral paralysis and/or pronounced hunchback; grade 4 (red), moribund mice.

cells [16, 32-40] or irradiated FT-necrotic cells [41-49]. Retrospective analysis of primary GBM patients' survival data with a Karnofsky performance score (KPS) of more than 70 revealed a trend towards prolonged overall survival in patients vaccinated with DCs fed with irradiated (IR) FT-necrotic GBM cells (FT+IR-DC vaccine, n = 27, median survival of 33.5 months) as compared to patients treated with DCs fed with FT-necrotic GBM cells (FT-DC vaccine, n = 34, median survival of 22.5 months, data not shown). These results have to be interpreted with due caution, as a more stringent and better powered meta-analysis is required to correctly compare the two treatment groups. Insufficient data were available for comparison of immunogenicity-related parameters.

In conclusion, this literature survey showed that several clinical trials utilized FT-DC vaccine and FT+IR-DC vaccine *ad libitum* for anti-HGG immunotherapy. Preliminary survival analysis hints towards giving preference to the use of irradiated necrotic lysate for loading DCs; however the two treatment regimens were indiscernible at the level of immunoscoring parameters.

Irradiation of necrotic cells potentiates DC vaccine-induced overall survival in glioma-challenged mice

Since we were unable to reach a consensus on immunogenicity-related differences between the FT-DC vaccine and the FT+IR-DC vaccine based on above analysis, we decided to conduct preclinical experiments to directly compare the efficacy of these two DC vaccine 'types'.

Using a prophylactic treatment strategy we observed a significant increase ($p < 0.05$) in the median survival of mice vaccinated with the FT+IR-DC vaccine (53.5 days) as compared to mice treated with the FT-DC vaccine (34 days) (Figure 1A). Moreover, treatment with FT+IR-DC vaccine protected 5 of 14 animals (36 %) from tumor development, while only 2 of 14 (14 %) mice that received FT-DC vaccine were protected. Of note, in line with our previously published data, vaccination with FT-DC vaccine induced a significant improvement in median survival ($p < 0.01$) in comparison to untreated animals (34 *versus* 23 days, respectively).[50]

Consistent with the survival data, graphical representation of the tumor-induced neurological deficit scores over time revealed a delay in the onset of clinically-relevant symptoms and a less pronounced clinical manifestation in mice treated with the FT+IR-DC vaccine compared to FT-DC vaccine immunized animals (Figure 1B-D).

As irradiation is a known enhancer of tumor cell immunogenicity [51, 52], we also wished to address if IR on its own or IR treatment prior to FT of the GL261 cells could impact the *in vivo* immunogenicity of the tumor cell preparations. Both treatment groups (IR-DC vaccine and IR+FT-DC vaccine, respectively) however failed to induce significant survival benefit as compared to untreated mice or FT+IR-DC vaccine treated mice (Suppl. Figure 1). Interestingly, to achieve a 90 % cell killing rate the GL261 cells had to receive an 800 Gy X-ray dose, underlining the high radiotherapy resistance of this HGG cell line (data not shown). Of note, because of the requirement of 100 % tumor cell avitality for clinical translation of DC vaccines, the IR-DC vaccine cannot be considered as a clinically relevant alternative for the FT+IR-DC vaccine.

Increased potency of DCs pulsed with irradiated FT-necrotic cells is associated with a favorable shift in brain immune contexture

Next, we investigated whether the increased *in vivo* immunogenicity of the FT+IR-DC vaccine was associated with an immunostimulatory shift in the brain immune contexture. To this end, brain-infiltrating immune cells were analyzed by flow cytometry, 20 days after intracranial GL261 tumor inoculation (Figure 2A). Each graphical point (depicting one mouse) was given a color according to its neurological deficit score at the moment of sacrifice (Figure 2B-K). Evaluating the adaptive immune compartment, a significantly increased infiltration of $CD3^+$ T cells was observed in mice vaccinated with the FT+IR-DC vaccine as compared to both untreated and FT-DC vaccine treated animals ($p < 0.05$, Figure 2B). We observed no significant differences in the percentages of total $CD4^+$ brain-infiltrating T cells between the different groups (Figure 2C). Moreover, FT+IR-DC vaccine injected mice showed a reduced infiltration of $CD4^+FoxP3^+$ Tregs and an increase in the clinically relevant ratio of $CD8^+$ T cells to $CD4^+FoxP3^+$ Tregs ($p < 0.05$, Figure 2D and 2E, respectively). As IFN-γ production by T cells is considered crucial for the induction of Th1 polarization-based antitumor immunity,[53] we evaluated the intracellular levels of this cytokine in the brain-infiltrating lymphocytes. Only in FT+IR-DC vaccine treated mice the frequency of $IFN\text{-}\gamma^+CD4^+$ T cells i.e. Th1 cells and $IFN\text{-}\gamma^+CD8^+$ T cells i.e. cytotoxic T lymphocytes (CTLs) was significantly higher as compared to untreated control mice ($p < 0.05$ and $p < 0.01$ respectively, Figure 2F and 2G, respectively). In addition to the adaptive

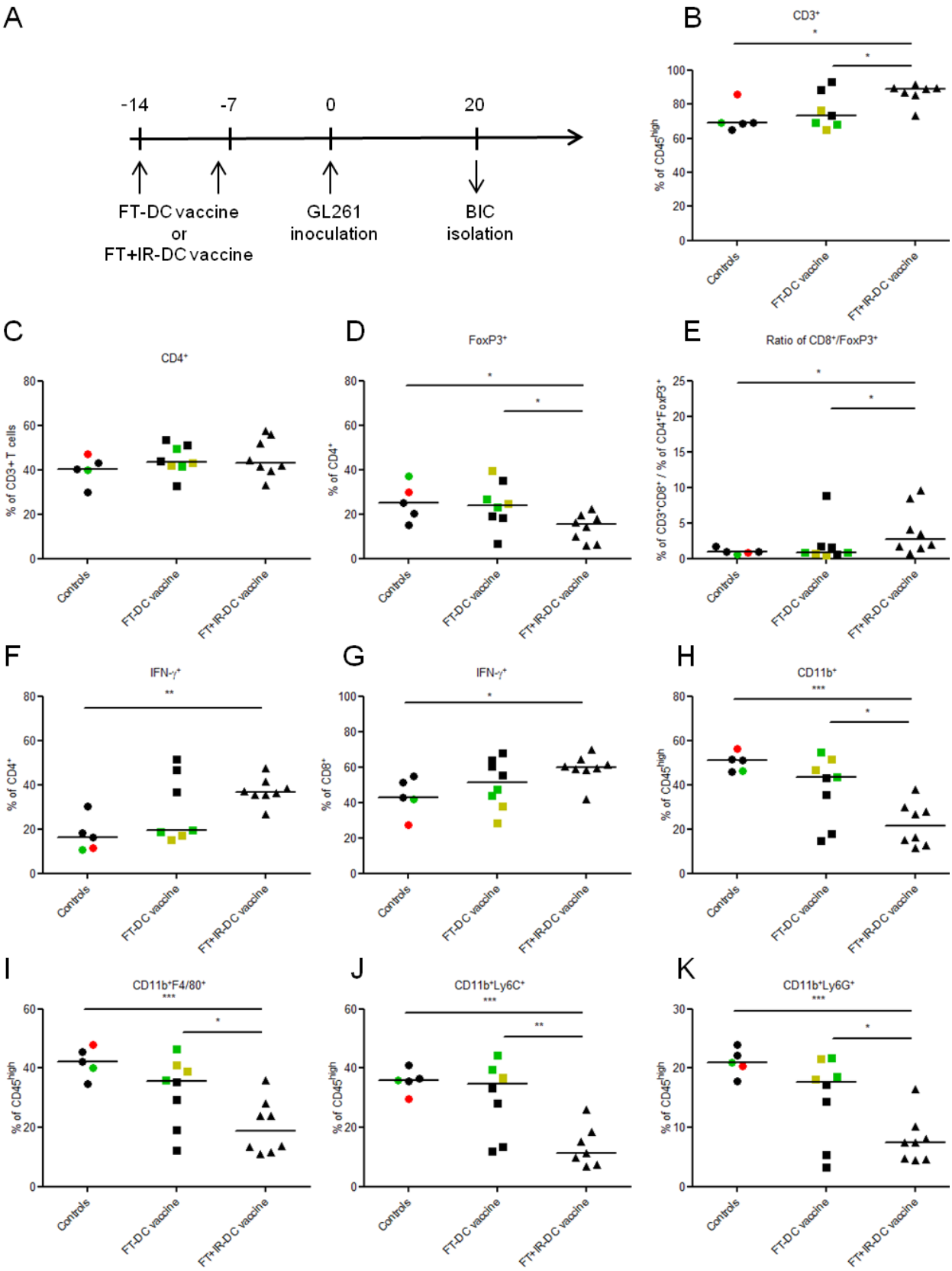
A
-14
-7
0
20
FT-DC vaccine
or
FT+IR-DC vaccine
GL261
inoculation
BIC
isolation
B
CD3+
% of CD45high
C
CD4+
% of CD3+ T cells
D
FoxP3+
% of CD4+
E
Ratio of CD8+/FoxP3+
% of CD3+CD8+ / % of CD4+FoxP3+
F
IFN-γ+
% of CD4+
G
IFN-γ+
% of CD8+
H
CD11b+
% of CD45high
I
CD11b+F4/80+
% of CD45high
J
CD11b+Ly6C+
% of CD45high
K
CD11b+Ly6G+
% of CD45high
Controls
FT-DC vaccine
FT+IR-DC vaccine

Figure 2. Prophylactic vaccination with FT+IR-DC vaccines favorably impacts the brain immune contexture. (A) Timeline depicting the prophylactic treatment protocol. Mice received two weekly i.p. vaccinations with either the FT-DC vaccine (n = 8) or the FT+IR-DC vaccine (n = 8). One week after the last vaccination, mice were intracranially inoculated with GL261 cells. Mice not immunized with vaccines were used as controls (n = 5). The brain-infiltrating immune cells (BICs) were isolated on day 20 post GL261 tumor inoculation. (B-K) Flow cytometry of the mononuclear BICs. Each point (representing data from one mouse) is given a color according to its neurological deficit score: grade 0 (black), healthy mice; grade 1 (green), slight unilateral paralysis; grade 2 (yellow), moderate unilateral paralysis and/or beginning hunchback; grade 3 (orange), severe unilateral or bilateral paralysis and/or pronounced hunchback; grade 4 (red), moribund mice. (B) Percentages of $CD3^+$ T cells ($CD45^{high}$-gated), (C) $CD4^+$ T cells ($CD3^+$-gated), (D) $FoxP3^+$ Tregs ($CD4^+$-gated) and (E) the ratio of $CD8^+$ T cells ($CD3^+$-gated) over $FoxP3^+$ Tregs ($CD4^+$-gated). (F-G) Percentages of IFN-γ^+ cells in the (F) $CD4^+$ and (G) $CD8^+$ T cell populations, analyzed by intracellular IFN-γ staining. (H) Percentages of $CD11b^+$ myeloid cells, (I) $CD11b^+F4/80^+$ macrophages, (J) $CD11b^+Ly6C^+$ mononuclear MDSCs and (K) $CD11b^+Ly6G^+$ granulocytic MDSCs within the $CD45^{high}$ gate, thereby excluding $CD45^{int}CD11b^+$ microglia. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (one-way ANOVA). Data are presented as medians.

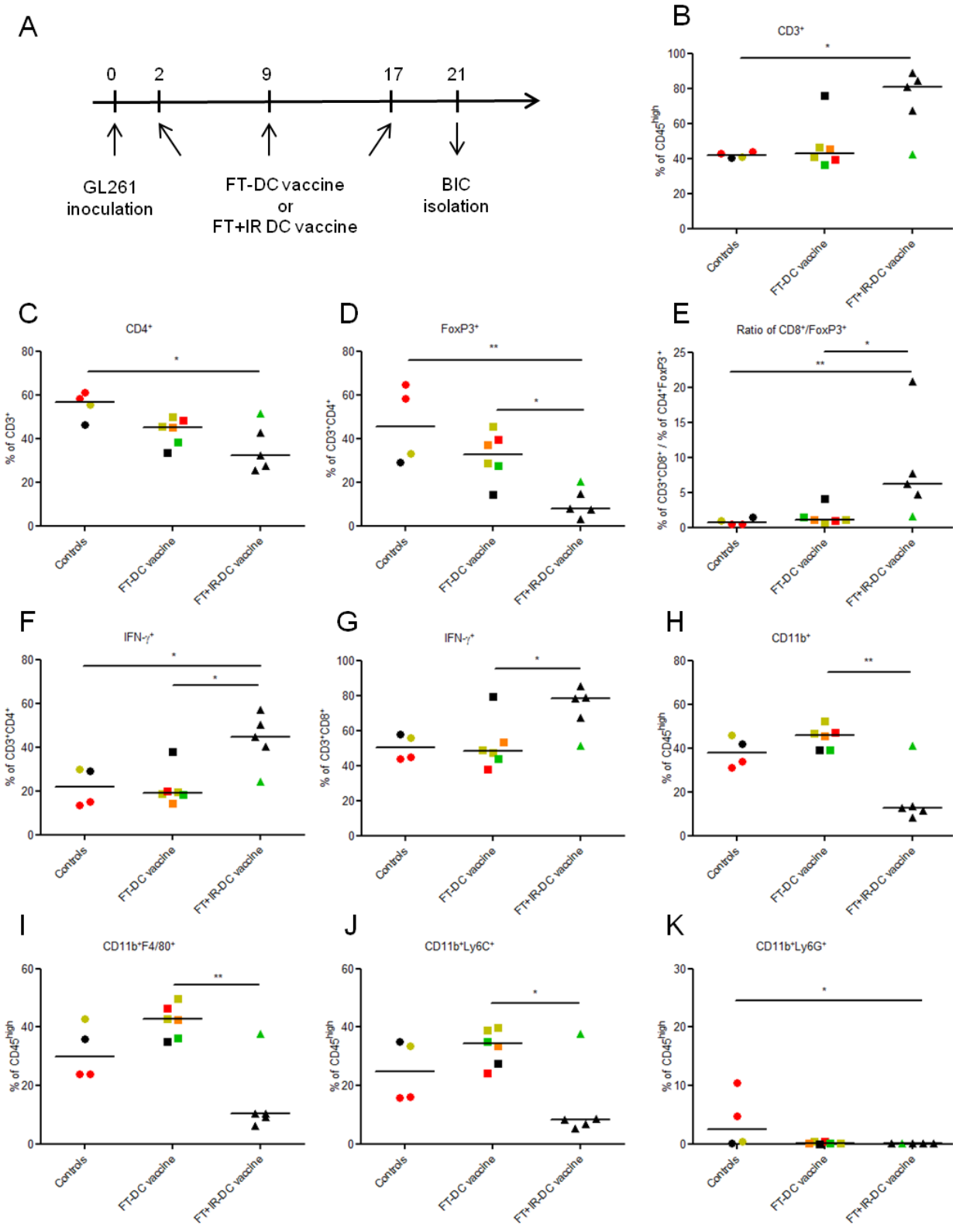
A
0
2
9
17
21
GL261 inoculation
FT-DC vaccine or FT+IR DC vaccine
BIC isolation
B
CD3+
% of CD45high
C
CD4+
% of CD3+
D
FoxP3+
% of CD3+CD4+
E
Ratio of CD8+/FoxP3+
% of CD3+CD8+ / % of CD4+FoxP3+
F
IFN-γ+
% of CD3+CD4+
G
IFN-γ+
% of CD3+CD8+
H
CD11b+
% of CD45high
I
CD11b+F4/80+
% of CD45high
J
CD11b+Ly6C+
% of CD45high
K
CD11b+Ly6G+
% of CD45high
Controls
FT-DC vaccine
FT+IR-DC vaccine

Figure 3. Curative vaccination with the FT+IR-DC vaccine induces a pro-inflammatory shift in brain immune contexture. (A) Timeline depicting the curative treatment protocol. Treated mice received three i.p. vaccinations with either FT-DC vaccines (n = 6) or FT+IR-DC vaccines (n = 5) on days 2, 9 and 17 post intracranial GL261 inoculation. Mice not immunized with vaccines were used as controls (n = 4). The brain-infiltrating immune cells (BICs) were isolated on day 21 post GL261 tumor inoculation. (B-K) Flow cytometry of the mononuclear BICs. Each point (representing data from one mouse) is given a color according to its neurological deficit score: grade 0 (black), healthy mice; grade 1 (green), slight unilateral paralysis; grade 2 (yellow), moderate unilateral paralysis and/or beginning hunchback; grade 3 (orange), severe unilateral or bilateral paralysis and/or pronounced hunchback; grade 4 (red), moribund mice. (B) Percentages of $CD3^+$ T cells ($CD45^{high}$-gated), (C) $CD4^+$ T cells ($CD3^+$-gated), (D) $FoxP3^+$ Tregs ($CD4^+$-gated) and (E) the ratio of $CD8^+$ T cells ($CD3^+$-gated) over $FoxP3^+$ Tregs ($CD4^+$-gated). (F-G) Percentages of IFN-γ^+ cells in the (F) $CD4^+$ and (G) $CD8^+$ T cell populations, analyzed by intracellular IFN-γ staining. (H) Percentages of $CD11b^+$ myeloid cells, (I) $CD11b^+F4/80^+$ macrophages, (J) $CD11b^+Ly6C^+$ mononuclear MDSCs and (K) $CD11b^+Ly6G^+$ granulocytic MDSCs within the $CD45^{high}$ gate, thereby excluding $CD45^{int}CD11b^+$ microglia. *, $p < 0.05$; **, $p < 0.01$ (one-way ANOVA). Data are presented as medians.

immune compartment, we also evaluated the brain infiltration of different myeloid cell populations in tumor-inoculated mice (Figures 2H-K). Analysis of the percentages of brain-infiltrating myeloid cells ($CD11b^+$) revealed a significantly reduced influx of these cells in mice treated with the FT+IR-DC vaccine compared to both untreated mice ($p < 0.05$) and FT-DC vaccine immunized mice ($p < 0.01$, Figure 2H). A more detailed phenotypic characterization of the brain-infiltrating myeloid cells showed a significant decrease in the percentage of TAMs ($CD11b^+F4/80^+$, Figure 2I), mononuclear MDSCs ($CD11b^+Ly6C^+$, Figure 2J) and granulocytic MDSCs ($CD11b^+Ly6G^+$, Figure 2K) in mice receiving the FT+IR-DC vaccine in comparison to both FT-DC vaccine injected ($p < 0.05$, $p < 0.01$ and $p < 0.05$, respectively) and untreated animals ($p < 0.001$ for all three populations). Although there was a decreased infiltration of these myeloid cell populations in FT-DC vaccine treated mice as compared to untreated mice, this did not reach statistical significance.

Of note, mice experiencing neurological deficits (grade 1-4) were only present in the untreated and FT-DC vaccine treated groups. These mice in general showed the lowest infiltration of Th1 cells and CTLs, the highest infiltration of the different myeloid populations (especially in the FT-DC vaccine group) and the highest Treg levels (Figure 2).

Together, these data suggest that prophylactic vaccination with FT+IR-DC vaccines leads to an increased accumulation of immunostimulatory, IFN-γ producing $CD4^+/CD8^+$ T cells while simultaneously impeding the infiltration of immunosuppressive Tregs, TAMs and MDSCs.

Curative treatment with DC vaccines fed with irradiated necrotic cells establishes a shift towards Th1 and CTL driven antitumor immunity

As prophylactic treatment does not really represent the clinical *status quo*, we next evaluated the *in vivo* immunogenicity of the two vaccine 'types' in a curative vaccination setup. Treated mice received three weekly DC vaccines and all mice were sacrificed four days after the last vaccination to analyze the brain-infiltrating immune cell populations (Figure 3A). This study revealed an increased infiltration of $CD3^+$ T lymphocytes and a reduced infiltration of $CD4^+$ T cells in FT+IR-DC vaccine injected mice as compared to control mice ($p < 0.05$ for both populations, Figure 2B and 2C). However, this reduced infiltration of general $CD4^+$ T cells might be explained by the strong significant reduction in Treg levels in these mice as compared to both untreated mice ($p < 0.01$) and FT-DC vaccine treated mice ($p < 0.05$, Figure 3D). Moreover vaccination with the FT+IR-DC vaccine resulted in a significant increase in the $CD8^+$ T cell to Treg ratio ($p < 0.05$ *versus* FT-DC vaccine and $p < 0.01$ *versus* controls, Figure 3E) and this was paralleled by an enhanced infiltration of Th1 cells and CTLs (Figure 3F and 3G, respectively; $p < 0.05$ in comparison to FT-DC vaccine treated mice for both populations). With respect to the myeloid cell populations, we observed a significantly reduced infiltration of total $CD11b^+$ myeloid cells (Figure 3H), $F4/80^+$ TAMs (Figure 3I) and $Ly6C^+$ MDSCs (Figure 3J) in FT+IR-DC vaccine injected mice as compared to FT-DC vaccine immunized mice ($p < 0.01$, $p < 0.01$ and $p < 0.05$, respectively). Although $Ly6G^+$ MDSCs were almost absent in the brains of both vaccinated groups, statistical analysis revealed a lower infiltration of these granulocytic MDSCs only in the FT+IR-DC vaccine treated mice in comparison to untreated animals (Figure 3K, $p < 0.05$). Interestingly, also in this setting, mice injected with FT-DC vaccine showed no significant differences as compared to untreated mice for any of the studied immune cell populations.

Of note, in accordance with the results obtained in the prophylactic setting, mice showing neurological deficits (predominantly present in the untreated and FT-DC vaccine group) in general showed the highest infiltration of Tregs and myeloid cell populations, while having a less evident infiltration of IFN-γ producing effector T cells (Figure 3).

In conclusion, these remarkable results indicate that the productive/pro-inflammatory immune interphase between the tumor and its immune contexture is maintained in the clinically relevant setting where vaccination with FT+IR-DC vaccine is applied in a pre-established tumor setting.

Pulsing of DCs with irradiated FT-necrotic cells does not impact DC maturation

Different tumor lysate preparations have been found to impact the phenotypic and functional maturation of DCs interacting with them.[18, 20] To this end, we examined whether the pulsing of DCs with FT+IR cells could directly modify the DC phenotype. As shown in figure 4A, we observed no differences in the expression levels of the costimulatory proteins CD80, CD86 and CD40 and the MHC class I and II molecules (H-2Kb and I-A/I-E, respectively) between DCs constituting the FT-DC vaccine and the FT+IR-DC vaccine.

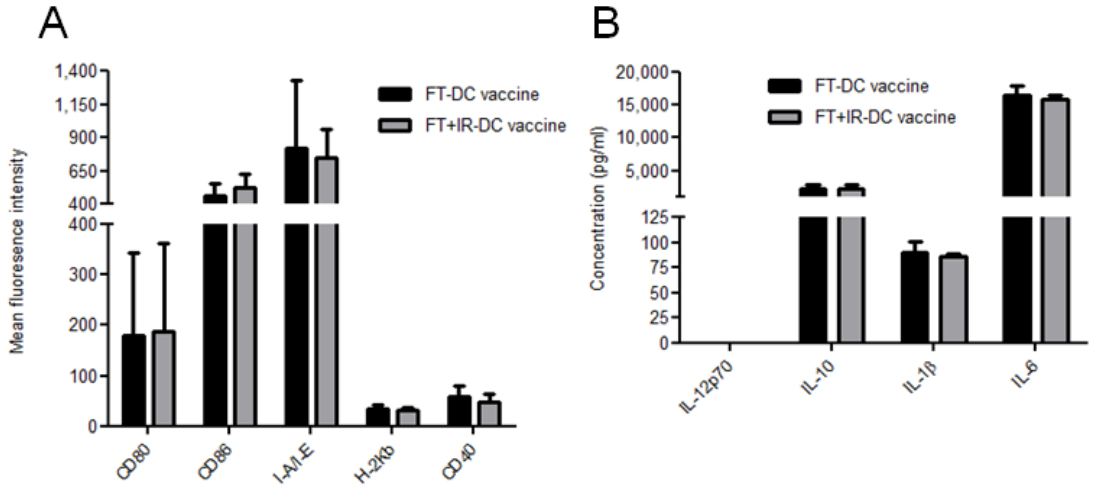

Figure 4. Loading of DCs with irradiated GL261 tumor lysate does not impact DC maturation. (A) Surface expression of CD80, CD86, I-A/I-E, H-2Kb and CD40 on the FT-DC vaccine and the FT+IR-DC vaccine was evaluated by means of flow cytometry. Data are presented as the mean fluorescent intensity ± SD of 6 independent experimental determinations. (B) The conditioned media of the FT-DC vaccines and the FT+IR DC vaccines were collected followed by analysis for concentrations of IL-12p70, IL-10, IL-1β and IL-6. Data are presented as mean ± SD of 2 independent experiments, each performed in triplicate.

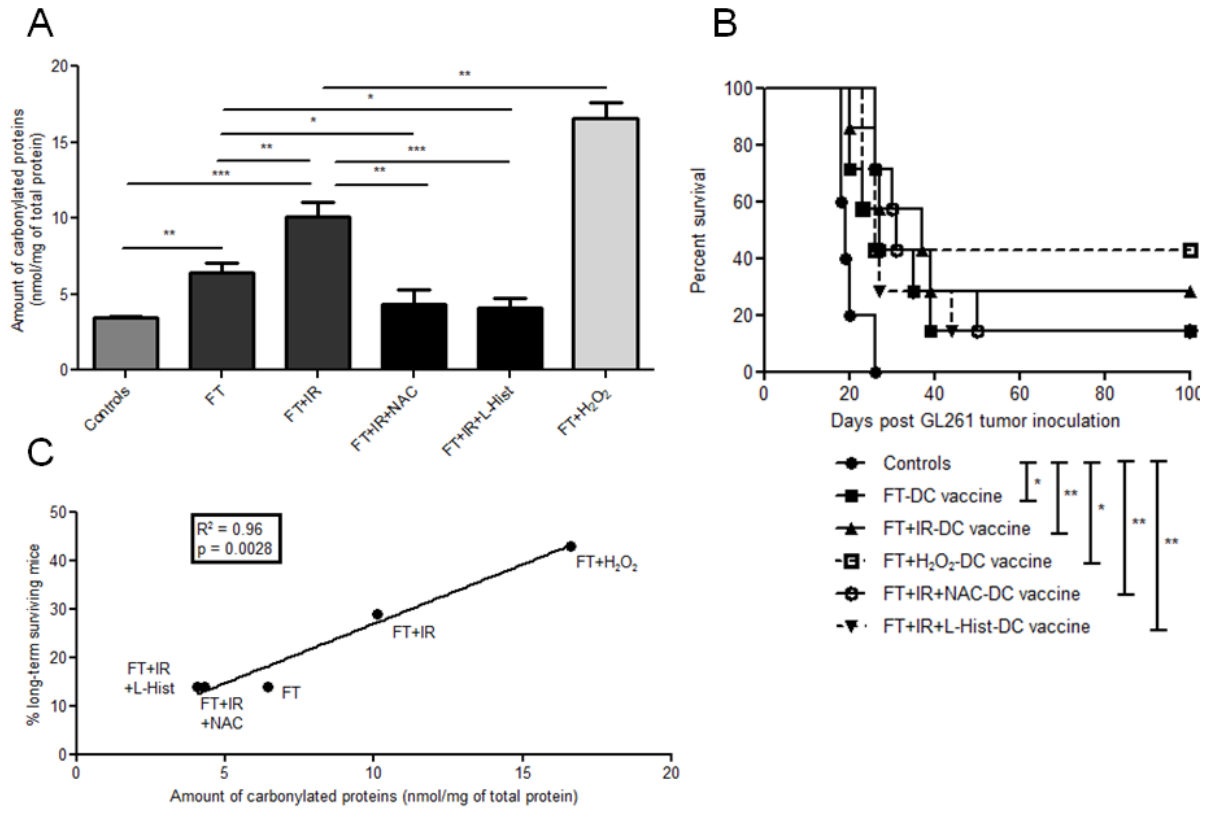

Figure 5. Oxidation-induced protein carbonylation in GL261 tumor lysates correlates with *in vivo* tumor rejection. The carbonyl content (calculated as nmols of carbonylated proteins per mg of total proteins) was evaluated in untreated GL261 cells (controls) and in the following lysate conditions: FT, FT+IR, FT+IR+NAC, FT+IR+L-Hist and FT+H_2O_2. Data are presented as mean values ± SD from one experiment performed in triplicate. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (Student's t-test). (B) Kaplan-Meier graph of an experiment in which GL261 inoculated mice were either left untreated (controls, ●, n = 5) or were immunized with FT-DC vaccines (■, n = 7), FT+IR-DC vaccines (▲ , n = 7), FT+IR+NAC-DC vaccines (○ , n = 7), FT+IR+L-Hist-DC vaccines (▼ , dashed line, n = 7) or FT+H_2O_2-DC vaccines (□, dashed line, n = 7). *, $p < 0.05$; **, $p < 0.01$ (Log-rank test). (C) Linear correlation between the amount of protein carbonylation in the different lysate preparations (as depicted in A) and the percentages of long-term surviving animals that were vaccinated with DCs pulsed with the respective lysate preparations ($p = 0.0028$, $R^2 = 0.96$, linear regression analysis).

In order to gain more insight into the functional status of the lysate-pulsed DCs, we evaluated the conditioned media of the FT-DC vaccine and the FT+IR-DC vaccine for the pattern of certain important cytokines like IL-10, IL-12p70, IL-6 and IL-1β (Figure 4B). The exposure of DCs to both lysate preparations did not induce production of IL-12p70. Moreover, we did not detect any differences in the expression levels of IL-10, IL-1β and IL-6 between the two vaccines. Conditioned media of the GL261 lysates (in the absence of DCs) did not contain detectible levels of any of the cytokines (data not shown).

Together, these data suggest that irradiation of FT-necrotic cells to pulse DCs does not influence the phenotypic and functional maturation of DCs.

Oxidation-induced protein carbonylation in tumor lysates *in vitro*, correlates with DC vaccine's ability to induce tumor rejection *in vivo*

Given the well described ROS-inducing features of irradiation,[22] and the increased immunogenicity observed with OAMPs accumulation,[54] we wondered whether ROS-induced OAMPs (like protein carbonylation) might contribute to the increased *in vivo* immunogenicity of FT+IR GL261 cells.

To this end, we first decided to measure whether IR treatment can increase accumulation of protein carbonylation, an indicator of irreversible protein oxidation.[55] Indeed, the level of protein carbonylation was significantly higher in FT+IR treated cells compared to FT treated cells ($p < 0.01$, Figure 5A). Moreover, as positive control, we employed the *bona fide* oxidizing agent hydrogen peroxide (H_2O_2)[56] and as expected, H_2O_2 induced even higher levels of protein carbonylation compared to FT+IR treated cells ($p < 0.01$, Figure 5A). Next, we wondered whether the increase in the levels of carbonylated proteins was caused by ROS production induced by exposing the FT-necrotic cells to IR. To prove this, we decided to employ well-established anti-oxidants like N-acetylcysteine (NAC)[57] and L-histidine (L-Hist)[55, 58] in combination with the FT+IR treatment. We observed that the presence of these ROS-scavengers in combination with IR treatment of FT-necrotic cells reduced the protein carbonyl content, back to the level of untreated GL261 cells (Figure 5A). This result proved that, IR of FT-necrotic cells is associated with increased ROS-based biomolecule modifications.

Next, we decided to employ our prophylactic DC vaccination model to address whether the increased OAMPs were mediating the *in vivo* immunogenicity of the FT+IR-DC vaccine (Figure 5B). Reduction of the protein oxidative burden in the tumor lysates used to pulse the DCs was able to reduce (albeit not significant) the median survival of FT+IR+L-Hist-DC vaccine treated mice (26 days) and FT+IR+NAC-DC vaccine treated mice (31 days) as compared to mice vaccinated with the FT+IR-DC vaccine only (37 days).

Interestingly though, the percentage of mice that became long-term survivors was the same in the anti-oxidant treated groups as in the FT-DC vaccine treated group (14 %), while 29 % of mice became long-term survivors in the FT+IR-DC vaccine injected group. Mice vaccinated with the FT+H_2O_2-DC vaccine showed the highest percentage of tumor-rejecting mice (43 %). Intriguingly, the amount of protein carbonylation in the different lysate preparations showed a highly significant linear correlation with the percentage of long-term surviving mice ($R^2 = 0.96$, $p = 0.0028$, Figure 5C). These data clearly indicate that the levels of OAMPs, like carbonylated proteins, in lysates used to pulse DCs, are highly associated with (and may partly mediate) long-term immunity in this DC vaccination model for murine HGG.

Discussion

In this study, we show the superiority of DC vaccines pulsed with X-ray irradiated FT-necrotic glioma cells as compared to DC vaccines pulsed with FT-necrotic glioma cells only, in terms of improving the survival of HGG-bearing mice. Irradiation of necrotic glioma cells did not impact DC maturation but did, however, increase the amount of protein carbonylation in the lysate preparation. Most intriguingly, the protein carbonyl content in the tumor lysate preparations used to pulse the DC vaccines correlated significantly with the percentage of long-term surviving HGG-inoculated animals.

Meta-analysis of clinical data has indicated that subtle differences in the production process of DC vaccines, like DC maturation status, DC dose and use and non-use of adjuvants can impact clinical parameters in human melanoma, prostate cancer and renal cell cancer patients.[59, 60] Moreover, a meta-analysis of 173 vaccination trials in a wide range of cancers concluded that patients vaccinated with whole tumor antigen vaccines had a higher rate of clinical responses compared to those vaccinated with defined tumor antigens.[61] Indeed, whole tumor vaccine strategies have the advantage of not relying on selected haplotypes such that immune responses are raised against a plethora of tumor antigens (including neo-antigens, if any), thereby avoiding the risk of immune escape variants.[62]

Our literature overview of clinical studies reporting the use of whole tumor lysate-pulsed DCs in HGG patients (both adults and children) revealed that there is no consensus yet in how FT-necrotic tumor lysates ought to be prepared (Table I). However, based on our study, it can be hypothesized that FT-necrotic cells/lysates, if utilized for DC vaccines, should be preferably combined with high oxidation-inducing modalities.

Moreover, to the best of our knowledge, this is the first preclinical study evaluating the tumor immune contexture in a prophylactic as well as in a curative DC vaccination setup. This study revealed a reduced brain infiltration of Tregs, TAMs and MDSCs in GL261-inoculated mice previously immunized with the FT+IR-DC vaccine in both settings. All three of the abovementioned immunosuppressive leukocyte populations have been known to accumulate in the circulation and/or in the tumor microenvironment of HGG patients.[63] Accumulation of $CD4^+FoxP3^+$ Tregs in the peripheral blood of GBM patients tends to correlate with impairment of T cell proliferation and the frequency of tumor-infiltrating Tregs has been shown to correlate with tumor grade in astrocytomas.[10, 64] Moreover, transient depletion of Tregs in the GL261 glioma model is capable of rescuing all tumor-challenged animals, however without the induction of immunological memory upon GL261 rechallenge.[17] Similarly, in human GBM, increased percentages of circulating MDSC ($CD15^+CD33^+HLA\text{-}DR^-$) have been reported.[65] Nevertheless, TAMs are the predominant innate immune cells infiltrating gliomas, far outweighing the T cells, and their (TAMs) number has been shown to correlate with the tumor grade.[66] Depletion of $CD11b^+$ cells in GL261-bearing mice has been shown to slow down glioma progression, further indicating that the majority of tumor-infiltrating $CD11b^+$ cells serve a tumor-promoting role in the context of HGG.[67] Whether vaccination of GL261-inoculated mice with FT+IR-DC vaccine can also influence the functional status of Tregs, TAMs and MDSCs remains an open question. Additional *ex vivo* functional studies are required to address this question.

In addition, we observed an enriched population of $CD3^+$ T cells and a stronger infiltration of Th1 cells and CTLs in mice receiving the FT+IR-DC vaccine. This finding suggests that this vaccination therapy can tackle the suppressive immune compartment, while simultaneously promoting the immune stimulating arm. This shift in immune balance towards a more antitumorigenic immune contexture is further demonstrated by the increased ratio of $CD8^+$ T cells to Tregs in our FT+IR-DC vaccination set-up. Sayour and colleagues recently disclosed a positive correlation between the CD8+ to Treg ratio in primary GBM patients and survival time. [68]

Radiotherapy is a known inducer of immunogenic cell death (ICD) due to its combined action of generating ROS and inducing collateral endoplasmatic reticulum stress.[51, 52] This ICD-inducing feature of radiotherapy however does not apply in the setting where FT-necrosis is applied before X-ray irradiation (thereby making them 'signaling incompetent' from ICD's perspective). On the other hand, the ROS-inducing features of radiotherapy are still applicable in this situation. Accumulating research reveals that OAMPs like carbonylated proteins can act as danger signals.[25-29] Irradiation of the necrotic GL261 cells upregulated the levels of protein carbonylation. Moreover, treatment of irradiated FT-cells with two different anti-oxidants completely abolished this effect. Of note, anti-oxidants based abrogation of irradiation-induced protein carbonylation (partially but not significantly) reduced overall median survival as well as the percentage of long-term surviving mice in our prophylactic vaccination model. The lack of significance between the different treatment conditions in this *in vivo* experiment (Figure 5B) might be due to the small survival benefit observed with the FT+IR-DC vaccine (as compared to the FT-DC vaccine), leaving a very stiff window for intervention. DC vaccines pulsed with tumor lysate treated with the chemical oxidizer H_2O_2 showed the highest level of glioma protection. These data are in line with a recent study of Chiang and colleagues in the ID8 ovarian cancer model.[18] They observed an increased *in vivo* efficacy of DCs pulsed with hypochlorous acid-oxidized tumor lysate as compared to DCs pulsed with FT-lysate. These findings suggest that the level of irreversible protein carbonylation in lysates - a convenient surrogate of the overall oxidative/OAMPs burden - used to pulse DC vaccines associates with (and possibly partially mediates) long-term antitumor immunity in this GL261 glioma model.

Interestingly, irradiation of the GL261 cells prior to freeze-thawing was shown to negatively affect the vaccine's *in vivo* immunogenicity. This could be explained by the fact that freeze-thawing is described to destroy carbonyl adducts.[69] This is in accordance with our own observation of lower protein carbonyl content in the IR+FT treated cells in comparison to FT+IR treated cells (data not shown).

Various oxidatively damaged components caused by the irradiation, including lipid (per-)oxidation products, which have not been investigated here, may also ultimately contribute to the heightened protein carbonylation status

of the lysates.[70] This might be paralleled by formation of high-molecular weight aggregates of unfolded proteins (i.e. oxidation-induced aggregation), which can further enhance immunogenicity.[71]

All together, these data warrant the use of X-ray irradiated FT necrotic lysates, over the use of FT necrotic lysates alone, as an antigen source to pulse DCs in clinical DC vaccination studies. Moreover, these data foster the preclinical investigation of strong oxidizing or OAMPs-inducing compounds to further increase the potency of whole tumor antigen-pulsed DC vaccinations.[72]

Materials and methods

Mice and tumor cell line

Adult, female (8 to 10 weeks old) C57BL/6J mice were purchased from Harlan (Horst, The Netherlands). The mice were housed in conventional pathogen-free conditions. All animal experiments were approved by the bioethics committee of the KU Leuven that follows international guidelines.

Methylcholanthrene-induced murine C57BL/6J syngeneic GL261 glioma cells were kindly provided by Dr. Eyüpoglu (University of Erlangen, Germany). The cells were maintained in DMEM supplemented with 10 % heat-inactivated FCS (Sigma-Aldrich, F7524), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (both from Lonza, BE17-605E and DE17-602E, respectively). Cell morphology was evaluated by microscopic examination multiple times per week.

Orthotopic glioma model and DC immunotherapy

The mice were intracranially injected with 5 x 10^5 GL261 tumor cells as previously described.[17] Briefly, mice were anesthetized and fixed in a stereotactic frame (Kopf Instruments, Tujunga, CA), which allows for accurate injection at 2 mm lateral and 2 mm posterior from the bregma and at 3 mm below the dura mater. Stereotactic inoculation was performed under sterile conditions.

DC immunotherapy was applied in the prophylactic setting by intraperitoneal (i.p.) injection of 1 x 10^6 lysate-pulsed mature DCs on day 14 and 7 prior to GL261 tumor inoculation. The mice were defined as long-term survivors when their survival exceeded three times the median survival of the untreated control animals. In the curative treatment set-up, mice received three i.p. administered DC vaccines on days 2, 9 and 17 after intracranial GL261 tumor inoculation.

Preparation of GL261 lysates

GL261 cells were harvested, washed and suspended in phosphate-buffered saline (DPBS, Lonza) supplemented with 1% FCS. Seven different GL261 lysate formulations were prepared; GL261 cells subjected to six consecutive freeze-thaw cycles (FT, 3 min in liquid nitrogen and 3 min on 56 °C, respectively), GL261 cells subjected to 6 freeze-thaw cycles and followed by 60 Gy X-ray irradiation (FT+IR), GL261 cells exposed to 60 Gy X-ray irradiation prior to 6 freeze-thaw cycles (IR+FT), GL261 cells subjected to 800 Gy X-ray irradiation only (IR), GL261 cells exposed to the anti-oxidants NAC (5 mM, Sigma-Aldrich, A9165) or L-Hist (250 mM, Sigma-Aldrich, 53319) before, during and after the freeze-thaw and irradiation treatment (FT+IR+NAC and FT+IR+L-Hist, respectively) and GL261 cells incubated with the strong oxidizer H_2O_2 for 24 hours (750 µM, Sigma, H1009) after the freeze-thaw cycles (FT+H_2O_2). Protein concentrations were determined via colorimetric assay (Bradford protein assay) according to the manufacturer's instructions (Bio-Rad).

Generation and characterization of DCs

DCs were derived from bone marrow progenitor cells as described.[17] In short, bone-marrow progenitor cells were cultured for 7 days in the presence of GM-CSF (20 ng/ml, Peprotech, 312-03). Medium was refreshed on day 3 and day 5. On day 7 immature DCs were harvested using a cell scraper. For lysate loading, immature DCs were incubated with GL261 lysates at 2 mg protein per 10 x 10^6 DCs per ml culture medium at 37 °C for 90 minutes. Immediately after loading, the DCs were transferred to culture flasks for 24 hours in DC medium containing 20 ng/ml GM-CSF and 1 µg/ml *Escherichia coli*-derived lipopolysaccharide (LPS, Sigma-Aldrich, L6529-1MG) to induce maturation. Twenty-four hours later, the mature lysate-loaded DCs were harvested, counted and resuspended to 1 x 10^6 DCs in 100 µl PBS for i.p. injection.

Surface expression of DC maturations markers was assessed on lysate-pulsed mature DCs by flow cytometry using the following monoclonal antibodies (mAbs): FITC-conjugated anti-H-2Kb (Becton Dickinson (BD), 553569), PE-conjugated anti-I-A/I-E and anti-CD40 (BD, 557000 and 553791, respectively) and PE-conjugated anti-CD80 and anti-CD86 (eBioscience, 12-0862-83 and 12-0114-83, respectively). For each staining the appropriate isotypes were used. Analysis was performed using the

Cellquest software on a FACSort cytometer (BD, Erembodegem, Belgium). The conditioned media derived from the lysate-loaded mature DCs were collected and checked for cytokine levels of IL-10 (88-7104-88), IL-12p70 (88-7121-88), IL-1β (88-7013-88) and IL-6 (88-7064-88) by means of ELISA kits from eBioscience, according to the manufacturer's instructions. Cytokine levels in GL261 lysates were also evaluated to rule out GL261 cell-derived cytokine production.

Isolation and characterization of brain-infiltrating immune cells

Brain-infiltrating immune cells were isolated from GL261-inoculated mice as previously described. [17] Two different staining panels were used respectively for the prophylactic and curative setup. The staining protocol was however exactly the same for the two experiments. In the prophylactic setting surface staining was performed with anti-CD11b PE (eBioscience, 12-0112-83), anti-CD4 PE and anti-CD8 PE (BD, 553049 and 553033), anti-F4/80 FITC (AbD Serotec, MCA497DB), anti-Ly6G FITC (eBioscience, 11-5931-83), anti-CD3 FITC, anti-Ly6C FITC (BD, 553062 and 553104, respectively) and anti-CD45 PerCP-Cy5.5 mAbs (eBioscience, 45-0451-82). Intracellular FoxP3 was detected using a FoxP3-Alexa Fluor 488 staining kit (eBioscience, 73-5776) according to the manufacturer's protocols. For intracellular IFN-γ staining, cells were stimulated for 4 hours *in vitro* with 100 ng/ml phorbol myristate acetate, 1 µg/ml ionomycin and 0.7 µg/ml monensin (all from Sigma-Aldrich, P8139-1MG, I0634-1MG and M5273-500MG, respectively). After restimulation, surface staining for CD4, CD8 and CD45 was performed and cells were washed with a permeabilisation buffer containing 0.5 % saponin and 0.5 % bovine serum albumin (BSA). Intracellular staining was performed with an anti-IFN-γ FITC mAb (BD, 554411). Data acquisition was performed on a FACSort flow cytometer (BD) and the Cellquest software was used for data analysis.

In the curative setting surface staining was performed with anti-Ly6C Alexa Fluor® 647 (Bio-Rad, MCA2389A6457), anti-CD11b BV421, anti-Ly6G FITC, anti-CD8 BV421 (all three from BD, 562605, 551460 and 563898, respectively), anti-CD45 Alexa Fluor® 700, anti-F4/80 PE, anti-CD3 FITC and anti-CD4 PerCP-Cyanine5.5 mAbs (all four from eBioscience, 56-0451-82, 12-4801-80, 11-0031-82 and 45-0042-82, respectively). Intracellular FoxP3 was detected using an anti-FoxP3 PE staining set (eBioscience, 72-5775). For intracellular IFN-γ staining, surface staining of stimulated cells was performed with anti-CD45 Alexa Fluor® 700, anti-CD3 PE, anti-CD4 APC-eFluor® 780 (all three from eBioscience, 56-0451-82, 12-0031-82 and 47-0042-82, respectively) and anti-CD8 BV421 mABs (BD), followed by intracellular staining performed with an anti-IFN-γ PerCP-Cyanine5.5 mAb (BD, 560660). Data acquisition was performed on a LSRFortessa flow cytometer (BD Biosciences) and the FlowJo software was used for data analysis.

Protein carbonylation detection assay

The detection of protein carbonyl derivatives was performed as previously described.[55] In brief, 50 µl of GL261 cell lysates or untreated GL261 cells, corresponding to a total protein concentration of 5 to 10 mg/ml were incubated with an equal volume of 200 µM fluorescein-5-thiosemicarbazide (FTC, Sigma-Aldrich, 46985-100MG-F) overnight at room temperature in the dark. Twenty-four hours later, proteins were precipitated and centrifuged after which the supernatant was discarded and consequently the protein precipitates were washed three times with acetone. The acetone supernatant was discarded and the precipitates were air-dried. These precipitates were then solubilized with 50 µl of guanidine hydrochloride (GuHCl, 6M) and diluted with 450 µl of NaH_2PO_4 (pH 7.0). Subsequently, the protein concentrations in these samples were measured via the bicinchoninic (BCA) protein assay kit from Thermo Scientific (23225). Fifty microliters of these samples were aliquoted in a black microtiter plate and fluorescence was measured with a FlexStation 3 microplate reader (Molecular Devices, Berkshire, United Kingdom, 0310-5627). A standard curve that was prepared using different concentrations of FTC allowed for the calculation of the nanomoles of FTC-reacted carbonyls. These values were then divided by the respective protein concentrations to derive the amount of protein carbonyls expressed as nmol/mg protein.

Literature search of tumor lysate-pulsed DC vaccination trials in HGG patients

The studies listed in table I were identified through an electronic search of the PubMed database, reference lists of published trials and relevant review articles. The search strategy included the following search terms: "glioma" AND "dendritic cells" AND "tumor lysate". We only included studies that performed freeze-thaw cycles to obtain tumor lysates and excluded case reports, articles not published in English and protocol studies.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 software (San Diego, USA). Survival analysis was performed using the log-rank test. Student's t-test was performed for statistical analysis with significance level set at $p < 0.05$. Wherever applicable (with respect to comparison between multiple data-sets), a one-way ANOVA analysis was performed. Linear regression analysis was performed to investigate a possible association between protein carbonylation levels and the percentages of long-term surviving mice.

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Table I: Autologous tumor lysate-pulsed DC vaccination studies in HGG patients

Author	Year	No. of patients (type of trial)	Grade III/IV	ND/R	Lysate preparation	Injection route	Treatment schedule	Immune response	Clinical response
Yamanaka et al. [32]	2003	10 (phase I-II)	III /IV	R	FT	ID and/or IT	1-10 vaccinations at 3-week intervals	Increase in NK cells in PBMCs (5/5); positive DTH reaction (3/6); increased IFN-γ ELISPOT (2/5)	MR (2/6)
Wheeler et al. [33]	2003	17 (phase I)	IV	ND/R	FT	NS	3 vaccinations at 2- week intervals	NS	NS
		17 (phase II)	IV	ND/R	FT		3 vaccinations at 2-week intervals; 4th vaccination 6 weeks later		
Wheeler et al. [34]	2004	5 (phase I) and 12 (Phase II)	III/IV	ND/R	FT	NS	3 vaccinations at 2-week intervals (phase I) and 4th vaccination 6 weeks later (phase II)	In GBM patients receiving post-vaccine chemotherapy, CD8+ TRECs predicted longer chemotherapy responses	Vaccinated patients receiving subsequent chemotherapy exhibited longer times to tumor recurrence after chemotherapy
Yu et al. [35]	2004	14 (phase I-II)	III/IV	ND/R	FT	SC	3 vaccinations at 2-week intervals; 4th vaccination 6 weeks later	Increased IFN-γ ELISPOT (6/10); expansion of CD8+ antigen specific T cell clones (4/9); systemic T cell cytotoxicity against tumor (1/1)	OS: 133 weeks in DC-group and 30 weeks in control-group (recurrent GBM)
Rutkowski et al. [41]	2004	12 (phase I)	IV	R	FT + IR	ID	2-7 vaccinations; 2nd vaccine 2 weeks after 1st , then monthly vaccines	Positive DTH reaction (6/8)	PR (1/12); tumor-free survival 5 year after vaccination (2/12); PFS: 3 months; OS: 105 months

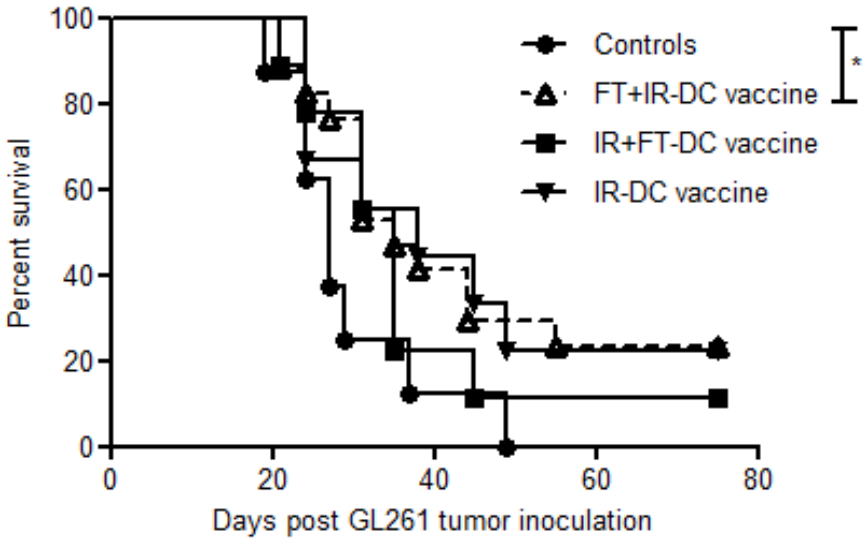
Yamanaka et al. [36]	2005	24 (phase I-II)	III/IV	R	FT	ID or ID + IT	1-10 vaccinations at 3-week intervals	Positive DTH reaction (8/17); positive IFN-γ ELISOT (7/16); positive immune monitoring predicts good clinical outcome	PR (1/24); MR (3/24); SD (10/24); significantly increased MS
Wheeler et al. [37]	2008	34 (phase II)	III/IV	ND/R	FT	SC	3 vaccinations at 2-week intervals; 4th vaccination 6 weeks later	Increased IFN-γ ELISPOT (17/34); DTH-test resulted in cutaneous GBM in 1 patient (DTH was subsequently discontinued)	Significant positive correlation between post-vaccine response magnitude and TTS; 2-year OS: 41 % in vaccine responders *versus* 7 % in non-responders; patients relapsing after vaccination showed increased chemosensitivity.
De Vleeschouwer et al. [42]	2008	56 (phase I-II)	IV	R	FT + IR	ID	Cohort comparison	Positive DTH (9/21 at time of diagnosis and 9/17 after 2 vaccinations)	PFS: 3 months; OS: 9.6 months; 2-year OS: 14.8 %; total resection is a predictor for better PFS; younger age and total resection are predictors for better OS in univariable analysis; tendency towards improved PFS when faster DC vaccination schedule with tumor lysate boosting was applied
Ardon et al. [43]	2010	8 (pilot)	IV	ND	FT + IR	ID	4 weekly vaccines, 3 monthly vaccines, then 3-month intervals	Increased IFN-γ ELISPOT (5/8), positive DTH reaction (3/6)	6-month PFS: 75 %; OS: 24 months; PFS: 18 months
Ardon et al. [44]	2010	33 children (phase I/II)	III/IV	R	FT + IR	ID	Depending on the cohort	NS	6-month PFS: 42 %; PFS: 4.4 months; OS: 13.5 months

Fadul et al. [45]	2011	10 (phase I/II)	IV	ND	IR + FT	IN	3 vaccines at 2-week intervals	Increased IFN-γ ELISPOT (4/10)	PFS: 9.5 months; OS: 28 months
Prins et al. [38]	2011	23 (phase I)	IV	ND/R	FT	ID	3 vaccines at 2–week intervals, booster vaccines every 3 months (in combination with Imiquimod/Poly-ICLC)	Mesenchymal tumors had a higher number of CD3+ and CD8+ tumor-infiltrating lymphocytes.	OS: 31.4 months and a 1-, 2- and 3-year survival rate of 91 %, 55 % and 47 %. Better survival in patients having a mesenchymal gene signature.
Elens et al. [46]	2012	39 (phase I/II)	III	R	FT + IR	ID	Depending on the cohort	NS	Median PFS/OS were 3.4/20.5 months (AA), 3.4/18.8 months (AOD) and 7.8/13.3 months (AOA)
Ardon et al. [47]	2012	77 (phase I/II)	IV	ND	FT + IR	ID	4 weekly vaccines, 3 monthly vaccines, then 3-month intervals	Immunological profiling could not predict clinical outcome.	6-month PFS: 70.1 %; median OS: 18.3 months; OS depending on RPA classification
De Vleeschouwer et al. [48]	2012	117 (phase II)	III/IV	R	FT + IR	ID	Changing per cohort	NS	OS: 6-48.4 months (according to HGG-immuno RPA classification)
Fong et al. [39]	2012	24 (two phase I trials): 19 (DC-ATL) and 5 (DC-GAA)	IV	ND/R	FT	ID	3 vaccinations at 2-week intervals	Decreased Treg frequency and decreased expression of CTLA-4 on T cells after DC vaccination was associated with better survival	OS: 33.8 months (DC-ATL), 14.5 months (DC-GAA); TTP: 13.9 months (DC-ATL), 9.6 months (DC-GAA)
Valle et al. [49]	2012	5 (pilot)	IV	ND	FT + IR	NS	1st vaccine one week before radiotherapy, 2nd 3 weeks after radiotherapy, 2 monthly vaccines and 4 every 2 months and later quarterly	PBMC proliferation (3/3); positive IFN-γ ELISA (3/3); positive IFN-γ ELISPOT (0/3)	PFS: 16.1 months; OS: 27 months

Lasky et al. [40]	2013	7 (pilot, children)	III/IV	ND/R	FT	ID	2-week intervals	Cytokine production upon *in vitro* stimulation (1/3)	MR: 1/3; CR: 2/3
Prins et al. [16]	2013	28 (phase I)	III/IV	ND/R	FT	ID	3 vaccines at 2-week intervals, booster vaccines every 3 months	Significant correlation between decreased Treg ratio (post/pre-vaccination) and OS	PFS: 18.1 months; OS: 34.4 months

AA, anaplastic astrocytoma; AOD, anaplastic oligodendroglioma; AOA, anaplastic oligoastrocytoma; ATL, autologous tumor lysate; CR, complete response; CTL, cytotoxic T lymphocyte; CTLA-4, cytotoxic T lymphocyte-associated protein 4; DC, dendritic cell; DTH, delayed-type hypersensitivity; ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunosorbent spot assay; FT, freeze-thaw; GAA, glioma-associated antigens; GBM, glioblastoma multiforme; IFN-γ, interferon-γ; ID, intradermal; IN, intranodal; IR, irradiation; IT, intratumoral; MR, minor response; MS, median survival; ND, newly diagnosed; NK, natural killer; NS, not specified; OS, overall survival; PBMC, peripheral blood mononuclear cell; PFS, progression-free survival; PR, partial response; R, relapsed; RPA, recursive partitioning analysis; SC, subcutaneous, SD, stable disease; TREC, T cell receptor excision circle; Treg, regulatory T cell; TTP, time to progression; TTS, time to survival.

Supplemantal figure and its legend



Suppl. Figure 1. Applying radiotherapy alone or prior to inducing FT-necrosis in the GL261 cells does not improve DC vaccine-induced antitumor immunity. Kaplan-Meier graph depicting survival of mice prophylactically immunized on days 14 and 7 prior to intracranial GL261 tumor inoculation with FT+IR-DC vaccines (Δ, dashed line, n = 17), IR+FT-DC vaccines (■, n = 9) and IR-DC vaccines (▼ , n = 9). Mice not immunized with vaccines were used as controls (●, n = 9). *, $p < 0.05$ (Log-rank test).

4. RESULTS – PART II

"A scientist in his laboratory is not a mere technician: he is also a child confronting natural phenomena that impress him as though they were fairytales."

- Marie Curie

Immunotherapy with dendritic cell vaccines based on immunogenic cell death elicit danger signals and T cells-driven rejection of high-grade glioma

Our research described in the previous chapter has highlighted the crucial contribution of oxidation, and more specifically OAMPs like carbonylated proteins, to the immunogenicity of whole tumor cell-based DC vaccines. This finding drove us to hypothesize that other very strong oxidizing treatment modalities, preferably non-chemical treatments, could further potentiate the immunogenicity of dying cancer cells in the setting of DC vaccines. In this regard, Hyp-PDT might constitute an extremely promising cancer cell killing regimen. Besides the strong ER-directed oxidation it confers, it is considered a prototypical inducer of ICD. Its capacity to emit the highest diversity and amounts of DAMPs as well as its favorable safety profile and many other advantages as compared to known ICD inducers (as described in chapter 1.11.2 of this work) make Hyp-PDT the ideal candidate to test for application in preclinical ICD-based DC vaccines.

Based on these observations, we wished to address the following aims in this research project:

I. To study the potential of Hyp-PDT to induce ICD in GL261 cancer cells;
II. To evaluate if Hyp-PDT-based DC vaccines can induce protective immunity in a prophylactic vaccination setup in the GL261 model;
III. To study the contribution of different ICD-related molecular and cellular players (i.e. adaptive immune cells, OAMPs, selective DAMPs, cancer cell's interface with DCs, TLR signaling) to the vaccine's immunogenicity;
IV. To develop a clinically relevant version of the Hyp-PDT-based DC vaccine that still induces potent protective immunity against HGG;
V. To study the brain immune-contexture of mice prophylactically vaccinated with the Hyp-PDT-based DC vaccine and its clinically relevant version;
VI. To evaluate the potency of Hyp-PDT-based DC vaccines in a curative, more clinically relevant setup, alone and in combination with the standard-of-care chemotherapeutic TMZ and to study the brain immune-contexture in this setting;

The research paper describing the results associated with this section and the corresponding supplementary information is presented on the following pages.

Dendritic cell vaccines based on immunogenic cell death elicit danger signals and T cell-driven rejection of high-grade glioma

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One-sentence summary: The first translational study combining a single-agent immunogenic cell death inducer with dendritic cell immunotherapy in a preclinical model of orthotopic high-grade glioma.

Keywords: DC immunotherapy, immune-contexture, anti-tumor immunity, prophylactic vaccination, curative vaccination, cancer cell death, patient prognosis.

Abbreviations: ATP – adenosine triphosphate; BBB – blood brain barrier; CD – cluster of differentiation; CNTR – control; CRT – calreticulin; CTL – cytotoxic T lymphocytes; DAMPs – damage-associated molecular patterns; DC – dendritic cells; eIF2α – eukaryotic initiation factor 2-alpha; ER – endoplasmic reticulum; F/T – freezing/thawing or freeze/thaw or freeze/thawed; GM – GentleMACS®; HGG – high grade glioma; HMGB1 – high-mobility group box 1; HSP – heat shock proteins; Hyp-PDT – Hypericin-based photodynamic therapy; ICD – immunogenic cell death; IFN – interferon; IL – interleukin; L-Hist – L-Histidine; LN – lymph node(s); LNC – lymph node cells; MHC – major histocompatibility complex; NAC – N-acetylcysteine; RT – radiotherapy; SPL – splenocytes or splenocytic; Th1 or Th17 cells – T helper type-1 polarized cells or T helper IL17A secreting cells; TMZ – temozolomide; TUDCA - Tauroursodeoxycholic acid; Vacc – vaccine;

Abstract

Promise of dendritic cell (DC)-based immunotherapy has been established by two decades of translational research. Of the four malignancies mostly targeted with clinical DC immunotherapy, high-grade glioma (HGG) has shown the highest susceptibility. However, HGG-induced immunosuppression is an anti-immunotherapy roadblock that necessitates application of Th1 immunity-biased, next-generation, DC immunotherapy. To this end, we combined DC immunotherapy with immunogenic cell death (ICD; a modality shown to induce Th1-immunity), induced by Hypericin-Photodynamic Therapy. In an orthotopic-HGG mouse model involving prophylactic/curative set-ups, both biologically and clinically-relevant versions of ICD-based DC vaccines provided strong anti-HGG survival benefit. We found that the ability of DC vaccines to elicit HGG rejection was significantly blunted if cancer cell-associated ROS and emanating danger signals were blocked (either singly or in concomitantly; showing hierarchical effect on immunogenicity i.e. extracellular-HMGB1>extracellular-ATP>surface-calreticulin) or if DCs, DCs-associated MyD88 signal or the adaptive immune system (especially $CD8^+$ T cells) were depleted. In curative setting, ICD-based DC vaccines synergized with standard-of-care chemotherapy (temozolomide) to increase survival of HGG-bearing mice by ~300% resulting in ~50% long-term survivors. Additionally, DC vaccines also induced an immunostimulatory shift in brain immune-contexture from Tregs to Th1/cytotoxic T lymphocytes/Th17 cells.

Analysis of the TCGA glioblastoma-cohort confirmed that increased intra-tumor prevalence of Th1/cytotoxic T lymphocytes/Th17 cells-linked genetic signatures associated with good patient prognosis. This is the first translational study combining a single-agent ICD inducer with DC immunotherapy against orthotopic HGG. We believe that, pending final preclinical-checks, ICD-based vaccines can be clinically translated for glioma treatment.

Introduction

Anti-cancer immunotherapy has emerged as an important therapeutic paradigm in recent times (1-3). Of the plethora of immunotherapies currently available, remarkable efforts have been invested in the development of dendritic cell (DC)-based vaccines (4). This is primarily because DCs are the main sentinel antigen-presenting cells positioned at a crucial interface between the innate and adaptive immune system (4). In therapeutic sense, DC vaccines largely act by stimulating tumor-specific cytotoxic T lymphocytes (CTLs) that recognize and eliminate malignant cells (3, 5).

The promise of DC-based immunotherapy is backed by two decades of clinical studies, frequently showing positive association between patient survival and DC vaccine-induced immunity (5). DC immunotherapy has been applied across a wide range of cancer-types but most prevalently in patients of melanoma, prostate cancer, high-grade glioma (HGG) and renal cell cancer (5). A recent meta-analysis showed that of these four, the highest positive objective responses (15.6% of patients) were observed for HGG patients (5, 6), suggesting that HGG might be susceptible to highly efficacious immunotherapy (5, 6).

It is noteworthy that existing multimodal treatments fail to improve HGG patient prognosis (7, 8). As many as 160 FDA-approved oncology drugs have been applied for anti-HGG treatment, yet only a few (like temozolomide or TMZ) are used as standard-of-care (8, 9). Such standard-of-care anti-HGG therapies, although capable of improving patients' prognosis to a certain extent, eventually fail due to HGG's brain localized invasion and recurrences (9). This alarmingly negative outlook means that HGG is in dire need for novel therapeutic interventions like "next-generation" DC-based immunotherapy (7).

Several preclinical and clinical studies have successfully employed anti-HGG DC immunotherapy (10-20). However, current anti-HGG DC immunotherapies mostly employ specific antigen-peptides/RNA for pulsing DCs (10-12, 17-19) or whole-glioma tumor cells (autologous or allogeneic) (13, 14, 16, 20) killed via freeze/thawing (F/T)-based necrosis (7, 15, 21, 22). Here, while the former methodology might exhibit low efficacy due to the high antigenic-heterogeneity of HGG, the latter procedure is associated with poor immunogenic potential (7, 22). Thus, clearly there is a need to improve the immunogenicity of the dying/dead cancer cells used to pulse the DCs to foster development of next-generation anti-HGG vaccines. Moreover, it is currently recognized that the next-generation DC vaccines need to be biased towards induction of Th1-immunity, since Th1 responses have superior capacity in eliciting CTLs-based anti-cancer cytotoxicity (5). To this end, we envisaged that for HGG, a next-generation DC vaccine could be produced by pulsing DCs with HGG cells undergoing immunogenic cell death (ICD) (1).

Cancer cells undergoing ICD exhibit superior immune-stimulatory capacity owing to the spatiotemporally-defined exposure/release of damage-associated molecular patterns (DAMPs) acting as potent danger signals (23, 24). DAMPs most crucial for ICD include, surface-exposed calreticulin (CRT), surface-exposed heat shock proteins (HSP)-70/90, secreted ATP and passively released high mobility group box 1 (HMGB1) protein (23, 24). ICD is associated with highly efficacious anti-cancer vaccination effect that is largely biased towards the Th1-immunity (24, 25). Several ICD inducers have been characterized recently (23, 24) however very few prototypical ICD inducers exist thus far (26). One such prototypical ICD inducer is, Hypericin-based Photodynamic Therapy (Hyp-PDT) (27). Hyp-PDT treated cancer cells expose/release the highest amounts and diversity of DAMPs, at a rapid pace, compared to chemotherapy (27-32). Hyp-PDT induced ICD elicits fully mature immunogenic human DCs, which in turn activate clonal proliferation of IFN-γ producing $CD4^+$/$CD8^+$ human T cells (28, 29, 31). Moreover, cancer cells undergoing Hyp-PDT induced ICD act as potent anti-cancer vaccines in various mice vaccination models [e.g. CT26 cells-BALB/c mice (31), B78 cells-C57BL/6 mice (29) and K1735 cells-C3H mice (unpublished data)].

Figure 1:

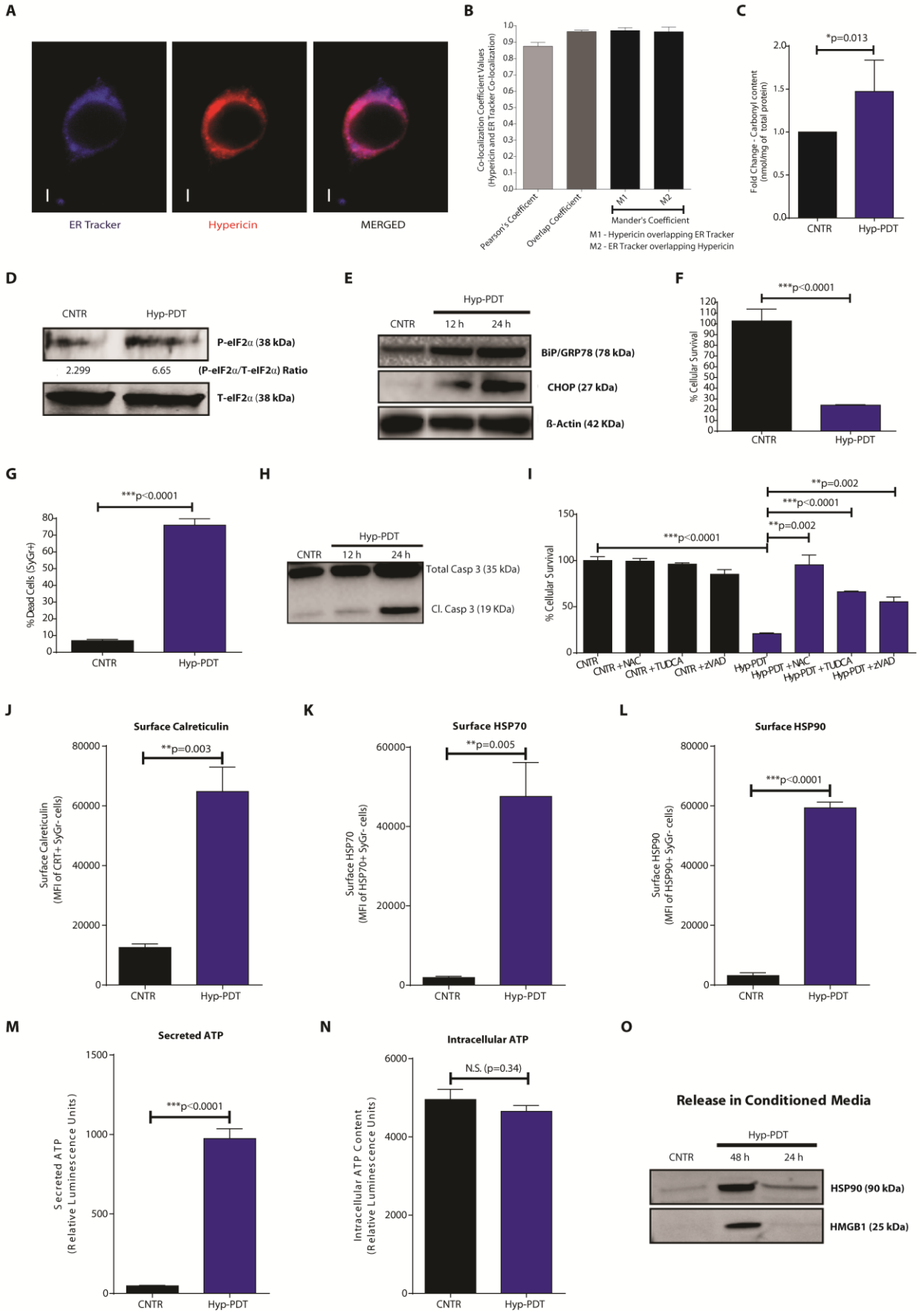

A
ER Tracker
Hypericin
MERGED
B
Co-localization Coefficient Values (Hypericin and ER Tracker Co-localization)
Pearson's Coefficient
Overlap Coefficient
M1
M2
Mander's Coefficient
M1 - Hypericin overlapping ER Tracker
M2 - ER Tracker overlapping Hypericin
C
*p=0.013
Fold Change - Carbonyl content (nmol/mg of total protein)
CNTR
Hyp-PDT
D
CNTR
Hyp-PDT
P-eIF2α (38 kDa)
2.299
6.65
(P-eIF2α/T-eIF2α) Ratio
T-eIF2α (38 kDa)
E
Hyp-PDT
CNTR
12 h
24 h
BiP/GRP78 (78 kDa)
CHOP (27 kDa)
ß-Actin (42 KDa)
F
***p<0.0001
% Cellular Survival
CNTR
Hyp-PDT
G
***p<0.0001
% Dead Cells (SyGr+)
CNTR
Hyp-PDT
H
Hyp-PDT
CNTR
12 h
24 h
Total Casp 3 (35 kDa)
Cl. Casp 3 (19 KDa)
I
**p=0.002
***p<0.0001
**p=0.002
***p<0.0001
% Cellular Survival
CNTR
CNTR +NAC
CNTR +TUDCA
CNTR +zVAD
Hyp-PDT
Hyp-PDT +NAC
Hyp-PDT +TUDCA
Hyp-PDT +zVAD
J
Surface Calreticulin
**p=0.003
Surface Calreticulin (MFI of CRT+ SyGr- cells)
CNTR
Hyp-PDT
K
Surface HSP70
**p=0.005
Surface HSP70 (MFI of HSP70+ SyGr- cells)
CNTR
Hyp-PDT
L
Surface HSP90
***p<0.0001
Surface HSP90 (MFI of HSP90+ SyGr- cells)
CNTR
Hyp-PDT
M
Secreted ATP
***p<0.0001
Secreted ATP (Relative Luminescence Units)
CNTR
Hyp-PDT
N
Intracellular ATP
N.S. (p=0.34)
Intracellular ATP Content (Relative Luminescence Units)
CNTR
Hyp-PDT
O
Release in Conditioned Media
Hyp-PDT
CNTR
48 h
24 h
HSP90 (90 kDa)
HMGB1 (25 kDa)

Figure 1. Hyp-PDT induces ROS/ER stress-based apoptosis and major ICD-associated DAMPs in murine glioma cells. (A,B) GL261 cells were loaded with 200 nM Hypericin (Hyp; for 2 h), followed by co-localization analysis of Hyp (red) versus ER Tracker Blue-White (blue) *via* fluorescence microscopy **(A)**; extended to co-localization defining coefficients i.e. Pearson's coefficient, Overlap coefficient and Mander's coefficients (M1/M2) (n=5, mean±s.e.m.) **(B)**. **(C-O)** GL261 were treated with Hyp-PDT (incubation of 2 h - 200 nM Hyp, light fluence - 4.05 J/cm^2), followed by: **(C)** recovery of cells 1 h post-PDT and estimation of carbonylated proteins (fold change relative to CNTR; n=3, mean±s.d.); **(D,E)** immunoblotting analysis of whole cell lysate at 1 h post-PDT using the respective antibodies as indicated; **(F)** recovery of cells 24 h post-PDT and estimation of % cellular survival *via* MTS assay (n=4; mean±s.d.); **(G)** recovery of cells 24 h post-PDT and estimation of % dead SyGr$^+$ cells *via* FACS (n=3; mean±s.d.); **(H)** immunoblotting of whole cell lysate at indicated time-points for casapase-3 cleavage; **(I)** recovery of cells 24 h post-PDT, as indicated the cells were pre-incubated for 1 h with, NAC (5 mM), TUDCA (500 µg/ml) or zVAD-fmk (25 µM) followed by estimation of % cellular survival (n=3; mean±s.e.m.); **(J-L)** recovery of cells 1 h post-PDT and FACS-based analysis for surface-calreticulin **(J)**, surface-HSP70 **(K)** and surface-HSP90 **(L)** in non-permeabilized cells (data presented as mean fluorescence intensity (MFI), n=3; mean±s.e.m.); **(M,N)** recovery of cells 1 h post-PDT and analysis for relative amounts of extracellular **(M)** and intracellular **(N)** ATP levels, measured in the conditioned media and cell lysates, respectively (n=6; mean±s.e.m.); and **(O)** recovery of cells at indicated time-points post-PDT, and immunoblotting analysis of concentrated conditioned media using the indicated antibodies. CNTR samples in these experiments were incubated with hypericin, but did not receive PDT treatment. In this figure, Student's t-test was used for statistics, *p<0.05/**p<0.01/***p<0.001 as indicated by bars; N.S. – not significant.

Of note, Hyp-PDT has been successfully applied in the past, for clinical treatment of patients with non-melanoma skin cancer (33), cutaneous T-cell lymphoma (34) and basal/squamous cell carcinoma (35).

Here, we performed a preclinical study employing a single-agent ICD inducer-based DC vaccine in an orthotopic HGG mouse model and tested its efficacy as a next-generation anti-HGG immunotherapy (21, 36). We also present a systematic assessment of the key molecular and signaling steps required to elicit immunogenicity, from cancer cell-associated signals to DC-associated as well as T cells-orchestrated immunity. Finally, we accounted for various stiff clinical operational and regulatory requirements (37), to produce a "clinically-relevant version" of our ICD-based DC vaccines.

Results

Murine glioma cells exhibit ICD-associated molecular determinants following Hyp-PDT

Considering the recent observations of contextual differences in ICD induction (23, 36), it was necessary to re-examine the ability of Hyp-PDT to elicit ICD-associated determinants in murine GL261 glioma cells. (27). As found in other cellular models (31, 38, 39), Hypericin strongly localized at the endoplasmic reticulum (ER) (Fig 1A-B) and upon light-activation, caused an accumulation of reactive oxygen species (ROS)-induced (40) carbonylated proteins (Fig 1C); along with ER stress signatures (eIF2α phosphorylation↑ BiP↑ CHOP↑), culminating into loss of viability and apoptosis (e.g. caspase-3 cleavage (Fig 1D-H)). Moreover, attenuating ROS (via the anti-oxidant NAC), ER stress (via the chemical chaperone, TUDCA (29)) or apoptosis (via pan-caspase inhibitor, zVAD-fmk (29)) increased the survival of GL261 cells following Hyp-PDT (Fig 1I) thereby substantiating the crucial pro-death role of these processes (38, 39).

On the level of danger signals/DAMPs, Hyp-PDT caused rapid, (within 1 h post-treatment) surface exposure of CRT (Fig 1J), HSP70 (Fig 1K) and HSP90 (Fig 1L) accompanied by a rapid secretion of ATP (Fig 1M), in the absence of any changes in intracellular ATP levels (Fig 1N). All this culminated into an extracellular exodus of HSP90 and HMGB1, at later time-points (e.g. 48 h), post-treatment (Fig 1O). Moreover, murine DCs co-incubated with GL261 cells dying in response to Hyp-PDT exhibited significantly increased phenotypic maturation (i.e.CD80^{high}CD86^{high}CD40^{high}MHC-I^{high}MHC-II^{high}) (Suppl. Fig S1).

Interestingly, another photosensitiser i.e. 5-aminolevulinic acid (5-ALA, Gliolan®) is frequently used for clinical fluorescence-guided resection of HGG (41). Considering its mitochondrial (but not ER) sub-cellular localization (42) we compared the DAMPs-inducing capabilities of 5-ALA-based PDT with Hyp-based PDT. At similar apoptosis-inducing doses (Suppl. Fig S2A), we found no differences in surface-HSP70 (Suppl. Fig S2B) or secreted ATP (Suppl. Fig S2C) between these photosensitizers. Yet, only Hyp-PDT induced significant surface-CRT (Suppl. Fig S2D), surface-HSP90 (Suppl. Fig S2E) and passively released-HMGB1 (Suppl. Fig S2F). This, along with other studies (29, 31, 43, 44), reiterates that higher therapeutic ER-targeting may translate into higher DAMPs induction.

Taken together, these results show that GL261 murine glioma cells succumb to ICD induced by Hyp-PDT.

ICD-based DC vaccines provide significant protective immunity against HGG

We next tested whether Hyp-PDT induced ICD-based DC vaccines can induce anti-glioma protective immunity by utilizing the prophylactic vaccination strategy (45) (Fig 2A). Immunocompetent, syngeneic C57BL/6 mice were vaccinated with either Hyp-PDT induced ICD-based DC vaccines or F/T-necrosis-based DC vaccines (the latter being a control for non-ICD and a method used pre-dominantly for vaccine production), twice, intra-peritoneally (with an interval of 7-8 days between vaccinations). Mice not immunized with the vaccines were used as controls (CNTR). Thereafter, immunized and non-immunized mice were inoculated with live GL261 glioma cells, intra-axially (i.e. in the brain), 7-8 days after vaccination. Following the tumor-inoculation, mice were monitored for HGG-induced neurological deficit symptoms (45). Remarkably, approximately 70% of mice vaccinated with ICD-based DC vaccines resisted orthotopic glioma challenge and showed significantly higher median survival as compared to CNTR mice or mice vaccinated with F/T necrosis-based DC vaccines (Fig 2B). Consistent with this, graphical representation of the HGG-induced neurological deficit scores/grades revealed a considerable delay in the onset of clinically-relevant symptoms in mice treated with ICD-based DC vaccines, compared to other cohorts (Fig 2C-E). We next decided to substantiate this data with magnetic resonance imaging (MRI)-based non-invasive monitoring of brain-associated glioma (Fig 2F-U). We procured the relevant MRI-scans of brains (Fig 2F, J, N, R) followed by 3D rendition of the brain (Fig 2G, K, O, S), the gliomas (wherever visible) (Fig 2L, P) and the anterior/middle

ventricles (Fig 2I, M, Q, U) (respective representatives are shown). On day 28 post-glioma-inoculation, we observed that in comparison to naïve mice (Fig 2F-I), CNTR mice (Fig 2J-M) and mice vaccinated with F/T necrosis-based DC vaccine (Fig 2N-Q), exhibited palpable HGG masses that were capable of distorting the morphology of the ventricles. However, most mice vaccinated with ICD-based DC vaccines exhibited absence of visible HGG masses and normal brain/ventricles morphology (Fig 2R-U). We also used the brain 3D renditions to estimate the arbitrary alpha-blending based relative brain volumes. Interestingly, as compared to naïve mice, mice vaccinated with ICD-based DC vaccines did not exhibit any strong increase in brain volume (Suppl. Fig S3); whereas CNTR mice (to a significant extent) and F/T necrosis-based DC vaccinated mice (to a variable hence non-significant extent), exhibited increase in brain volumes (Suppl. Fig S3).

HGGs exhibit high heterogeneity on multiple levels, including cellular (basal) major-histocompatibility complex (MHC)-based immunogenicity (46-48). GL261-tumors are partially immunotherapy-susceptible due to GL261's detectable MHC-based immunogenicity (46, 47). Thus, we tested the potential of ICD-based DC vaccine against CT2A, a well-known immunotherapy-evasive glioma model (47). As reported previously (47), we found that CT2A cells exhibited a very different morphology compared to GL261 cells (Suppl. Fig S4A). Moreover, they expressed significantly less MHC-I levels (even after IFN-γ stimulation; Suppl. Fig S4B) and almost negligible MHC-II levels (that couldn't be up-regulated by IFN-γ treatment; Suppl. Fig S4C), compared to GL261 cells. These observations substantiate that CT2A have very low immunogenicity (47, 48). Despite this disparity, CT2A cells in response to Hyp-PDT, exposed/released all the main ICD-related DAMPs (surface-CRT, surface-HSP90, surface-HSP70, secreted ATP and released-HMGB1) efficiently (Suppl. Fig S4D-H). Of note, mice vaccinated with ICD-based DC vaccines and orthotopically challenged with live CT2A cells, showed significantly higher median survival as compared to CNTR mice (although not to the same extent as in GL261 model) (Suppl. Fig S4I).

Altogether, this demonstrates that ICD-based DC vaccines are capable of inducing potent protective immunity against HGG in both immunotherapy-susceptible and to a certain extent immunotherapy-evasive cancer models.

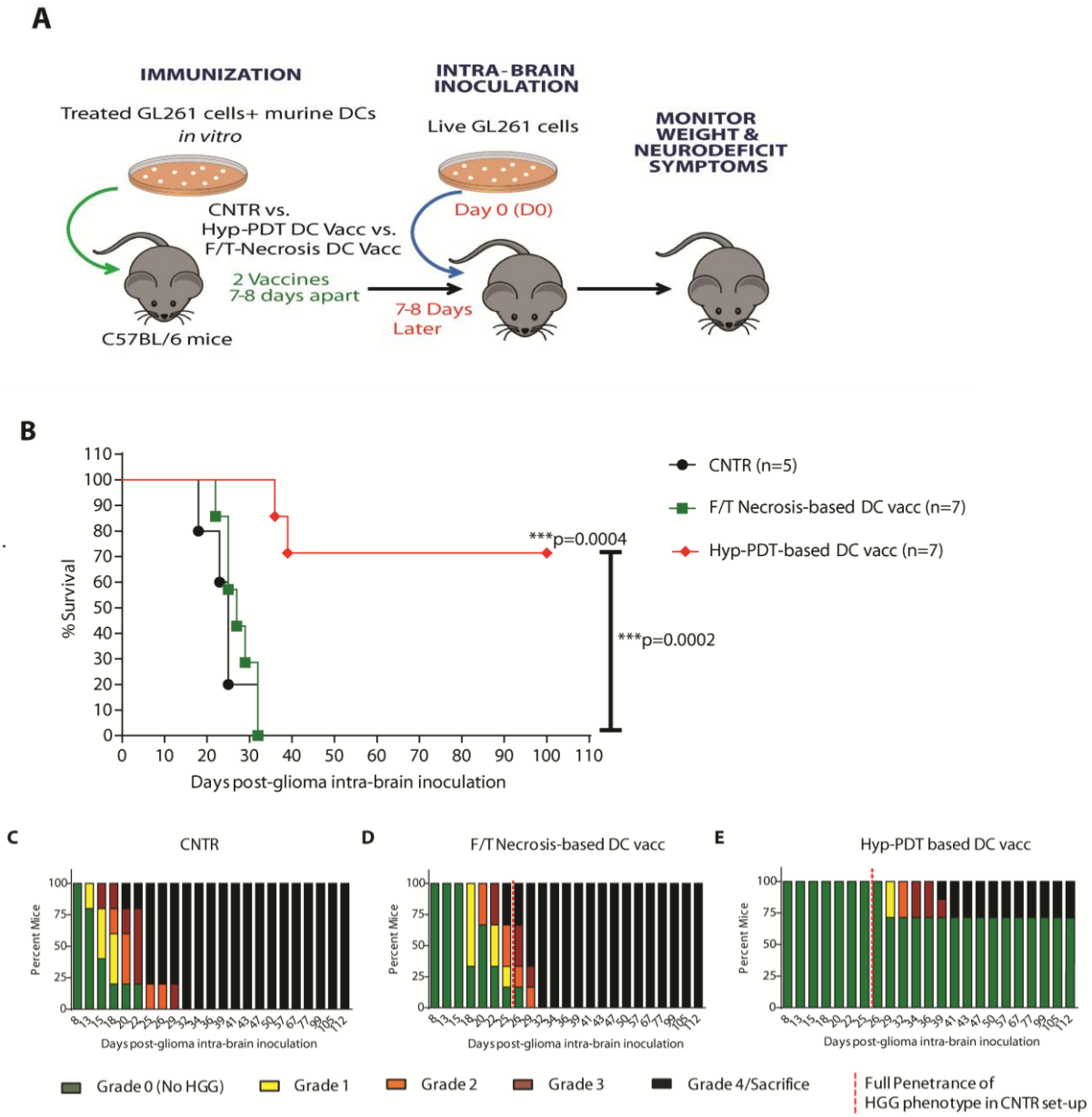
Anti-tumor immunity induced by ICD-based DC vaccines depends on ROS, danger signals, DCs, intact adaptive immune system and CD8$^+$ T cells

The immunogenic impact of major ICD-associated molecular/cellular determinants (ROS and danger signals/DAMPs) has never been comparatively tested in an orthotopic HGG model (49). To this end, we employed relevant blockade strategies to assess the relevance of key molecular steps in the signaling cascade eliciting ICD, for the generation of efficient DC-based vaccines, as depicted in Fig 3A. Presence of ROS-scavenging molecules, NAC/L-Hist (40), significantly attenuated the immunogenic potential of ICD-based DC vaccine (Fig 3B-C). Similarly, blocking different DAMPs reduced the vaccine's immunogenic potential in a hierarchical manner (extracellular-HMGB1>extracellular-ATP>surface-CRT) (Fig 3B-C). Extracellular-HMGB1 ablation by blocking antibodies significantly reduced both % median survival (Fig 3B) and % long-term survivors (Fig 3C); whereas degradation of extracellular-ATP by apyrase only significantly ablated the latter (Fig 3B-C). Surface-CRT blockade by blocking antibodies reduced both albeit insignificantly (Fig 3B-C). Remarkably, concurrent ablation of all three DAMPs additively reduced the mice median survival (30 days), more than individual DAMPs blockade, to nearly the same level as CNTRs (26 days) (Fig 3B-C).

Cellular adjuvant like DCs (3) are often considered dispensable for ICD in a vaccination set-up (25, 50); since the cancer cells dying via ICD are considered to be sufficiently immunogenic (31). However in our current set-up, vaccination with dead/dying cancer cells undergoing ICD alone, without DCs, drastically reduced the vaccine's immunogenic potential (Fig 3D-E). Since extracellular-HMGB1 emerged as a predominant DAMP in this model, we decided to ablate its toll-like receptor (TLR)-agonist function (51), which is conveyed through the TLR-adaptor, myeloid differentiation primary response gene 88 (MyD88) for innate immune signaling (52). Notably, utilizing *Myd88*$^{-/-}$ DCs for making ICD-based DC vaccine, resulted in the severe ablation of both % median survival (in a slightly delayed fashion) and especially the % long-term survivors parameters (Fig 3D-E), thus indicating the crucial adjuvant role of proper DC-based signaling in these settings.

The immunogenic potential of ICD and DC vaccines relies upon an intact adaptive immune system and especially on CD8$^+$ T cell-activity (2, 5). Remarkably, mice lacking an intact adaptive immune system (due to lack of recombination-activating gene 1 or *Rag1*$^{-/-}$)

Figure 2:


A
IMMUNIZATION
Treated GL261 cells+ murine DCs
in vitro
CNTR vs.
Hyp-PDT DC Vacc vs.
F/T-Necrosis DC Vacc
2 Vaccines
7-8 days apart
C57BL/6 mice
INTRA-BRAIN
INOCULATION
Live GL261 cells
Day 0 (D0)
7-8 Days
Later
MONITOR
WEIGHT &
NEURODEFICIT
SYMPTOMS
B
% Survival
Days post-glioma intra-brain inoculation
CNTR (n=5)
F/T Necrosis-based DC vacc (n=7)
Hyp-PDT-based DC vacc (n=7)
***p=0.0004
***p=0.0002
C
CNTR
D
F/T Necrosis-based DC vacc
E
Hyp-PDT based DC vacc
Percent Mice
Days post-glioma intra-brain inoculation
Grade 0 (No HGG)
Grade 1
Grade 2
Grade 3
Grade 4/Sacrifice
Full Penetrance of
HGG phenotype in CNTR set-up

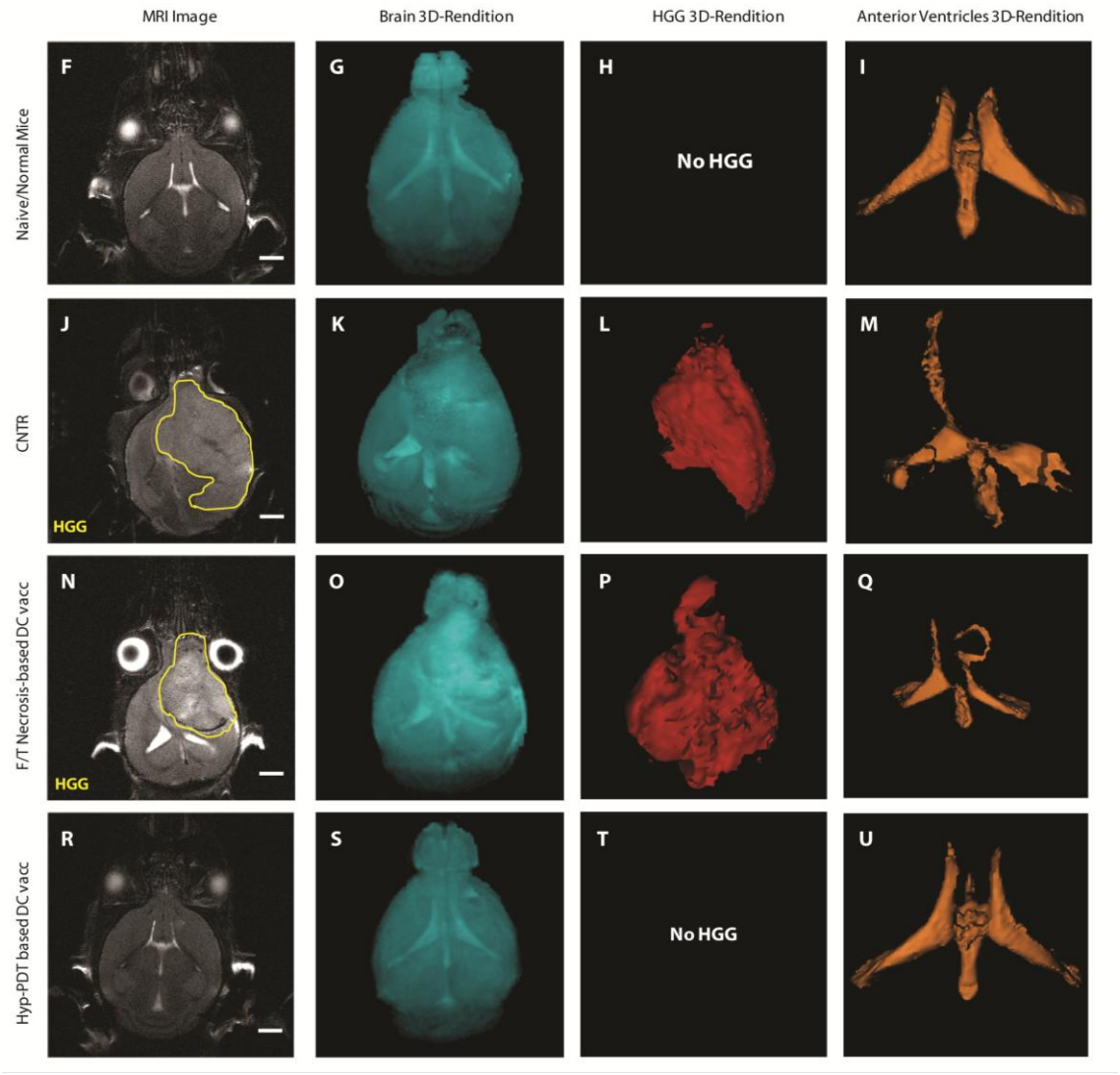


Figure 2. DC vaccines based on Hyp-PDT-induced ICD, elicit highly efficient HGG-rejecting immunity in an orthotopic model. (A) GL261 cells were treated to undergo ICD with Hyp-PDT and recovered 24 h post-PDT; thereafter they were co-incubated with murine DCs (for 24 h) to produce ICD-based DC vaccine (vacc). Alternatively, as applicable, DC vaccines were also produced with GL261 cells exposed to 6 cycles of F/T-based necrosis. Thereafter the vaccinations were carried out in a prophylactic vaccination set-up. **(B)** Kaplan-Meier curve depicts survival of three "cohorts" (CNTR, n=5; F/T Necrosis-based DC vacc, n=7; and Hyp-PDT-based DC vacc, n=7; Log-rank (Mantel-Cox) test; ***p<0.001 vs. CNTR (without bar) and bar indicates comparison between F/T Necrosis and Hyp-PDT-based DC vacc). **(C-E)** Graphs indicate the emergence of progressive HGG-induced neurological deficit symptoms (grade 1-3) before their sacrifice (grade 4) versus normal/healthy mice (grade 0), color-coded based on symptom severity. **(F-U)** Representative MRI-scans are shown for naïve/normal mice (**F**); i.e. mice not exposed to the procedure described in (**A**) and day 29 post-intra-axial GL261-inoculated mice from CNTR **(J)**, F/T Necrosis-based DC vacc **(N)** and Hyp-PDT-based DC vacc **(R)** "cohorts". Subsequently, these MRI-scans were used to create 3D renditions of the whole-brain **(G, K, O, S)**, visible HGG-tumor mass wherever available **(H, L, P, T)** and morphology of the anterior brain ventricles **(I, M, Q, U)**.

Figure 3:

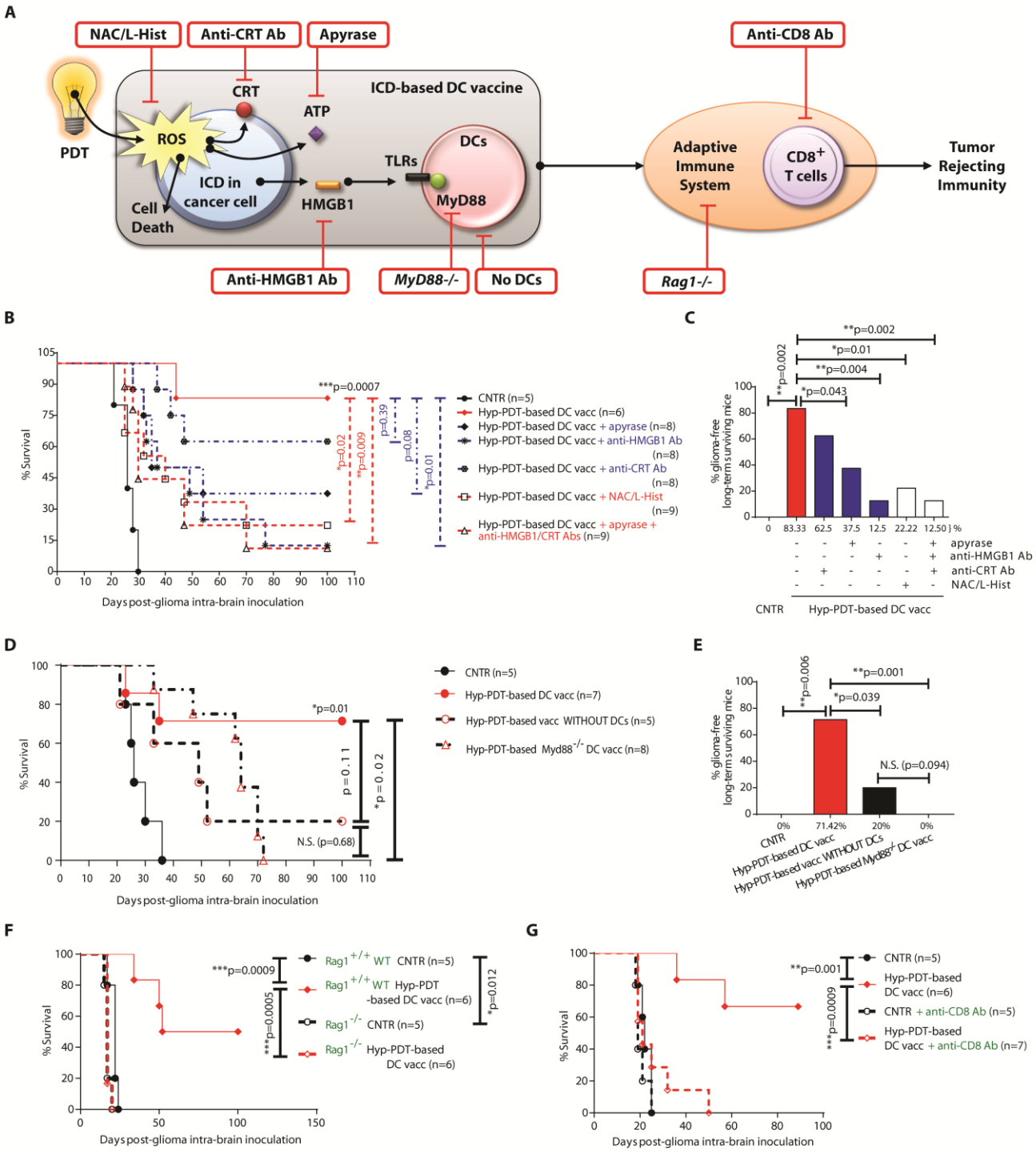

A
NAC/L-Hist
Anti-CRT Ab
Apyrase
Anti-CD8 Ab
PDT
ROS
CRT
ATP
ICD-based DC vaccine
ICD in cancer cell
Cell Death
HMGB1
TLRs
DCs
MyD88
Adaptive Immune System
CD8+ T cells
Tumor Rejecting Immunity
Anti-HMGB1 Ab
MyD88-/-
No DCs
Rag1-/-
B
% Survival
Days post-glioma intra-brain inoculation
***p=0.0007
*p=0.02
**p=0.009
p=0.39
p=0.08
*p=0.01
CNTR (n=5)
Hyp-PDT-based DC vacc (n=6)
Hyp-PDT-based DC vacc + apyrase (n=8)
Hyp-PDT-based DC vacc + anti-HMGB1 Ab (n=8)
Hyp-PDT-based DC vacc + anti-CRT Ab (n=8)
Hyp-PDT-based DC vacc + NAC/L-Hist (n=9)
Hyp-PDT-based DC vacc + apyrase + anti-HMGB1/CRT Abs (n=9)
C
% glioma-free long-term surviving mice
**p=0.002
*p=0.01
**p=0.004
*p=0.043
0 83.33 62.5 37.5 12.5 22.22 12.50 %
apyrase
anti-HMGB1 Ab
anti-CRT Ab
NAC/L-Hist
CNTR
Hyp-PDT-based DC vacc
D
% Survival
Days post-glioma intra-brain inoculation
*p=0.01
p=0.11
*p=0.02
N.S. (p=0.68)
CNTR (n=5)
Hyp-PDT-based DC vacc (n=7)
Hyp-PDT-based vacc WITHOUT DCs (n=5)
Hyp-PDT-based Myd88-/- DC vacc (n=8)
E
% glioma-free long-term surviving mice
**p=0.006
**p=0.001
*p=0.039
N.S. (p=0.094)
0% 71.42% 20% 0%
CNTR
Hyp-PDT-based DC vacc
Hyp-PDT-based vacc WITHOUT DCs
Hyp-PDT-based Myd88-/- DC vacc
F
% Survival
Days post-glioma intra-brain inoculation
***p=0.0009
***p=0.0005
*p=0.012
Rag1+/+ WT CNTR (n=5)
Rag1+/+ WT Hyp-PDT -based DC vacc (n=6)
Rag1-/- CNTR (n=5)
Rag1-/- Hyp-PDT-based DC vacc (n=6)
G
% Survival
Days post-glioma intra-brain inoculation
**p=0.001
***p=0.0009
CNTR (n=5)
Hyp-PDT-based DC vacc (n=6)
CNTR + anti-CD8 Ab (n=5)
Hyp-PDT-based DC vacc + anti-CD8 Ab (n=7)

Figure 3. Blockade of ROS, danger signals, DCs (or DC-associated MyD88), intact adaptive immune system and $CD8^+$ T cells abrogates the ICD-based DC vaccine-induced HGG-rejection. (A) Molecular (ROS, CRT, ATP, and HMGB1) and immunological determinants (DCs, DC-based HMGB1-MyD88 axis, adaptive immunity especially on $CD8^+$ T cells' level) were targeted through chemical, antibody or genetic means as specified below. **(B-G)** Respective vaccines (vacc) were injected twice in C57BL/6 mice. Here, the respective vacc were pre-treated/neutralized by NAC/L-Hist, anti-CRT or anti-HMGB1 antibodies/Abs, apyrase and a combination of the latter three **(B,C)**, or the DCs were either absent or derived from *Myd88*$^{-/-}$ mice **(D,E)** or the mice receiving the vacc were *Rag1*$^{-/-}$ **(F)** or depleted for $CD8^+$ cells **(G)**. Thereafter, the immunized and non-immunized (i.e. CNTR) mice were intra-axially inoculated with live GL261 cells. **(B,C)** Kaplan-Meier curve **(B)** depicts survival of seven "cohorts" (CNTR, n=5; Hyp-PDT based DC vacc, n=6; and Hyp-PDT-based DC vacc combined with - apyrase, n=8; anti-HMGB1 Ab, n=8; anti-CRT Ab, n=8; NAC/L-Hist, n=9; apyrase+anti-HMGB1/CRT Abs, n=9); while % glioma-free long-term surviving mice are depicted in **(C)**. **(D,E)** Kaplan-Meier curve **(D)** depicts survival of four "cohorts" (CNTR, n=5; Hyp-PDT based DC vacc, n=7; and Hyp-PDT-based vacc, without DC, n=5; or with *Myd88*$^{-/-}$ DCs, n=8); while % glioma-free long-term surviving mice are depicted in **(E)**. **(F)** Kaplan-Meier curve depicts survival of four "cohorts" (*Rag1*$^{+/+}$ WT CNTR, n=5; *Rag1*$^{+/+}$ WT Hyp-PDT-based DC vacc, n=6; *Rag1*$^{-/-}$ CNTR, n=5; *Rag1*$^{-/-}$ Hyp-PDT-based DC vacc, n=6). **(G)** Kaplan-Meier curve depicts survival of four "cohorts" (CNTR, n=5; Hyp-PDT-based DC vacc, n=6; CNTR + anti-CD8 Ab, n=5; Hyp-PDT-based DC vacc + anti-CD8 Ab, n=7). In this figure, Log-rank (Mantel-Cox) test **(B, D, F, G)** or Chi-square test **(C, E)** were used for statistics, *p<0.05/**p<0.01/***p<0.001 as indicated by bars or otherwise versus respective CNTRs; N.S.: not significant.

completely failed to resist intra-brain glioma-inoculation despite immunization with ICD-based DC vaccines (Fig 3F). Interestingly, even in CNTR settings, *Rag1*$^{-/-}$ mice (17 days) showed significantly, albeit marginally, reduced median survival as compared to *Rag1*$^{+/+}$ mice (22 days) (Fig 3F), an observation reminiscent of anti-glioma immunosurveillance (45, 53). Next, we depleted the CD8^{+} T cells through anti-CD8 antibodies (Suppl. Fig S5). Depletion of CD8^{+} T cells (but not CD4^{+} T cells) was confirmed in various immunological compartments like peripheral blood and (cervical/distal) lymph nodes (Suppl. Fig S5). Similar to *Rag1*$^{-/-}$ mice, mice depleted of CD8^{+} T cells, also failed to resist intra-brain glioma-inoculation despite immunization with ICD-based DC vaccines (Fig 3G). Of note, vaccinated mice depleted of CD8^{+} T cells (median survival: 21 days) (Fig 3G) survived only marginally better than similarly vaccinated *Rag1*$^{-/-}$ mice (median survival: 17 days) (Fig 3F).

In conclusion, HGG-rejecting immunity elicited by ICD-based DC vaccines is highly dependent upon ROS, danger signals (especially the released HMGB1-MyD88 axis), presence of DCs as adjuvant and a CD8^{+} T cell-driven adaptive immune response.

ICD-based DC vaccines are able to withstand the rigid clinically-relevant operational parameters

Two clinically-relevant operational parameters pose a significant challenge to the immunogenicity of anti-cancer vaccines i.e. avitalization (i.e. absolute absence of living cancer cells) and the possibility of tumor tissue material, to be used for vaccine preparation, being in frozen state (due to the clinical practice of snap-freezing the resected tumor) (54). In order to enable future translation towards the clinic we felt it was necessary to produce a clinically-relevant version of Hyp-PDT induced ICD-based DC vaccine capable of withstanding such rigid operational parameters.

We employed, in combination with Hyp-PDT, several avitalization strategies often applied in clinical vaccine preparation protocols i.e. high-dose radiotherapy (RT), mechanical necrosis (achieved here *via* GentleMACS® system i.e. GM necrosis) and the highly prevalent F/T necrosis. To confirm avitalization, we either estimated the percent cellular survival (Fig 4A) or percent clonogenic survival (Fig 4B). Hyp-PDT alone, Hyp-PDT+GM necrosis, Hyp-PDT+RT and Hyp-PDT+RT+GM necrosis, failed to achieve absolute avitalization (Fig 4A-B). On the other hand, Hyp-PDT+F/T necrosis and Hyp-PDT+RT+F/T necrosis were found to achieve complete avitalization (Fig 4A-B). An analysis in the prophylactic treatment set-up showed that only DC vaccines based on GL261 cells treated with Hyp-PDT+RT, Hyp-PDT+F/T necrosis and Hyp-PDT+RT+F/T necrosis showed survivals that were not significantly different than mice immunized with Hyp-PDT induced ICD-based DC vaccines (Fig 4C). Although it is worth noting that these avitalization strategies did reduce the overall number of long-term surviving mice (Fig 4A). Overall, the most minimal combination that achieved both absolute avitalization (Fig 4A-B) and significant anti-HGG protective immunity *in vivo* (Fig 4C) was Hyp-PDT+F/T necrosis-based DC vaccines; and hence these were used subsequently as a "clinically-relevant version" along with the original biologically-relevant vaccine.

Next, we tested the effect of the start-up cellular material having undergone a cycle of snap-freezing (overnight) and thawing (i.e. 1xF/T cycle, so called "frozen" cells, mimicking the clinical *status quo*) on vaccine immunogenicity. This F/T-step drastically reduced the survival of the residual cancer cells (however did not completely abrogate living cells) (Suppl. Fig S6). Hyp-PDT and Hyp-PDT+F/T-necrosis treatment on these "frozen" cells further reduced the cellular survival gradually towards avitalization (Suppl. Fig S6). Interestingly, for Hyp-PDT+F/T necrosis-based DC vaccine, it did not strongly matter whether the start-up cellular material was live or "frozen", as mice immunized with both vaccines exhibited similarly increased median survivals (live: 48.5 days vs. "frozen": 60 days; as compared to CNTR: 26 days) and long-term survivors (approximately 20-30%, as compared to 0% in CNTR setting) (Fig 4D). On the other hand, for Hyp-PDT alone, the state of the start-up cellular material made a significant difference (Fig 4D).

Taken together, our results show that ICD-based DC vaccines are capable of withstanding the rigid clinically-relevant operational parameters such that Hyp-PDT+F/T necrosis based DC vaccines represent the most clinically-relevant version.

ICD-based DC vaccines induce a prophylactic shift in brain immune-contexture from Tregs-to-Th1/CTLs/Th17 cells

Owing to the highly promising HGG-rejecting immunity induced by ICD-based DC vaccine and its clinically-relevant version, we wondered whether this *in vivo* immunogenicity was associated with an immune stimulatory shift in the brain immune-contexture. To this end, we analyzed the brain-infiltrating immune cells in mice that were prophylactically vaccinated (Hyp-PDT based or Hyp-PDT+F/T necrosis based, DC vaccines) or not (CNTR setting) and inoculated within the brain with

live GL261 glioma cells. Remarkably, we found that GL261 glioma-inoculated mice prophylactically vaccinated with Hyp-PDT or Hyp-PDT+F/T necrosis based DC vaccines, exhibited significant increase (as compared to CNTR mice) in brain infiltration of $CD3^+$ T lymphocytes (Fig 5A), $CD4^+$ T lymphocytes (Fig 5B) and $CD8^+$ T lymphocytes (Fig 5C). Moreover, GL261 glioma-inoculated mice prophylactically vaccinated with the respective ICD-based DC vaccines, but not CNTR mice, showed increased infiltration of Th1 cells i.e. IFN-γ^+CD4^+ T cells (Fig 5D), CTLs i.e. IFN-γ^+CD8^+ T cells (Fig 5E) and Th17 cells i.e. IL-$17A^+CD4^+$ T cells (Fig 5F). Interestingly, whereas CNTR mice showed a higher intra-brain infiltration of Treg cells i.e. $Foxp3^+CD4^+$ T cells, mice vaccinated with respective ICD-based DC vaccines, showed significantly reduced levels of intra-brain Tregs (Fig 5G). The two ICD-based vaccines did not reveal significant differences. However, mice vaccinated with Hyp-PDT+F/T necrosis based DC vaccines showed a trend towards slightly higher Tregs (Fig 5G) and reduced infiltration of Th17 cells compared to mice vaccinated with Hyp-PDT based DC vaccines (Fig 5F).

Next, it was imperative to confirm whether the immune stimulation observed above was only a brain-localized reaction or whether the immune stimulation was visible on a systemic level, the latter being crucial for long term immunity. We then analyzed whether mice splenocytic (SPL) T cells have the ability to exhibit "immune memory" responses upon antigen re-exposure. Hence SPL T cells were isolated from GL261 glioma-inoculated CNTR mice or mice subjected to prophylactic vaccination with respective ICD-based DC vaccines (Suppl. Fig S7). These SPL T cells were then re-stimulated with naïve (i.e. untreated) GL261 lysates. We observed that SPL T cells isolated from respective vaccinated mice showed significantly higher IFN-γ production upon re-stimulation as compared to CNTR mice (Fig 5H).

In conclusion, GL261-inoculated mice prophylactically vaccinated with ICD-based DC vaccines show a shift in brain contexture towards Th1/CTLs/Th17 cells and away from Tregs and display systemic anti-tumor immunity.

ICD-based DC vaccines in combination with chemotherapy exhibit the ability to cure HGG-bearing mice

Prophylaxis strategy of vaccination, although critical for mechanistic analysis of immunogenicity, does not represent the clinical *status quo* where vaccination is given in a curative setup (often in combination with standard-of-care chemotherapeutics like TMZ) (1, 5, 55). We then decided to test the immunogenicity of ICD-based DC vaccines in a curative set-up, either alone or in sequential combination with prior TMZ-based chemotherapy. Here, mice were first inoculated orthotopically with live GL261 cells, followed by their random division into 6 "cohorts" as detailed in the Fig 6A. Of note, TMZ did not induce any overt signs of toxicity, as evident by maintenance of a healthy weight by the treated mice (Suppl. Fig S8). In this highly clinically-relevant experimental set-up, we observed that both versions of the ICD-based DC vaccines (i.e. Hyp-PDT and Hyp-PDT+F/T) alone, increased the median survival of glioma-inoculated mice by approximately 34-41% (albeit statistically insignificantly), compared to CNTR mice (Fig 6B-C); but no long-term "cured" survivors were observed (Fig 6C). This reflects the well-known aggressiveness of this tumor model (56). On the other hand, TMZ-based chemotherapy alone significantly increased the median survival of glioma-inoculated mice by approximately 95% (Fig 6B-C) but failed to produce long-term "cured" survivors (Fig 6C). Remarkably, the sequential combination of TMZ treatment followed by immunization with the two respective ICD-based DC vaccines, not only strongly increased the median survival of glioma-inoculated mice by approximately 302-306% (Fig 6B-C) but also produced approximately 50% of long-term "cured" survivors (Fig 6C). These remarkable results were also confirmed by MRI based non-invasive analysis of the brain (Fig 6D-K). At day 23, whereas the CNTR mice showed large intra-brain glioma/HGG masses (Fig 6D) mice that received vaccination only (Fig 6E-F) or TMZ only (Fig 6G) exhibited relatively smaller but palpable glioma/HGG masses. On the other hand, MRI analysis of long-term "cured" survivors belonging to the "TMZ+vaccination" cohorts (Fig 6H-K), showed complete absence of any palpable glioma masses on days 55 (Fig 6H, J) and 90 (Fig 6I, K) after glioma-inoculation. Further analysis using the equations established by Robert Clarke (57) revealed that TMZ and the respective ICD-based DC vaccines synergized with each other to achieve this remarkable anti-glioma/HGG efficacy (Suppl. Fig 9). Notably, the long-term "cured" survivors from the respective "TMZ+vaccination" cohorts managed to survive significantly better (than age-matched CNTR mice) following a further re-challenge with intra-axial glioma/GL261 inoculation (Suppl. Fig 10). This observation proves the long-term anti-HGG immune memory induced by ICD-based DC vaccines.

In conclusion, a combination of chemotherapy and ICD-based DC vaccines provides remarkable survival benefit against HGG and produces long-term "cured" survivors with palpable anti-HGG immune memory.

Figure 4:

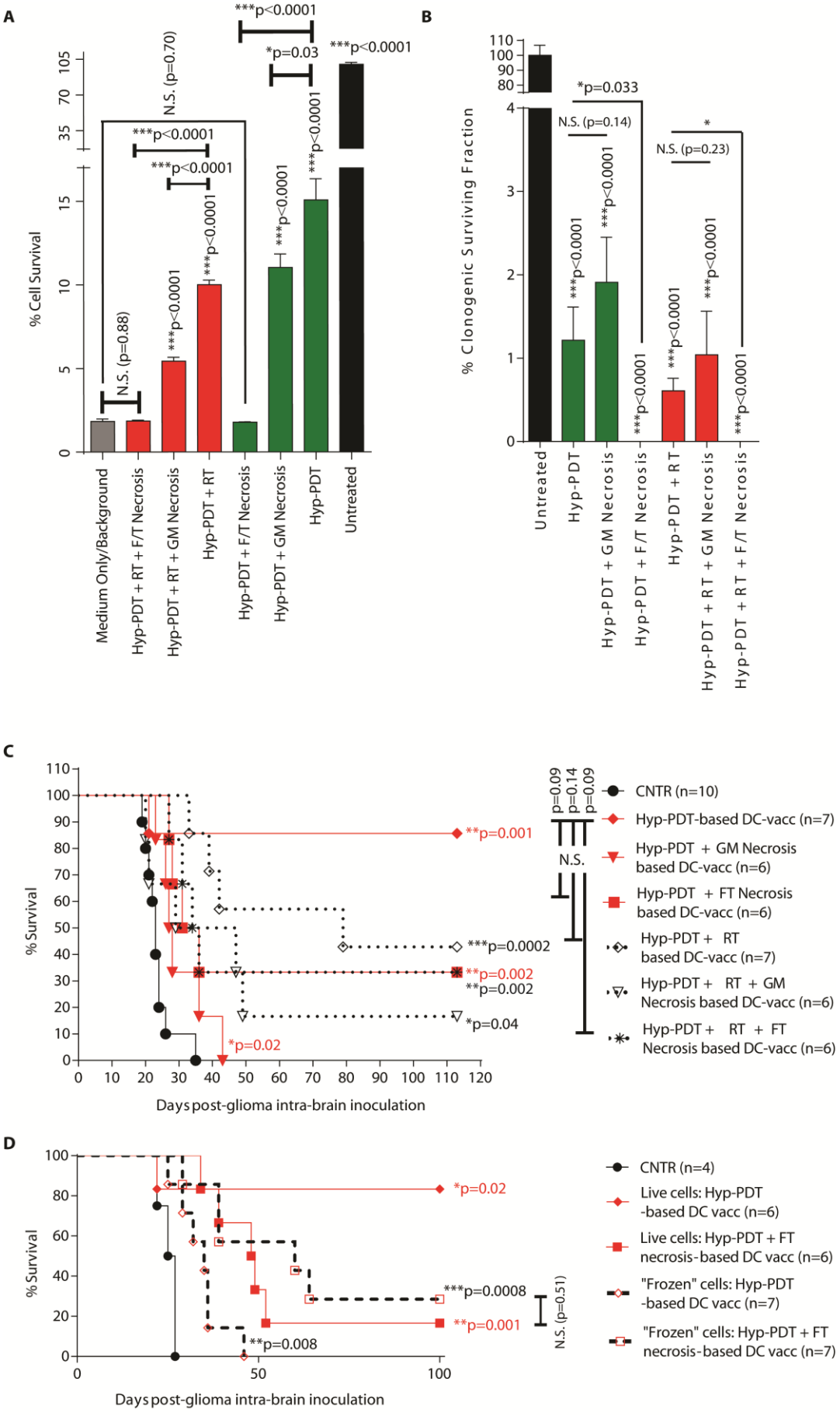

A
% Cell Survival
N.S. (p=0.88)
N.S. (p=0.70)
***p<0.0001
*p=0.03
Medium Only/Background
Hyp-PDT + RT + F/T Necrosis
Hyp-PDT + RT + GM Necrosis
Hyp-PDT + RT
Hyp-PDT + F/T Necrosis
Hyp-PDT + GM Necrosis
Hyp-PDT
Untreated
B
% Clonogenic Surviving Fraction
*p=0.033
N.S. (p=0.14)
N.S. (p=0.23)
Untreated
Hyp-PDT
Hyp-PDT + GM Necrosis
Hyp-PDT + F/T Necrosis
Hyp-PDT + RT
Hyp-PDT + RT + GM Necrosis
Hyp-PDT + RT + F/T Necrosis
C
% Survival
Days post-glioma intra-brain inoculation
**p=0.001
***p=0.0002
**p=0.002
**p=0.002
*p=0.04
*p=0.02
p=0.09
p=0.14
p=0.09
N.S.
CNTR (n=10)
Hyp-PDT-based DC-vacc (n=7)
Hyp-PDT + GM Necrosis based DC-vacc (n=6)
Hyp-PDT + FT Necrosis based DC-vacc (n=6)
Hyp-PDT + RT based DC-vacc (n=7)
Hyp-PDT + RT + GM Necrosis based DC-vacc (n=6)
Hyp-PDT + RT + FT Necrosis based DC-vacc (n=6)
D
% Survival
Days post-glioma intra-brain inoculation
*p=0.02
***p=0.0008
**p=0.001
**p=0.008
N.S. (p=0.51)
CNTR (n=4)
Live cells: Hyp-PDT -based DC vacc (n=6)
Live cells: Hyp-PDT + FT necrosis-based DC vacc (n=6)
"Frozen" cells: Hyp-PDT -based DC vacc (n=7)
"Frozen" cells: Hyp-PDT + FT necrosis-based DC vacc (n=7)

Figure 4. DC vaccines based on Hyp-PDT induced ICD plus F/T-necrosis, represent the most minimal combination that achieves absolute avitality coupled with efficient HGG-rejecting immunity. (A,B) GL261 cells were either left untreated or treated to undergo ICD *in vitro* with Hyp-PDT; thereafter they were exposed to various avitalization (i.e. live cell-eliminating) methodologies i.e. F/T-necrosis, GentleMACS® (GM)-necrosis, high-dose radiotherapy (RT, 60 Gy), RT+F/T-necrosis and RT+GM-necrosis. This was followed by either - **(A)** determination of percentage cellular viability through MTS assay, 10 days post-treatment (n=3; mean±s.e.m.) or **(B)** clonogenic assay after 10 days of growth (n=3; mean±s.d.). **(C)** Respective vaccines (vacc; +/- above avitalizations) were injected twice in C57BL/6 mice followed by intra-axial inoculation with live GL261 cells. Kaplan-Meier curve depicts survival of seven "cohorts" (CNTR, n=10; Hyp-PDT-based DC vacc, n=7; Hyp-PDT+GM-Necrosis-based DC vacc, n=6; Hyp-PDT+F/T-Necrosis-based DC vacc, n=6; Hyp-PDT+RT-based DC vacc, n=7; Hyp-PDT+RT+GM Necrosis-based DC vacc, n=6; Hyp-PDT+RT+F/T-Necrosis-based DC vacc, n=6). **(D)** Frozen and then thawed ("Frozen" cells) or live GL261 (Live cells) were treated with Hyp-PDT or Hyp-PDT+F/T-Necrosis as described above. DC vaccines based on these conditions were then injected in the orthotopic HGG mice model. Kaplan-Meier curve depicts survival of five "cohorts" (CNTR, n=4; Live cells: Hyp-PDT-based DC vacc, n=6; Live cells: Hyp-PDT+F/T-Necrosis-based DC vacc, n=6; "Frozen" cells: Hyp-PDT-based DC vacc, n=7; "Frozen" cells: Hyp-PDT+F/T-Necrosis-based DC vacc, n=7). In **A**,**B**, Student's t-test while in **C,D**, Log-rank (Mantel-Cox) test were used for statistics; *p<0.05/**p<0.01/***p<0.001 as indicated by bars or otherwise versus respective CNTRs/medium only/background; N.S. – not significant.

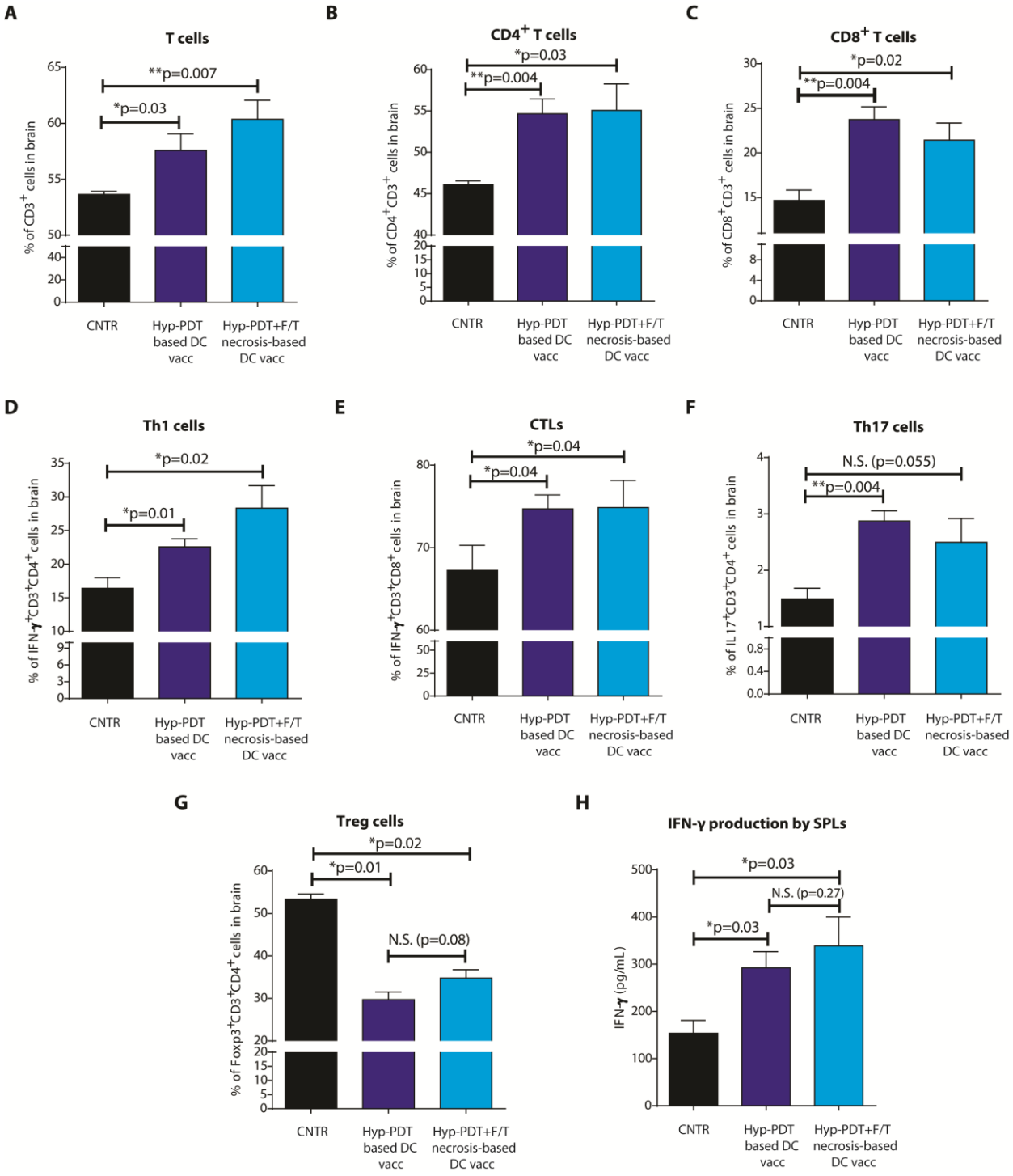
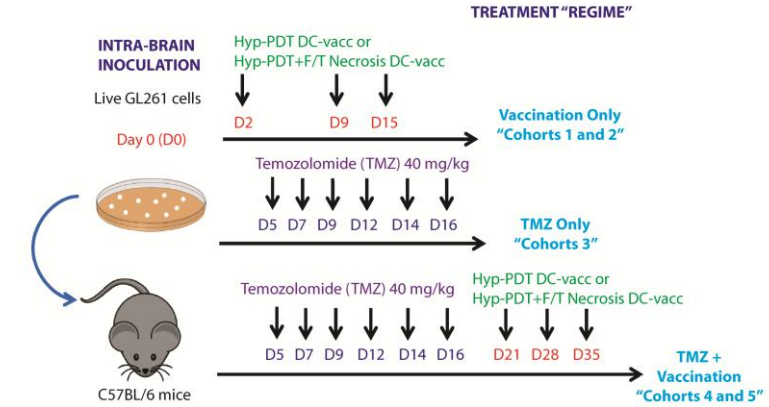


Figure 5. ICD-based DC vaccines cause increased brain-infiltration of Th1/Th17/CTLs and decreased infiltration of Tregs. Respective ICD-based DC vaccines (vacc) were injected in a prophylactic orthotopic HGG murine model. Thereafter the mice were sacrificed at day 16 post intra-axial GL261-inoculation. Subsequently, the brains from the respective mice were isolated and processed for FACS-based immunophenotyping analysis for **(A)** T cells ($CD3^+$ cells), **(B)** $CD4^+$ T cells ($CD4^+CD3^+$ cells), **(C)** $CD8^+$ T cells ($CD8^+CD3^+$ cells), **(D)** Th1 cells (IFN-γ intracellular staining in $CD4^+CD3^+$cells), **(E)** cytotoxic T cells/CTLs (IFN-γ intracellular staining in $CD8^+CD3^+$ cells), **(F)** Th17 cells (IL17A intracellular staining in $CD4^+CD3^+$ cells) and **(G)** Treg cells (Foxp3 intracellular staining in $CD4^+CD3^+$ cells). In **A-G**, n=4-6; mean±s.e.m. At the same time, spleens **(H)** were recovered from the above mice (enriched for T cells). Thereafter, T cell-enriched splenocytes were co-incubated with DCs (DC:T cells ratio of 1:5) pulsed with "naïve" lysates of GL261 cells (prepared via GentleMACS® based mechanical disruption) for 5 days followed by estimation of IFN-γ production in respective supernatants (n=4-5; mean±s.e.m.). In this figure, Mann-Whitney statistical test was used; *p<0.05/**p<0.01/***p<0.001 as indicated by bars or otherwise versus respective CNTRs; N.S. – not significant.

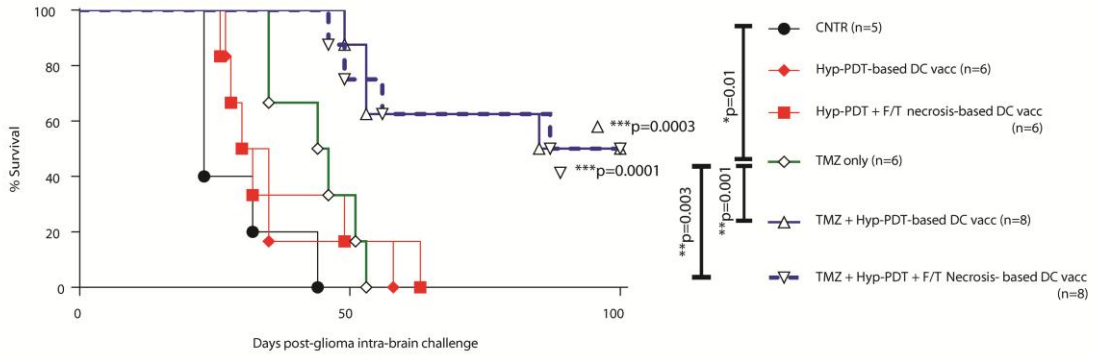
A



B

"COHORTS"	MEDIAN SURVIVAL (DAYS)	% CHANGE
CNTR	23	-
Hyp-PDT based DC vacc	32.5	41.3%
Hyp-PDT + F/T Necrosis DC vacc	31	34.78%
TMZ only	45	95.65%
TMZ + Hyp-PDT based DC vacc	92.5	302.17%
TMZ + Hyp-PDT + F/T Necrosis based DC vacc	93.5	306.52%

C



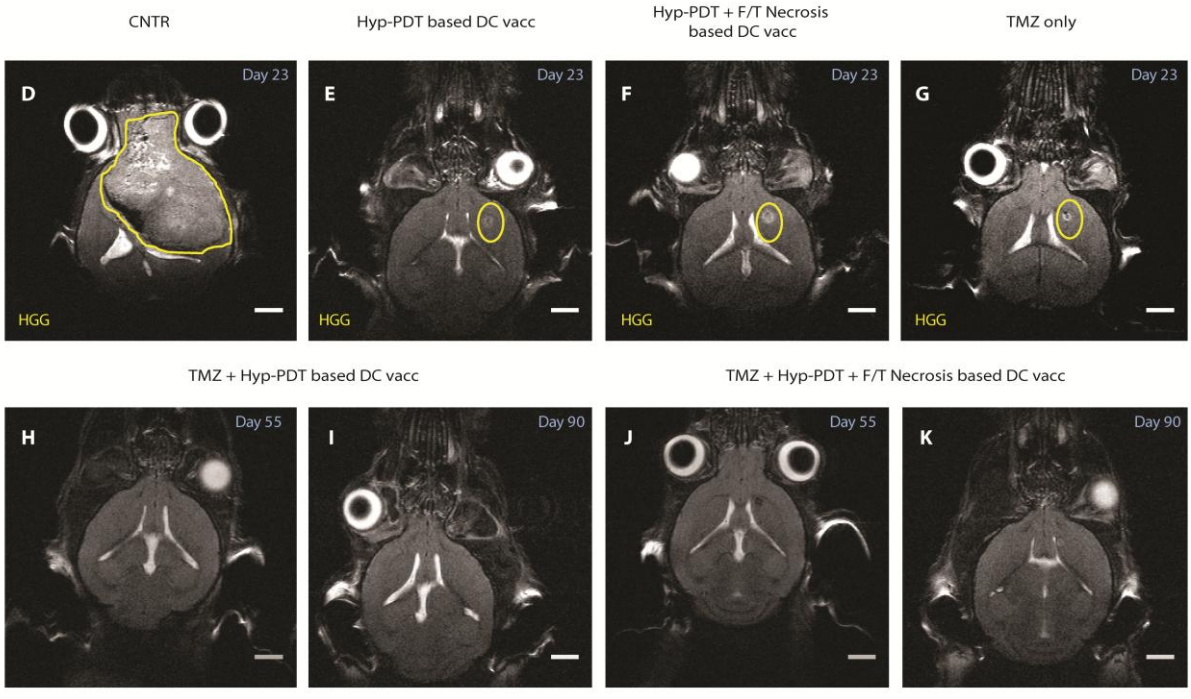


Figure 6. DC vaccines based on Hyp-PDT induced ICD in combination with the, chemotherapeutic drug, TMZ, provides very strong survival benefit in a curative or therapeutic HGG set-up. (A) Firstly, C57BL/6 mice were inoculated with live GL261 cells (Day 0), intra-axially and randomly divided into 6 "cohorts". Respective ICD-based DC vaccines (vacc) and TMZ were administered (40 mg/kg) as depicted. **(B,C)** Median Survival data and Percentage Change (%Δ) in median survival (Δ in median survival from CNTR group to treatment groups divided by CNTR group median survival and multiplied by 100) **(B)** and Kaplan-Meier curve **(C)** depict survival of six "cohorts"; Log-rank (Mantel-Cox) test; *p<0.05/**p<0.01/***p<0.001 vs. CNTR (without bar) and others are indicated by bars). **(D-K)** Representative MRI scans are shown for day 23 post-intra-axial GL261-inoculated mice; CNTR **(D)**, Hyp-PDT-based DC vacc only **(E)**, Hyp-PDT+F/T-Necrosis-based DC vacc **(F)** and TMZ only **(G)** "cohorts". Scans are also shown for day 55 **(H, J)** and day 90 **(I, K)** post-intra-axial GL261-inoculated mice from TMZ+Hyp-PDT-based DC vacc **(H-I)** and TMZ+Hyp-PDT+F/T-Necrosis-based DC vacc **(J,K)** "cohorts".

ICD-based DC vaccines partially overcome the immune-ablating effects of chemotherapy and favor a shift in brain immune-contexture from Tregs to Th1/CTLs/Th17 cells in curative settings

TMZ (at medium-to-high doses) has been traditionally found to exert immune-ablative effects on mononuclear immune cells leading to myelosuppression and/or lymphopenia (58, 59). Thus, it was imperative to confirm the effects of TMZ alone or in combination with vaccines on brain immune-contexture. In line with previous observations, TMZ treatment indeed severely reduced the absolute levels of intra-brain mononuclear immune cells (Fig 7A). However, combined treatment with respective ICD-based DC vaccines significantly rescued the TMZ immune-ablating effect (Fig 7A). On the lymphocytic level, TMZ treatment reduced the overall levels of intra-brain T cells (Fig 7B); however these lympho-ablative effects of TMZ were not targeted towards $CD4^+$ T cells (Fig 7C) but rather towards $CD8^+$ T cells (Fig 7D). TMZ-mediated intra-brain ablation of $CD8^+$ T cells was so severe that the vaccines could not significantly rescue it (Fig 7D). Notably, the combination of TMZ with ICD-based DC vaccines was still able to significantly reduce intra-brain levels of immunosuppressive Treg cells (Fig 7E) whereas TMZ alone failed to do so (Fig 7E). Considering the skewing of the baseline levels of intra-brain mononuclear cells or lymphocytes by TMZ treatment alone, we decided to further analyze the shift in brain immune-contexture by estimating immune cell ratios (60) of immunostimulatory T cells to Tregs (i.e. Th1/Tregs, CTLs/Tregs and Th17/Tregs) (61, 62). TMZ alone failed to increase the levels of Th1 (Fig 7F), CTLs (Fig 7G) or Th17 (Fig 7H) cells relative to Tregs in the brain. However, combining the respective vaccines with TMZ caused a significant increase in the Th1/Treg (Fig 7F), CTLs/Treg (Fig 7G) and Th17/Treg (Fig 7H) ratios.

In conclusion, in the curative settings, TMZ-induced immune-ablative effects are partially overcome by the combination with ICD-based DC vaccines, which are capable of eliciting a Treg to Th1/CTLs/Th17 shift in the brain immune-contexture.

Increased prevalence of Th1/CTLs/Th17 cells-associated genetic signatures, but not Tregs, correlates with good prognosis in glioblastoma patients

An immunostimulatory tumoral immune-contexture associates with improved prognosis in cancer patients (60). Our observations that Th1/CTLs/Th17 intra-brain infiltrates induced by ICD-based DC vaccines associate with improved survival of HGG-challenged mice, created an important precedence for future clinical translation. While clinical translation of these vaccines is beyond the scope of the current study, we were interested to ascertain the prognostic impact of Th1/CTLs/Th17-based immune-contexture in HGG/glioblastoma (GBM) patients. Two technologies can be utilized to analyze this i.e. the "classical" direct immunophenotyping of tumor-infiltrating lymphocytes or the more recently emerging method of utilizing (pre-established) lymphocyte subpopulation-specific mRNA signatures within the tumor (63-65). In the current study we utilized the latter, since this allowed us to analyze a very large, standardized and publicly-available cohort of >500 GBM patients (i.e. The Cancer Genome Atlas/TCGA-cohort of 541 newly diagnosed GBM patients) (66, 67). With the former technology such a large-scale analysis would be practically unfeasible (63).

Here, broad lymphocyte subtype-specific mRNA signatures available from previous studies (63-65) were further tailored for GBM by delineating groups of genes within these signatures showing strong co-expression (thereby acting as 'metagenes' (68)) centered on standard/specific T cell markers (64). Through this methodology (63, 64), we produced GBM-derived gene co-expression profiles (based on Pearson's correlation coefficients) of different T cell subpopulation-associated mRNA signatures (Suppl. Fig 11A-B, Fig 8A-D). We then delineated a collection of GBM-tailored, T cell subtype-associated metagenes (centered on specific markers in case of high co-expressional heterogeneity) e.g. $CD4^+$ T cells (uncentered; Suppl. Fig 11A), $CD8^+$ T cells (*CD8A*/*CD8B*-centered; Suppl. Fig 11B), Treg cells (*CD3D/CD3G/CD2*-centered; Fig 8A), Th1 cells (*IFNG*-centered; Fig 8B), CTLs (*CD8B*-centered; Fig 8C) and Th17 cells (*IL17A/RORC*-centered; Fig 8D).

Next, we estimated the prognostic impact of these metagenes' differential expression on patient overall survival (OS) in the TCGA GBM-cohort. This was further complemented with calculation of percent-change (%Δ) in median survival (MS) or %ΔMS between the high and low expression groups i.e. $((MS^{high} - MS^{low})/MS^{high}) \times 100$, as described previously (5). High expression of $CD4^+$ T cell-metagene associated with slightly reduced OS (Suppl. Fig 11C), as evident from negative %ΔMS of -8.37% and a hazard ratio (HR) of more than 1. However further analysis of $CD4^+$ T cell subtypes showed that while high expression of Treg-metagene associated with reduced OS (Fig 8E) (%ΔMS of -7.36%) yet high expression of Th1-associated metagene correlated with prolonged OS (Fig 8F) (%ΔMS of +8.73%). On the other

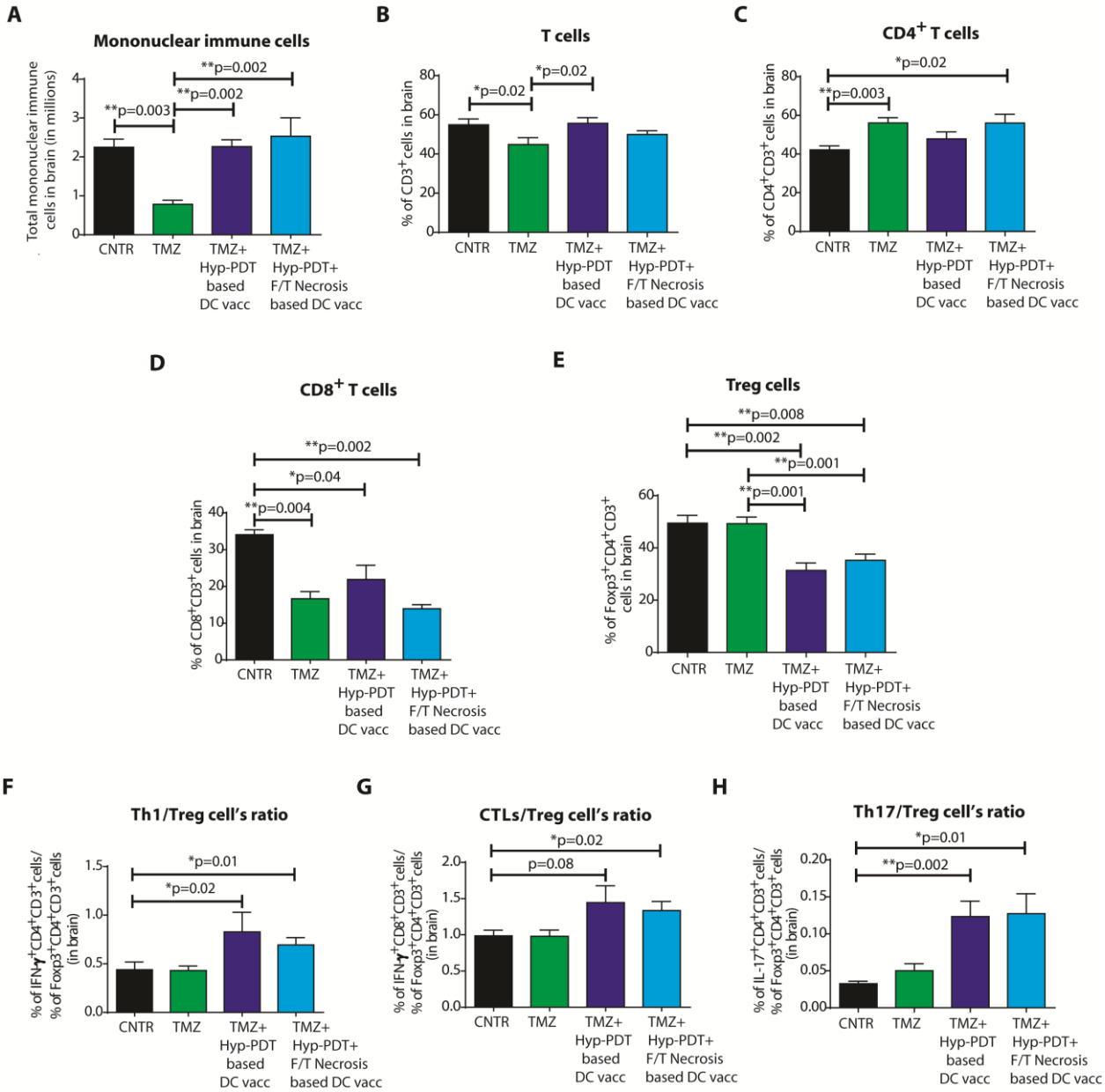
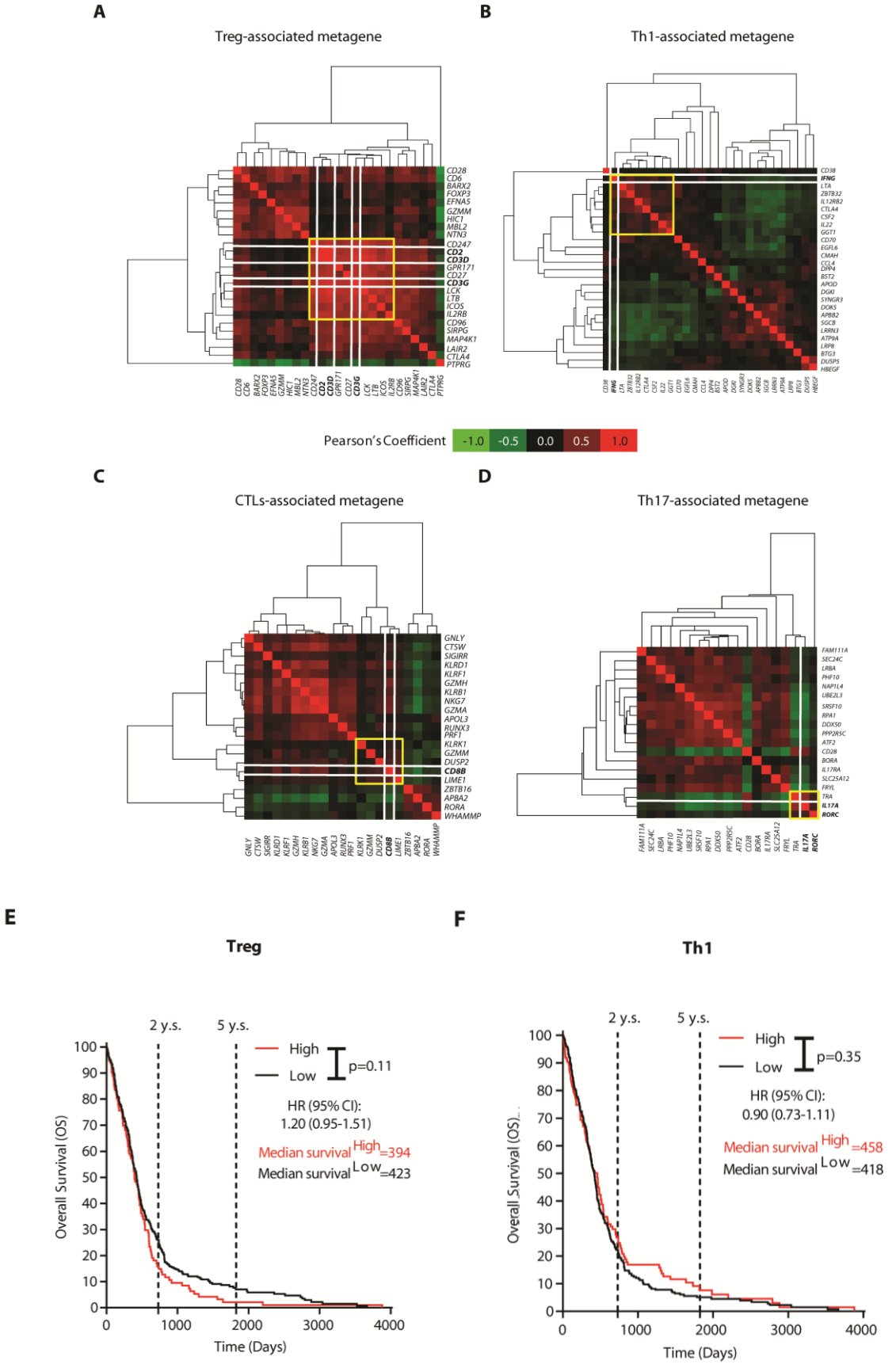


Figure 7. ICD-based DC vaccines cause a Treg-to-Th1/Th17/CTLs shift in brain-immunofiltrates after TMZ-based HGG chemotherapy treatment. Firstly, C57BL/6 mice were inoculated with live GL261 cells (Day 0), intra-axially and randomly divided into 4 "cohorts". Thereafter a cohort was left untreated (CNTR); a cohort received 6 cycles of TMZ only (40 mg/kg; Fig 6A) and other two received TMZ in sequential combination with respective ICD-based DC vaccines (vacc), as depicted in Fig 6A. Thereafter the mice were sacrificed within 10-20 days following end of respective treatment/intra-axial challenge, as applicable. Subsequently, the brains from the respective mice were isolated. Initially, **(A)**, total mononuclear immune cells were counted. Thereafter these were processed for FACS-based immunophenotyping analysis for **(B)** T cells ($CD3^+$ cells), **(C)** $CD4^+$ T cells ($CD4^+CD3^+$ cells), **(D)** $CD8^+$ T cells ($CD8^+CD3^+$ cells), **(E)** Treg cells (Foxp3 intracellular staining in $CD4^+CD3^+$ cells), **(F)** Th1-to-Treg cells ratio, **(G)** cytotoxic T cells/CTLs-to-Treg cells ratio, and **(H)** Th17 to Treg cells ratio. In this figure, n=5-6, mean±s.e.m., Mann-Whitney statistical test was used; *p<0.05/**p<0.01/***p<0.001 as indicated by bars.

Figure 8:


A
Treg-associated metagene
B
Th1-associated metagene
Pearson's Coefficient
-1.0
-0.5
0.0
0.5
1.0
C
CTLs-associated metagene
D
Th17-associated metagene
E
Treg
2 y.s.
5 y.s.
High
Low
p=0.11
HR (95% CI):
1.20 (0.95-1.51)
Median survival High=394
Median survival Low=423
Overall Survival (OS)
Time (Days)
F
Th1
2 y.s.
5 y.s.
High
Low
p=0.35
HR (95% CI):
0.90 (0.73-1.11)
Median survival High=458
Median survival Low=418
Overall Survival (OS)
Time (Days)

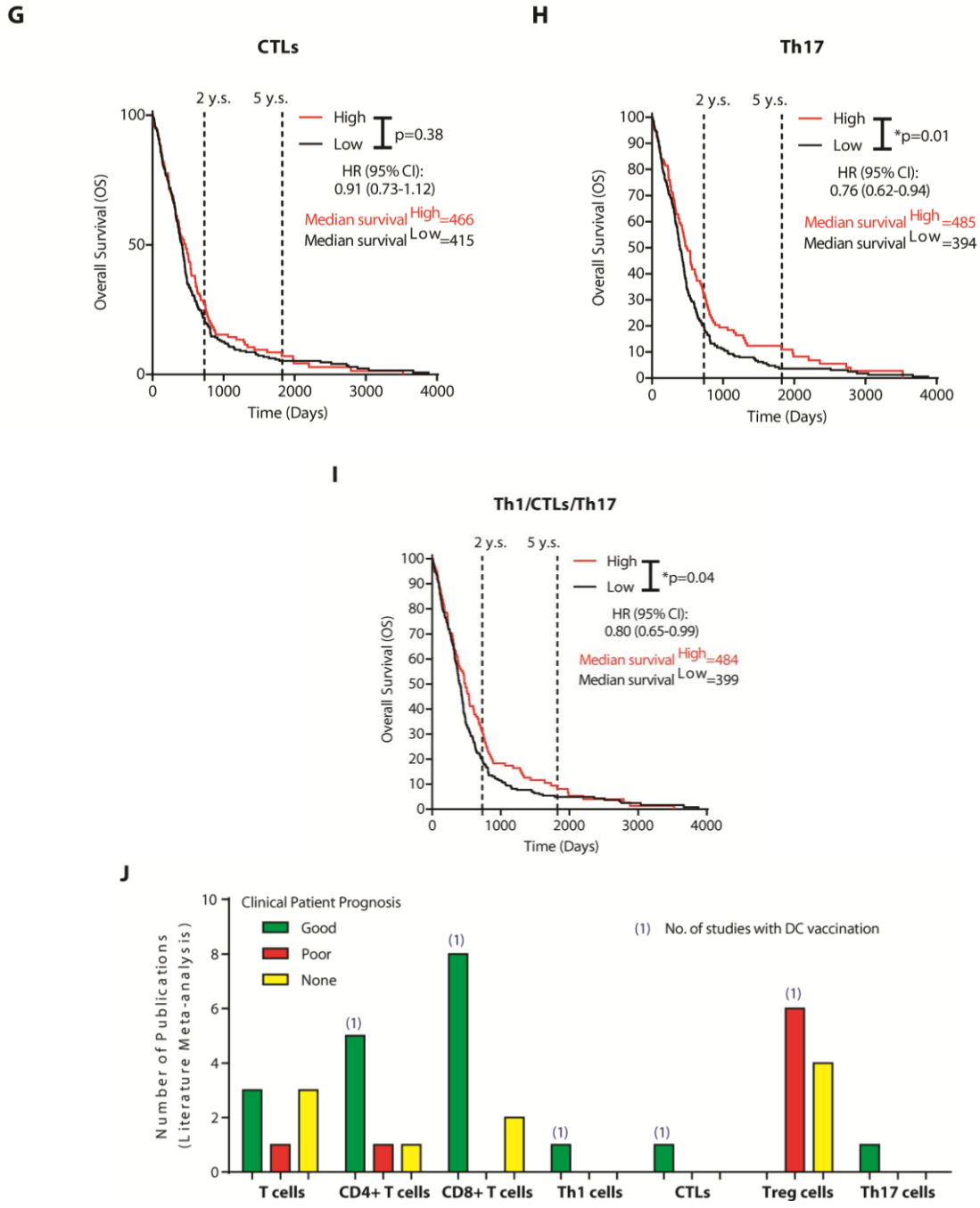


Figure 8. Increased tumoral expression of Th1/CTLs/Th17-associated metagenes, but not Treg-associated metagene, associate with prolonged overall survival in glioblastoma (GBM) patients. (A-D) Metagenes associated with specific T cell-types were established by generating gene co-expression profiles (from TCGA GBM patient data-sets, n=541) for independently established T cells-associated gene patterns (214, 215). Pearson's correlation coefficient (indicated in color-code legend) was used to analyze tendency of gene co-expression; and metagenes were characterized by centering the profiles (indicated by yellow line delineation) to specific T cell markers e.g. *CD3D/CD3G/CD2* (for Treg cells, **A**), *IFNG* (for Th1 cells, **B**), *CD8B* (for CTLs, **C**) and *IL17A/RORC* (for Th17 cells, **D**). **(E-I)** Publicly-available TCGA cohort of GBM patients (n=541) was stratified (at 75th percentile of gene expression) into "high expression" (red; n=136) or "low expression" (black; n=405) groups for each metagene; followed by Kaplan-Meier plotting of patient's overall survival (OS; Y-axis) versus follow-up duration in days (X-axis). This was done for metagene expressions associated with Treg (**E**), Th1 (**F**), CTLs (**G**), Th17 (**H**) and, combination of Th1/CTLs/Th17 (**I**). In all **E-I** graphs, respective log-rank (Mantel-Cox) test p-values and hazard ratios (HR; with its 95% confidence interval in parenthesis) are displayed. Two dotted lines represent approximate point of 2 and 5 year survival (y.s.) windows. **(J)** The analysis of 20 published articles studying the impact of T cell immune-infiltrates on the prognosis of glioma/GBM patients (Suppl. Table S1) is represented. 'Good' refers to increased infiltrates associating with favourable patient prognosis, 'Poor' refers to increased infiltrates associating with poor patient prognosis and 'None' means lack of conclusive correlation. Enlargements of figures A-D are shown in supplemental figure S12.

hand, high expression of $CD8^+$ T cell-metagene associated with slightly prolonged OS (Suppl. Fig 11D) (%ΔMS of +4.47%), a positive prognostic impact that improved further on CTLs-level. Indeed, high CTLs-metagene expression associated with more prolonged OS (Fig 8G) (%ΔMS of +10.94%). Although the above survival trends were not significant, interestingly, high expression of Th17-metagene alone (Fig 8I) (%ΔMS of +18.76%) or combined high expression of Th1/CTLs/Th17-metagenes (Fig 8H) (%ΔMS of +17.56%), associated with significant prolongation of OS. It is noteworthy that, in general, GBM patients showing more than 2 years of survival (2 y.s.; especially between 2 and 5 y.s. window) showed the highest positive/negative impact of respective T cell-associated metagenes.

Next, we decided to substantiate the above results with a literature meta-analysis of papers employing direct immunophenotyping of tumor-infiltrating lymphocytes in GBM/HGG tumor tissue (Suppl. Table S1), as described previously (60). This meta-analysis revealed that, high tumor-infiltration of $CD4^+$ T cells and $CD8^+$ T cells associated with good prognosis in HGG/GBM patients (Fig 8J). Similar trend was implied for GBM/HGG-infiltration of Th1, CTLs and Th17 cells, albeit covered by fewer studies (Fig 8J). On the other hand, high tumor-infiltration of Tregs associated with poor prognosis (Fig 8J).

In conclusion, our TCGA- and literature meta-analysis revealed that increased prevalence of molecular signatures corresponding to Th1/CTLs/Th17 cells, but not Tregs, associate with good prognosis in GBM/HGG patients.

Discussion

To the best of our knowledge, this is the first translational study combining a single-agent ICD inducer with DC immunotherapy against a vital organ-associated cancer in a preclinical, orthotopic model. Previously, ICD-based vaccines have only been applied in either heterotopic subcutaneous cancer models (49, 69) or in an orthotopic model of non-vital organ-associated cancer (e.g. shikonin against breast cancer (70)). This is also the first study to, on one hand, test the efficacy of ICD-based DC immunotherapy against a highly immunosuppressive HGG; and, on the other hand, directly compare the immunogenic impact of four different ICD-essential molecular determinants (ROS, CRT, ATP, HMGB1) in an orthotopic tumor model (45, 71). This study also adds to the significant anti-tumor immunity inducing capabilities demonstrated for Hyp-PDT, in other cancer models (27, 29, 31). Considering the previous application of Hyp-PDT for clinical cancer treatment (27, 72) and of Hypericin for clinical fluorescence-guided HGG resection (73), Hyp-PDT based anti-cancer vaccines can be translated towards the clinic in near future (pending final preclinical checks pertaining to safety and regulatory parameters). We currently envisage undertaking this step for HGG.

Our results confirm, that Hyp-PDT induced ICD-based DC vaccines have a high potency both in biologically- and clinically-relevant forms. The latter form integrated both absolute cellular avitalization and the possibility of treating “frozen” tumor material. These results are of general translational interest, because the rigid clinically-relevant operational parameters (if not accounted for, in early stages) may lead to failure of anti-cancer vaccine in later stages of development. Such stiff parameters have facilitated usage of the low-immunogenic but operational parameters-satisfying procedure of F/T necrosis for cell death preparations (5, 7, 22, 29, 31). The current study shows that sequential combination of Hyp-PDT treatment and F/T necrosis can achieve a productive balance between preserving immunogenicity (to a certain degree) and withstanding the negative impact of stringent operational parameters.

Remarkably, in prophylactic/curative set-ups, both biologically and clinically-relevant versions of the ICD-based DC vaccines provided considerable survival benefit against HGG. Especially in the latter case, in combination with the standard-of-care chemotherapeutic TMZ, both ICD-based DC vaccines synergized to increase the median survival by more than 300% relative to CNTR. The combination with TMZ-based chemotherapy was essential to provide strong survival benefit since the vaccines alone only increased median survival by 34-40%. This phenomenon has been described previously i.e. DC immunotherapies tend to function sub-optimally in the presence of pre-existing tumors, owing to the high tumor-induced immune-suppression (5, 74). Also clinically, an inverse association has been observed between tumor burden and immunotherapy effectiveness (74, 75). These observations have paved the way for combining DC immunotherapy with cyto/tumor-reductive anti-cancer chemotherapies like TMZ (5) – a rationale that we successfully applied in this study. It has also been postulated that chemotherapy preceding DC immunotherapy can make tumor cells more susceptible to immunotherapy (7, 76, 77) thereby providing synergistic survival benefit (78).

Interestingly, although in the curative set-up there was no significant difference between the efficacies of Hyp-PDT-based and Hyp-PDT+F/T necrosis-based DC vaccines yet in prophylactic set-up, there was a distinct difference in the % long-term survivors between these vaccines. Such differences could originate from the nature of prophylactic vaccination. While it is widely recognized that prophylactic vaccination model is an adequate approach for the experimental testing of a vaccine's *in vivo* immunogenicity and the underlying mechanisms (25, 29, 55, 79, 80) yet when it comes to clinical relevance it has many disadvantages e.g. the absence of a pre-established tumor-burden based immune-suppression (55), possible occurrence of split immunity (81) and absence of systemic tolerogenicity towards overexpressed cancer antigens (55, 79, 80). Moreover, it should be also taken into consideration that pre-established tumors (and/or its pre-treatment with chemotherapy) can change immunological responses induced by DC immunotherapy – a phenomenon that has also been reported in the clinic (5, 7, 76). Although our DC immunotherapies show potency in both prophylactic and curative set-up, the latter is much more important and relevant.

On the molecular level, our study show that ROS and danger signals (extracellular HMGB1>extracellular ATP>surface-CRT) are major cancer cell-autonomous determinants of vaccine's immunogenicity. Clearly in case of Hyp-PDT or PDT in general, ROS generation is the most apical signaling event triggering cell death associated signals crucial for ICD, including ER stress and exposure/release of some danger signals (surface-CRT/secreted-ATP) (27, 28, 31, 38). The immunogenic role of ROS may not be limited to PDT but might have very broad implications for all anti-cancer therapies operating through oxidative stress (40). Beyond ROS, concurrent blockade of all three ICD-relevant danger signals, almost completely ablated the median survival advantage provided by the vaccine. Thus, our studies show that these three danger signals account for most of the ICD's *in vivo* immunogenic potential in this set-up and further outline the predominant immunogenic role played by the HMGB1-MyD88 axis for generating a productive interface between dying cancer cells and DC. These results also outline that depending on the vaccination or therapeutic set-ups the immunogenic potential of one, or a few, DAMP may prevail on the others (49). It is possible that, in our DC- based vaccines, late-apoptotic danger signal like extracellular HMGB1 has a predominant role since the vaccines are largely composed of post-apoptotic cells; while a danger signal like surface-CRT may require higher prevalence of pre-/early-apoptotic cells (31, 82, 83). Irrespective of this, our study supports the decisive role of ICD-associated DAMPs in the generation of novel and more powerful DC-based vaccines.

The efficacy of ICD-based DC vaccines was highly dependent upon an intact adaptive immune system. Moreover, our studies identified $CD8^+$ T cells as the predominant adaptive immune mediators of anti-tumor immunity, although this does not rule out the role of other immune cells. ICD-based DC vaccines also induced an immune-stimulatory shift in the brain immune-contexture i.e. increased intra-brain infiltration of Th1/CTLs/Th17 cells relative to reduction in Tregs, in both prophylactic and curative set-ups (latter, in combination with TMZ). Of note, TMZ exerted specific (intra-brain) immune-ablative effects that were corrected to a certain extent by the vaccines to sustain a Treg-to-Th1/CTLs/Th17 immunostimulatory shift in the brain. Hence, while on the level of overall survival, TMZ and ICD-based DC vaccines might exhibit synergism, yet on the level of brain immune-contexture these two strategies engage in partially antagonistic interactions. This further substantiates the largely cytotoxic or tumor-reductive role of TMZ in this set-up. Thus, as expected from a next-generation DC immunotherapy (5), Hyp-PDT induced ICD-based DC vaccines biased the immune reactions in favor of Th1-type immunity. Moreover, our TCGA GBM patient analysis and literature meta-analysis showed that increased incidence of molecular signatures associated with Th1/CTL/Th17 relative to Tregs can have significant positive prognostic impact in GBM patients. Of note, across preclinical and clinical data, brain-infiltrating Th17 cells (or corresponding molecular signatures) associated well with good prognosis in both HGG mice model and GBM/HGG patients. Since the role of Th17 in GBM/HGG remains largely enigmatic, in future, it would be necessary to carry out detailed studies on the anti-HGG immunological effects of Th17 cells and its possible modulators emerging from ICD and/or DCs. Of note, the analysis of prognostic impact of Th1/CTLs/Th17 cells-associated metagene in GBM/HGG patients is of great significance, since now it joins few other cancer-types (melanoma, colorectal, breast and lung cancer) where such analysis has been accomplished (60). Such analysis may also be crucial in future for patient stratification exercises striving to delineate GBM/HGG patients most likely to respond to ICD-based DC vaccines (49, 84).

Last but not least, our comparison between GL261 and CT2A glioma models shows that responsiveness to ICD-based DC vaccines could be affected by MHC-level

heterogeneity in HGG tumors. It would be also crucial in future to characterize other immunotherapies that can be combined with ICD-based DC vaccines to overcome resistance posed by CT2A-like glioma cells in tumors (e.g. IDO inhibitors, anti-CTLA4/PD1/PD-L1 antibodies (1)). However, the results obtained in the current study, using the HGG model consolidate the strength of Hyp-PDT based anticancer vaccines observed in previous studies, using other cancer models (29, 31). Whether Hyp-PDT-induced ICD-based DC vaccines will prove to have similar effectiveness in other cancer-types, is a conjecture that needs to be experimentally tested in the near future.

Materials and Methods

Cell culture, ICD induction and DC vaccine production

GL261 (received as a gift from Dr. Eyupoglu, University of Erlangen, Germany) and CT2A mouse glioma cells were cultured at 37°C under 5% CO_2 in DMEM containing 4.5 g/L glucose and 0.11 g/L sodium pyruvate and supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 µg/L streptomycin and 10% fetal calf serum. For induction of Hyp-PDT elicited ICD, the GL261 cancer cells were incubated with 200 nM Hypericin (for 2 h in serum-free media) followed by light irradiation (4.05 J/cm^2) performed as described previously (85) and recovered 24 h post-treatment unless otherwise mentioned. Of note, Hypericin was prepared, purified and stored as detailed elsewhere (86). Wherever applicable, Hypericin co-localization analysis in the endoplasmic reticulum was performed via fluorescence microscopy as described previously (31, 38). For preparation of Hyp-PDT induced ICD-based DC vaccines, DCs derived from the bone marrow progenitor cells as described previously (45), were loaded with the above Hyp-PDT treated GL261/CT2A cancer cells (2 mg protein per 10 x 10^6 DCs per ml culture medium) at 37°C for 90 min. Wherever applicable, either the DCs were derived from *Myd88*$^{-/-}$ mice (Charles River Laboratories, France) or the respective Hyp-PDT treated cancer cells-associated components were neutralized with anti-CRT antibody (6 µg/10^6 cells post-PDT; Abcam), anti-HMGB1 antibody (10 µg/10^6 cells post-PDT; Abcam), apyrase (13 U/10^6 cells post-PDT; Sigma), a combination of these three or a combination of N-acetylcysteine (NAC, 5 mM; Sigma)/L-Histidine (25 mM; Sigma) at pre-/post-PDT stages. Thereafter, the loaded DCs were transferred to culturing flasks for 24 h in DC culturing medium (consisting of 20 ng/ml GM-CSF, from Peprotech; and 1 µg/ml *E.coli*-derived LPS, from Sigma-Aldrich). Thereafter, 24 h later, the loaded DCs were harvested, counted and resuspended (1 x 10^6 DCs in 100 µL PBS) for intra-peritoneal injection in mice. As applicable, surface expression of DC maturation markers was assessed on pulsed DCs via flow cytometry, using the following monoclonal antibodies (mAbs): FITC-conjugated anti-H-2Kb (Becton Dickinson, BD), PE-conjugated anti-I-A/I-E and anti-CD40 (BD), and PE-conjugated anti-CD80 and anti-CD86 (eBioscience). For each staining the appropriate isotypes were utilized. Analysis was performed using the Cellquest software on a FACSort cytometer (BD, Erembodegem, Belgium).

Orthotopic high grade glioma (HGG) mice model

For this model, female C57BL/6J mice (8 to 10 weeks old) were purchased from Harlan (Horst, Netherlands). They were housed in the conventional pathogen-free conditions and all the relevant experiments were carried out in accordance with the bioethics guidelines or regulations of the KU Leuven. DC immunotherapy and temozolomide (Schering-Plough, Brussels, Belgium; 40 mg/kg) administration schedules are described either in the figures as schemas or within the figure legends. Temozolomide (TMZ) was dissolved in PBS with equal amounts of L-histidine (w/w) to improve solubility (mixture was sonicated for 1 h and agitated every 20 min to avoid precipitation). To create intra-brain HGG, the mice were intra-cranially/intra-axially injected with 5 x 10^5 GL261 or CT2A glioma cells as detailed elsewhere (45). Briefly, mice were anesthetized, fixed in a stereotactic frame (Kopf Instruments, Tujunga, CA) and injected (under sterile conditions) with the GL261/CT2A cells at 2 mm lateral and 2 mm posterior from the bregma and at 3 mm below the dura mater. After intra-cranial/brain-inoculation, the mice were monitored 3 times per week and clinical symptoms were scored with a neurological deficit grading scale adapted from an experimental autoimmune encephalomyelitis model, as described previously (45). Mice were defined as long-term survivors if their survival exceeded 3 times the median survival of the untreated control (CNTR) mice. Wherever applicable, the long-term survivors were re-challenged with GL261 cells, orthotopically (as described above) in the contralateral hemisphere, along with age-matched naïve mice serving as CNTR. *Rag1*$^{-/-}$ mice were kindly provided by Dr. Guy Boeckxstaens (KU Leuven). Also, as applicable, some animals received CD8$^+$ T cells depleting antibodies, intra-peritoneally 1 day before (200 µg) and 1 day after (100 µg) intracranial glioma-inoculation. The hybridoma producing the anti-CD8 antibody was kindly provided by the University of Cambridge. As applicable, either representatives of two-three independent mice

experiments were shown and/or power analysis based on previous publications (45, 53, 71) was employed to reach necessary sample sizes in individual experiments.

MRI analysis and Image processing

MR images were procured on the 9.4 T Biospec small animal MR system (Bruker Biospin, Germany) using a 7 cm linearly polarized resonator for transmission and an actively decoupled dedicated mouse brain surface coil acting as receiver (Rapid Biomedical, Germany). For localization, two-dimensional axial T2-weighted images (spin echo, TEeff = 48 ms, TR = 3500 ms) and two-dimensional coronal T2-weighted images were obtained. Image acquisition was performed with the Paravision 5.0 (Bruker BioSpin). ImageJ software was used for further image processing, 3D rendering, and for alpha-blending based brain volume calculations.

Brain immune infiltration analysis

Brain-infiltrating immune cells were isolated from GL261-inoculated mice as detailed before (45). Surface staining was performed with anti-CD4 PerCP-Cyanine5.5, anti-CD3 eFluor® 450 and anti-CD8a eFluor® 605NC mAbs (all from eBioscience). Intracellular FoxP3 was detected using a FoxP3-PE staining kit (eBioscience) according to the manufacturer's protocols. For intracellular IL-17A and IFN-γ staining, cells were stimulated for 4 h *in vitro* with 100 ng/ml phorbol myristate acetate, 1 µg/ml ionomycin and 0.7 µg/ml monensin (all from Sigma-Aldrich). After restimulation, surface staining for CD4 APC-eFluor® 780, CD8a eFluor® 450 and CD3 PE (all from eBioscience) was performed and cells were washed with a permeabilization buffer containing 0.5% saponin and 0.5% bovine serum albumin (BSA). Intracellular staining was performed with anti-IFN-γ-PerCP-Cyanine5.5 and anti-IL-17A-APC mAbs (eBioscience). Data acquisition was performed on LSRFortessa flow cytometer (BD Biosciences) and the FlowJo software was used for data analysis.

Statistical Analysis

All statistical analyses were performed using either Prism software (GraphPad Software, USA) or GraphPad QuickCalcs online software (http://www.graphpad.com/quickcalcs/index.cfm).
Student's *t*-test, Log-rank (Mantel-Cox) test or Mann-Whitney statistical test were used for statistical analysis, as applicable and unless otherwise mentioned, with significance level set at $p<0.05$.

List of Supplementary Materials: (1) Supplementary methods: (a) Cell death, cell survival and clonogenic survival analysis, (b) 5-ALA-based PDT treatment, (c) Analysis of DAMPs, (d) Analysis of MHC-I and MHC-II expression levels, (e) Immunoblotting, (f) splenocyte analysis, (g) literature meta-analysis for prognostic impact of immune-infiltrates in glioblastoma patients and (h) construction of immune-infiltrate associated metagenes and their prognostic impact in TCGA glioblastoma patient data-set; **(2) Supplementary figures**: Supplementary Figure S1. DCs co-incubated with Hyp-PDT treated GL261 cells exhibit increased phenotypic maturation; Supplementary Figure S2. Hyp-PDT induces superior enrichment of DAMPs exposure/release than 5-ALA-PDT from murine glioma cells; Supplementary Figure S3. Mice treated with ICD-based DC vaccines maintain normal brain volume despite GL261-based HGG challenge; Supplementary Figure S4. The low immunogenic, immunotherapy-resistant, CT2A glioma can be significantly rejected by Hyp-PDT induced ICD-based DC vaccine; Supplementary Figure S5. Anti-CD8 antibody depletes $CD8^+$ T cells (but not $CD4^+$ T cells) in various immune-compartments like blood and (cervical/distal) lymph nodes; Supplementary Figure S6. Single Freezing-step does not completely abrogate the survival or clonogenic potential of murine glioma cells. Supplementary Figure S7. Splenocytes derived from mice are functionally competent; Supplementary Figure S8. Treatment of mice with the chemotherapeutic drug, temozolomide (TMZ), does not lead to general toxicity; Supplementary Figure S9. ICD-based DC vaccines synergize with the, chemotherapeutic drug, temozolomide (TMZ) in providing survival benefit in a curative or therapeutic HGG set-up; Supplementary Figure S10. Long-term survivors immunized previously by ICD-based DC vaccines tend to significantly reject re-challenge with orthotopic HGG; Supplementary Figure S11. Increased tumoral expression of $CD8^+$ T cell-associated metagenes, but not $CD4^+$ T cell-associated metagene, associate with prolonged overall survival in glioblastoma (GBM) patients. Supplemental Figure S12. Enlargements of the metagenes associated with specific T cell-types as presented in main figure 8. **(3) Supplementary Table S1.** Literature meta-analysis of, prognostic impact of, intra-tumoral T cell infiltration in high-grade glioma or glioblastoma (GBM) patients.

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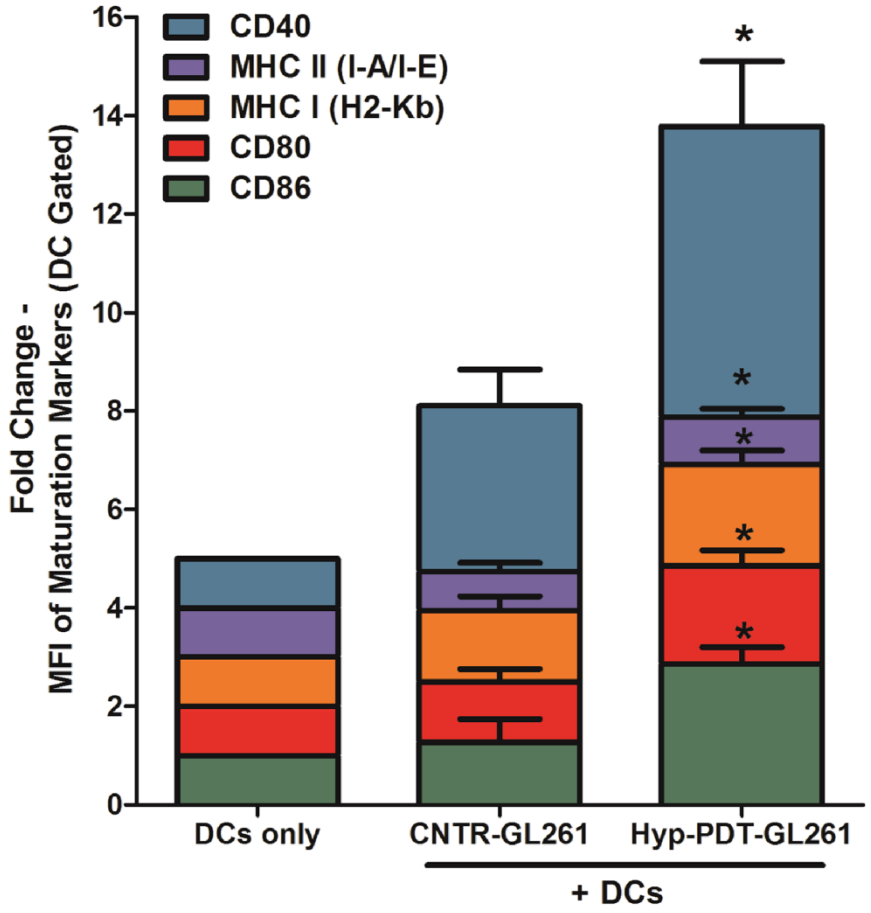
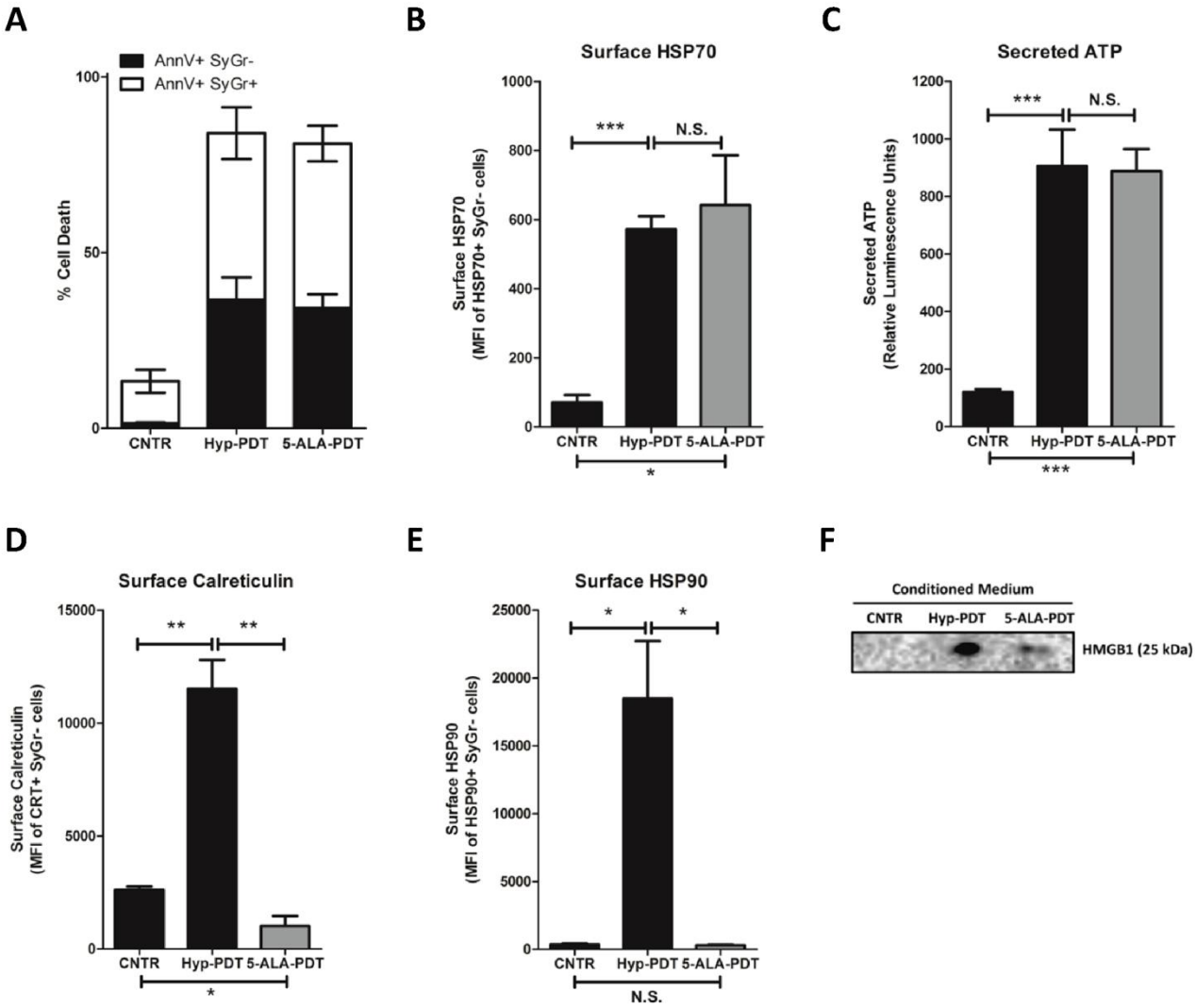
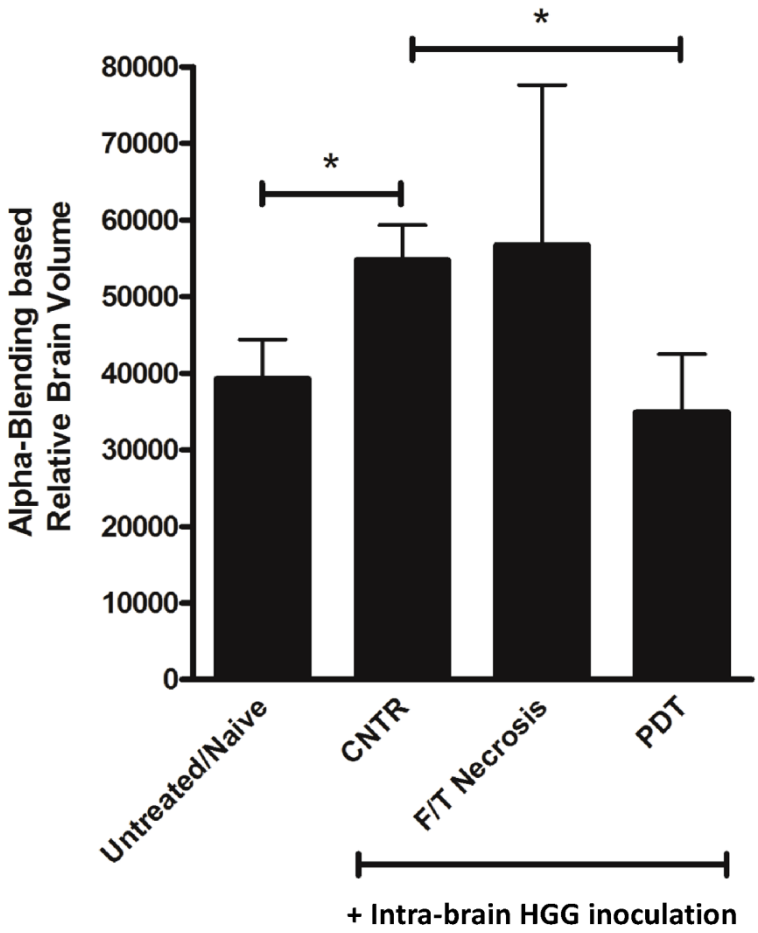


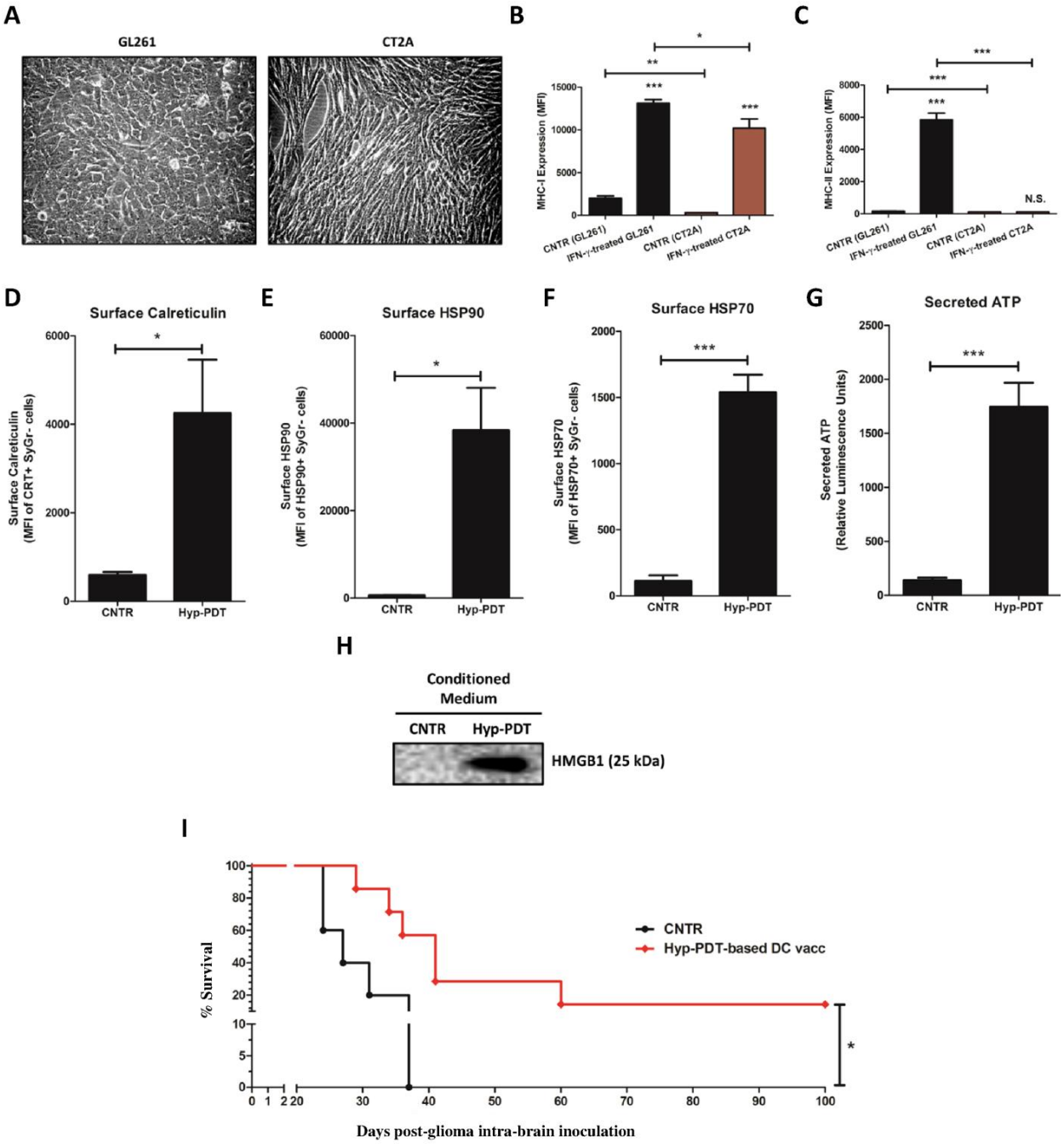
Supplementary Figure S1. DCs co-incubated with Hyp-PDT treated GL261 cells exhibit increased phenotypic maturation. GL261 cells were treated *in vitro* with Hyp-PDT (200 nM Hyp incubated for 2 h, followed by irradiation with light fluence of 4.05 J/cm^2), followed by their recovery 24 h post-treatment, and co-incubation with murine DCs for another 24 h. Thereafter the surface expression of CD40, MHC-II (I-A/I-E), MHC-I (H2-Kb), CD80 and CD86 were evaluated by FACS-based analysis (n=4; mean±s.e.m.; Student's paired t-test; *p<0.05/**p<0.01/***p<0.001 versus CNTR-GL261).



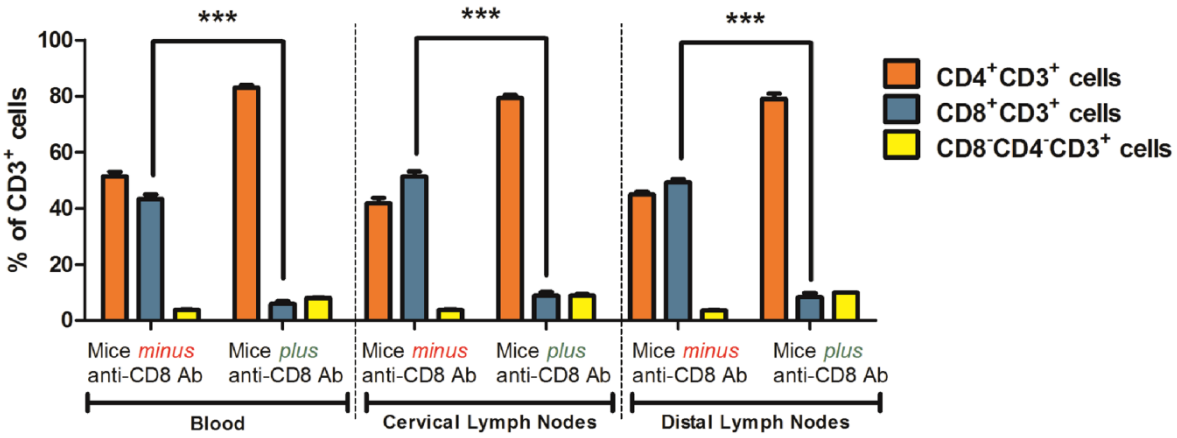
Supplementary Figure S2. Hyp-PDT induces superior enrichment of DAMPs exposure/release than 5-ALA-PDT from murine glioma cells. GL261 were treated with either Hyp-PDT (incubation of 2 h - 200 nM Hyp, light fluence – 2.70 J/cm^2) or 5-ALA-PDT (incubation of 2 h – 0.5 mM 5-ALA, light fluence – 2.13 J/cm^2), followed by: **(A)** recovery of cells 24 h post-PDT and estimation of % early-apoptotic (Ann-V$^+$/SyGr$^-$) or % late-apoptotic/necrotic (Ann-V$^+$/SyGr$^+$) cells *via* FACS (n=3; mean±s.d.); **(B-E)** recovery of cells 1 h post-PDT and FACS-based analysis for surface-calreticulin **(D)**, surface-HSP70 **(B)** and surface-HSP90 **(E)** in non-permeabilized cells (data presented as mean fluorescence intensity (MFI), n=3; mean±s.e.m.); **(C)** recovery of cells 1 h post-PDT and analysis for relative amounts of extracellular **(C)** ATP levels, measured in the conditioned media (n=5-6; mean±s.e.m.); and **(F)** recovery of cells at 48 h post-PDT, and immunoblotting analysis of concentrated conditioned media. In this figure, Student's t-test was used for statistics, *p<0.05/**p<0.01/***p<0.001 as indicated by bars; N.S. – not significant.



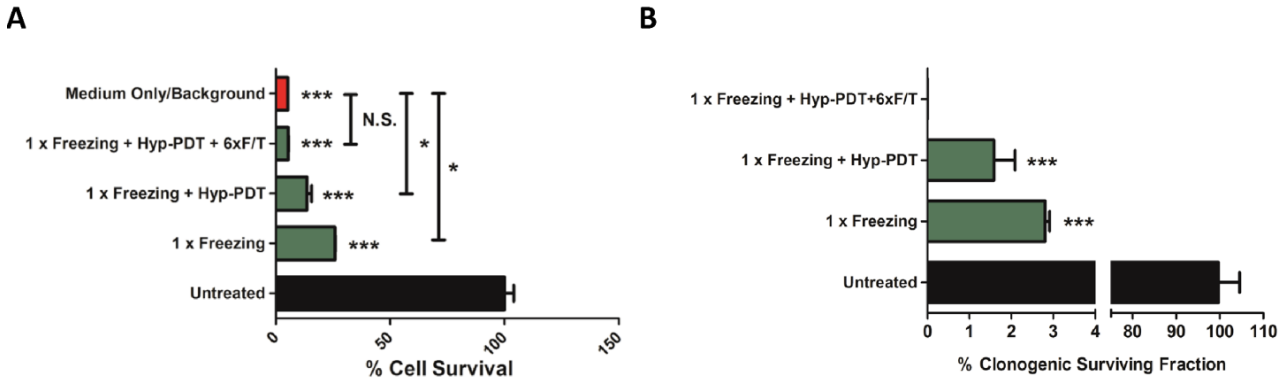
Supplementary Figure S3. Mice treated with ICD-based DC vaccines maintain normal brain volume despite GL261-based HGG challenge. GL261 cells were treated to undergo ICD *in vitro* with Hyp-PDT (200 nM Hyp incubated for 2 h, followed by irradiation with light fluence of 4.05 J/cm^2) and recovered 24 h post-PDT; thereafter they were co-incubated with murine DCs (for 24 h) to produce ICD-based DC vaccine. Alternatively, as applicable, DC vaccines were also produced for GL261 cells exposed to 6 cycles of freezing and thawing (F/T) based necrosis. These vaccines were injected twice in C57BL/6 mice intra-peritoneally, with an interval of 7-8 days between vaccinations. Mice not immunized with the vaccines were used as controls (CNTR). Thereafter, the immunized and non-immunized mice were inoculated with live GL261 cells, intra-axially (i.e. in the brain), 7-8 days after vaccination. Thereafter the mice were monitored for high-grade glioma (HGG)-induced neurological deficit symptoms. Thereafter, magnetic resonance imaging (MRI) scans were carried out for naïve/normal mice (i.e. mice not exposed to the procedure described above) and day 29 post-intra-axial GL261-inoculated mice from CNTR, F/T Necrosis-based DC vaccine and Hyp-PDT-based DC vaccine "cohorts". Subsequently, these MRI scans were used to create 3D renditions of the whole-brain and these 3D renditions were used to estimate alpha-blending based relative brain volume (n=3-4 mice; mean±s.d.; Student's t-test; *$p<0.05$ as indicated by the bars).



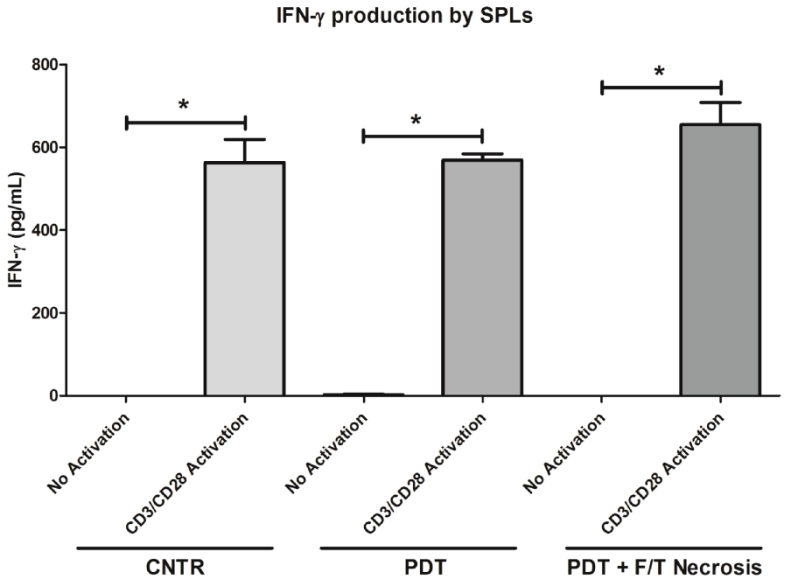
Supplementary Figure S4. The low immunogenic, immunotherapy-resistant, CT2A glioma can be significantly rejected by Hyp-PDT induced ICD-based DC vaccine. (A) A phase-contrast microscopic analysis of confluent cultures of GL261 and CT2A cells shows that while GL261 exhibit a more differentiated cellular phenotype yet, the CT2A cells exhibit a more undifferentiated mesenchymal-like cellular morphology. **(B-C)** GL261/CT2A cells were either left untreated or treated with recombinant murine IFN-γ, recovered 48 h post-treatment and analyzed for expression levels of MHC-I **(B)** or MHC-II **(C)** via flow cytometry (data presented as mean fluorescence intensity (MFI), n=5; mean±s.e.m.; two-way ANOVA). **(D-G)** CT2A were treated with Hyp-PDT (incubation of 2 h - 200 nM Hyp, light fluence – 4.05 J/cm^2), followed by: recovery of cells 1 h post-PDT and FACS-based analysis for surface-calreticulin **(D)**, surface-HSP90 **(E)** and surface-HSP70 **(F)** in non-permeabilized cells (data presented as mean fluorescence intensity (MFI), n=3; mean±s.e.m.); **(G)** recovery of cells 1 h post-PDT and analysis for relative amounts of extracellular ATP levels, measured in the conditioned media (n=5-6; mean±s.e.m.); and **(H)** recovery of cells at 48 h post-PDT, and immunoblotting analysis of concentrated conditioned media. In **D-G**, Student's t-test was used for statistics, *p<0.05/**p<0.01/***p<0.001 as indicated by bars; N.S. – not significant. **(I)** CT2A cells were treated to undergo ICD with Hyp-PDT and recovered 24 h post-PDT; thereafter they were co-incubated with murine DCs (for 24 h) to produce ICD-based DC vaccine (vacc). Thereafter the vaccinations were carried out in a prophylactic vaccination set-up. Kaplan-Meier curve depicts survival of two "cohorts" (CNTR, n=5; and Hyp-PDT-based DC vacc, n=7; Log-rank (Mantel-Cox) test; *p<0.05 vs. CNTR).



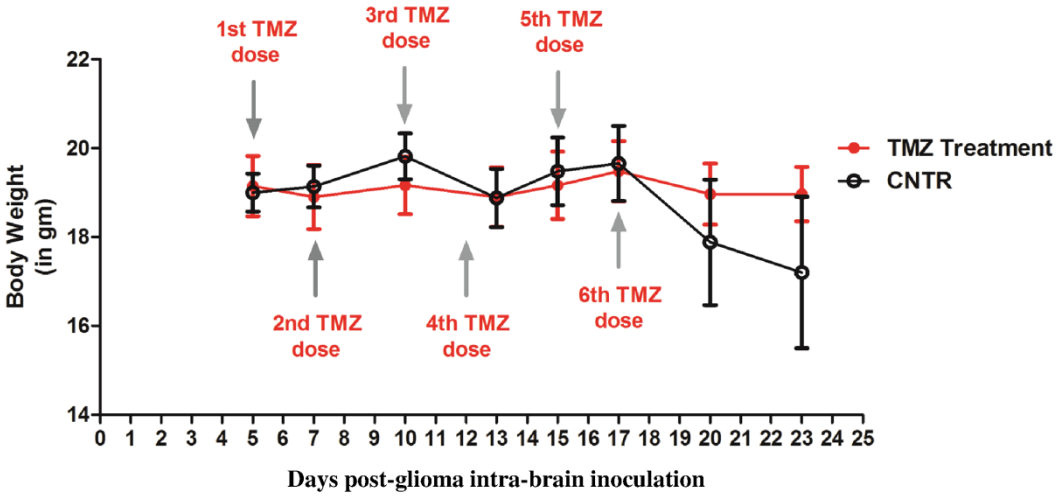
Supplementary Figure S5. Anti-CD8 antibody depletes CD8⁺ T cells (but not CD4⁺ T cells) in various immune-compartments like blood and (cervical/distal) lymph nodes. Mice were injected with anti-CD8 antibody (Ab) one day before (D-1) and one day after (D+1) the intra-axial inoculation with live GL261 cells. Thirteen days post-antibody injection, the mice blood, cervical lymph nodes and distal lymph nodes were isolated and processed for FACS-based immunophenotyping followed by detection of $CD4^+$ T cells ($CD4^+CD3^+$ cells), $CD8^+$ T cells ($CD8^+CD3^+$ cells) and double-negative T cells ($CD8^-CD4^-CD3^+$ cells) (n=2-3 mice; mean±s.e.m.; ***p<0.001 indicated by bars; Student's t-test).



Supplementary Figure S6. Single Freezing-step does not completely abrogate the survival or clonogenic potential of murine glioma cells. GL261 cells were either left untreated or treated with one freezing and thawing (F/T) cycle, F/T+Hyp-PDT and F/T+Hyp-PDT+6xF/T. This was followed by either - **(A)** determination of percentage cellular viability through MTS assay, 48 h post-treatment (n=4; mean±s.e.m.; one-way ANOVA) or **(B)** clonogenic assay after 10 days of growth (n=3; mean±s.d.; Student's t-test). In this figure, *p<0.05/**p<0.01/***p<0.001 is either vs. Untreated (without bars) or as indicated by bars; N.S. – not significant.

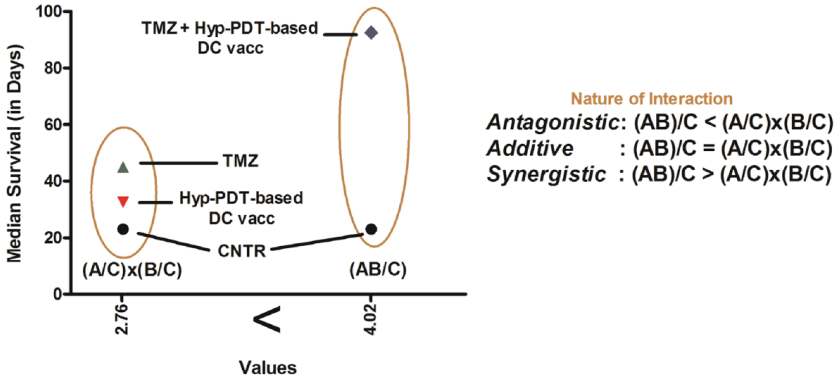


Supplementary Figure S7. Splenocytes derived from mice are functionally competent. GL261 cells were treated to undergo ICD *in vitro* with Hyp-PDT (200 nM Hyp incubated for 2 h, followed by irradiation with light fluence of 4.05 J/cm^2); thereafter in another case as applicable they were also exposed to avitalization via freeze/thawing (F/T) based necrosis. Subsequently, respective treated cancer cells were co-incubated with murine DCs (for 24 h) to produce respective ICD-based DC vaccines. These vaccines were injected twice in C57BL/6 mice intra-peritoneally, with an interval of 7-8 days between vaccinations. Mice not immunized with the vaccines were used as controls (CNTR). Thereafter, the immunized and non-immunized mice were inoculated with live GL261 cells, intra-axially (i.e. in the brain), 7-8 days after vaccination. Thereafter the mice were sacrificed at day 16 post intra-axial GL261-inoculation. Subsequently, spleens were recovered from the above mice, followed by their processing to enrich for T cells. Thereafter, the T cell-enriched splenocytes were either left without activation (negative control) or activated with beads associated with anti-CD3/anti-CD28 antibodies (positive control) for 5 days, followed by estimation of IFN-γ production in respective supernatants (n=4-5 mice; mean±s.e.m.; Mann-Whitney statistical test; *p<0.05 as indicated by bars; N.S. – not significant).



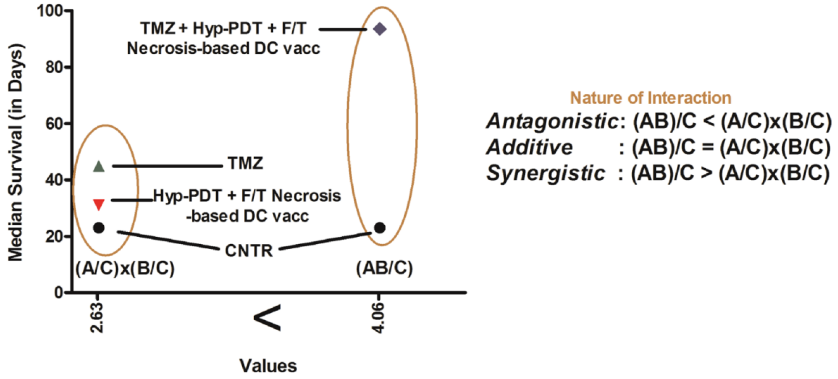
Supplementary Figure S8. Treatment of mice with the chemotherapeutic drug, temozolomide (TMZ), does not lead to general toxicity. C57BL/6 mice were inoculated with live GL261 cells (Day 0), intra-axially (i.e. in the brain). Consequently, these HGG-inoculated mice were either left untreated (CNTR) or treated with 6 cycles of orally-administrated (through gavage) chemotherapeutic, TMZ (40 mg/kg) at the indicated schedule. During TMZ treatment and until 1 week after TMZ treatment, the overall body weight of the CNTR mice and TMZ only mice were recorded and are presented in grams (gm) (CNTR, n=5; TMZ only, n=6; mean±s.e.m.).

A



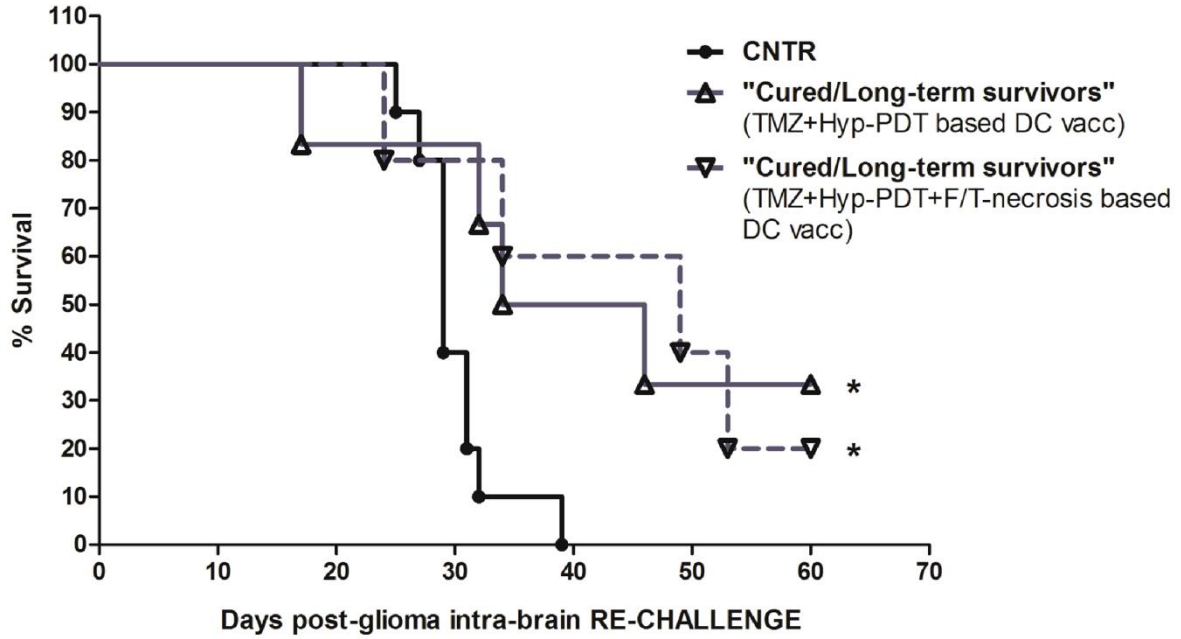
A – TMZ only
B – Hyp-PDT-based DC vacc
C – CNTR
AB – TMZ + Hyp-PDT-based DC vacc

B

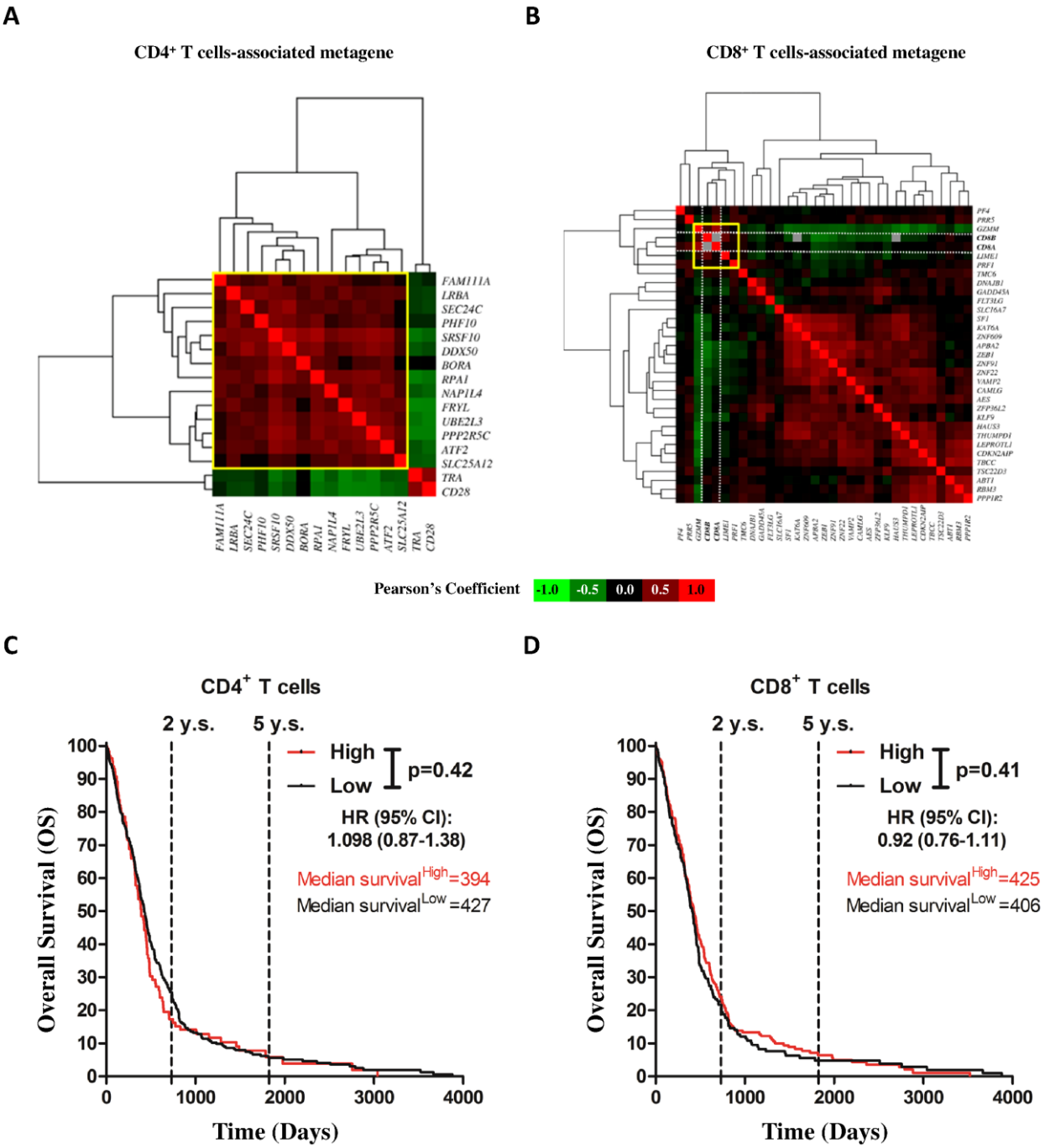


A – TMZ only
B – Hyp-PDT+F/T Necrosis-based DC vacc
C – CNTR
AB – TMZ + Hyp-PDT+F/T Necrosis-based DC vacc

Supplementary Figure S9. ICD-based DC vaccines synergize with the, chemotherapeutic drug, temozolomide (TMZ) in providing survival benefit in a curative or therapeutic HGG set-up. Firstly, C57BL/6 mice were inoculated with live GL261 cells (Day 0), intra-axially (i.e. in the brain). Consequently, GL261 cells were treated to undergo ICD *in vitro* with Hyp-PDT (200 nM Hyp incubated for 2 h, followed by irradiation with light fluence of 4.05 J/cm^2) and recovered 24 h post-PDT; thereafter in another case as applicable they were also exposed to avitalization via freeze/thawing (F/T) based necrosis; subsequently these cells were co-incubated with murine DCs (for 24 h) to produce respective ICD-based DC vaccines. Thereafter the intra-axially GL261-inoculated mice were randomly divided into 6 "cohorts" i.e. CNTR, where HGG-inoculated mice were not immunized with any vaccine; two cohorts ("vaccination only") where the above two vaccines were injected thrice in HGG-inoculated mice intra-peritoneally at the indicated schedule; one cohort ("TMZ only") where the HGG-inoculated mice were treated with 6 cycles of orally-administrated (through gavage) chemotherapeutic, TMZ (40 mg/kg) at the indicated schedule; and two cohorts ("TMZ+vaccination") where the HGG-inoculated mice were first treated with 6 cycles of orally-administrated (through gavage) chemotherapeutic, TMZ (40 mg/kg) at the indicated schedule and thereafter the above two vaccines were injected thrice, intra-peritoneally, at the indicated schedule. Thereafter the mice were monitored for HGG-induced neurological deficit symptoms. **(A-B)** Thereafter, based on respective median survival data, the calculations for antagonistic effect, additive effect and synergistic effect were carried out based on the Robert Clark equations (R Clarke, 1997, Breast Cancer Research and Treatment, 46:255-278) for Hyp-PDT-based DC vacc **(A)** and Hyp-PDT + F/T Necrosis-based DC vacc **(B)**.

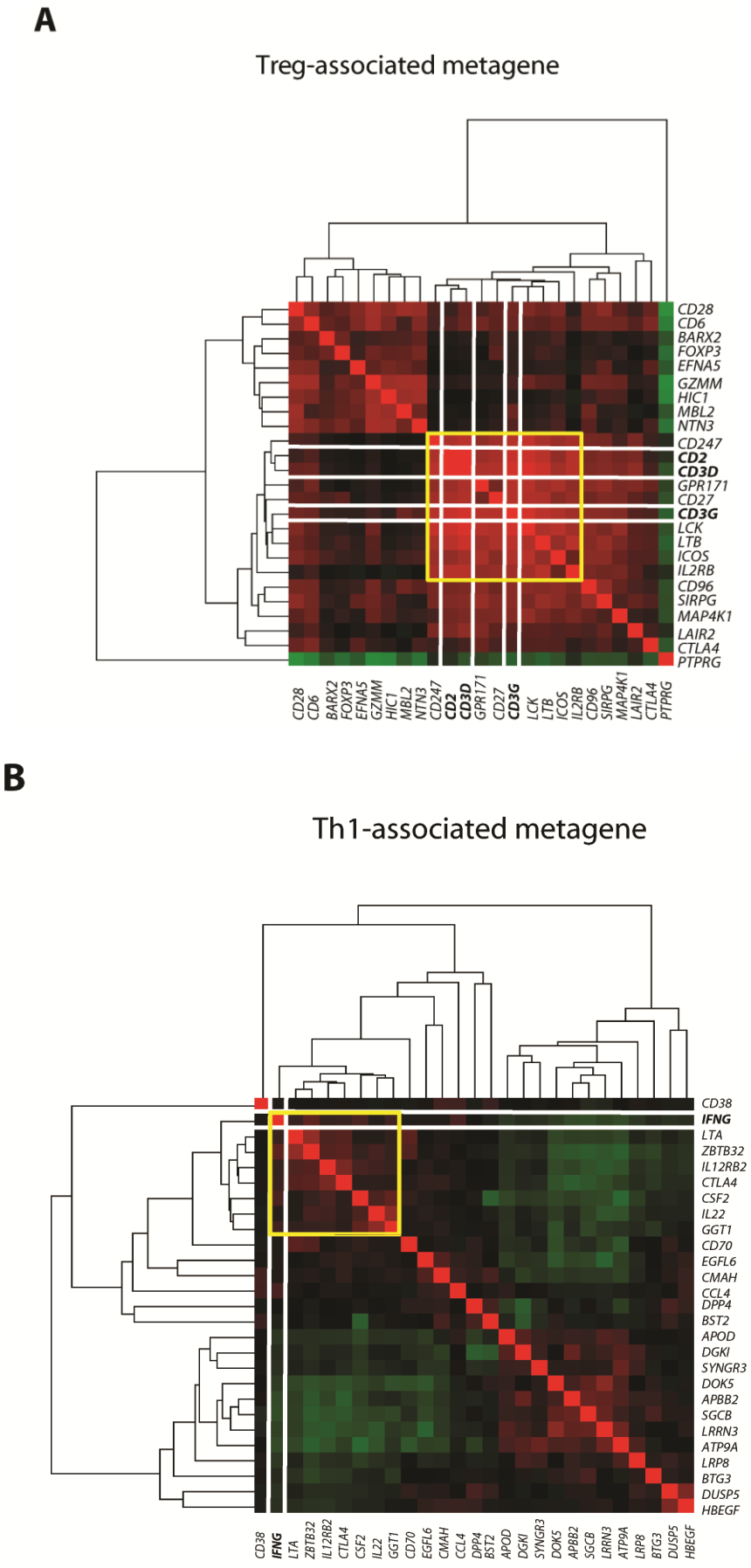


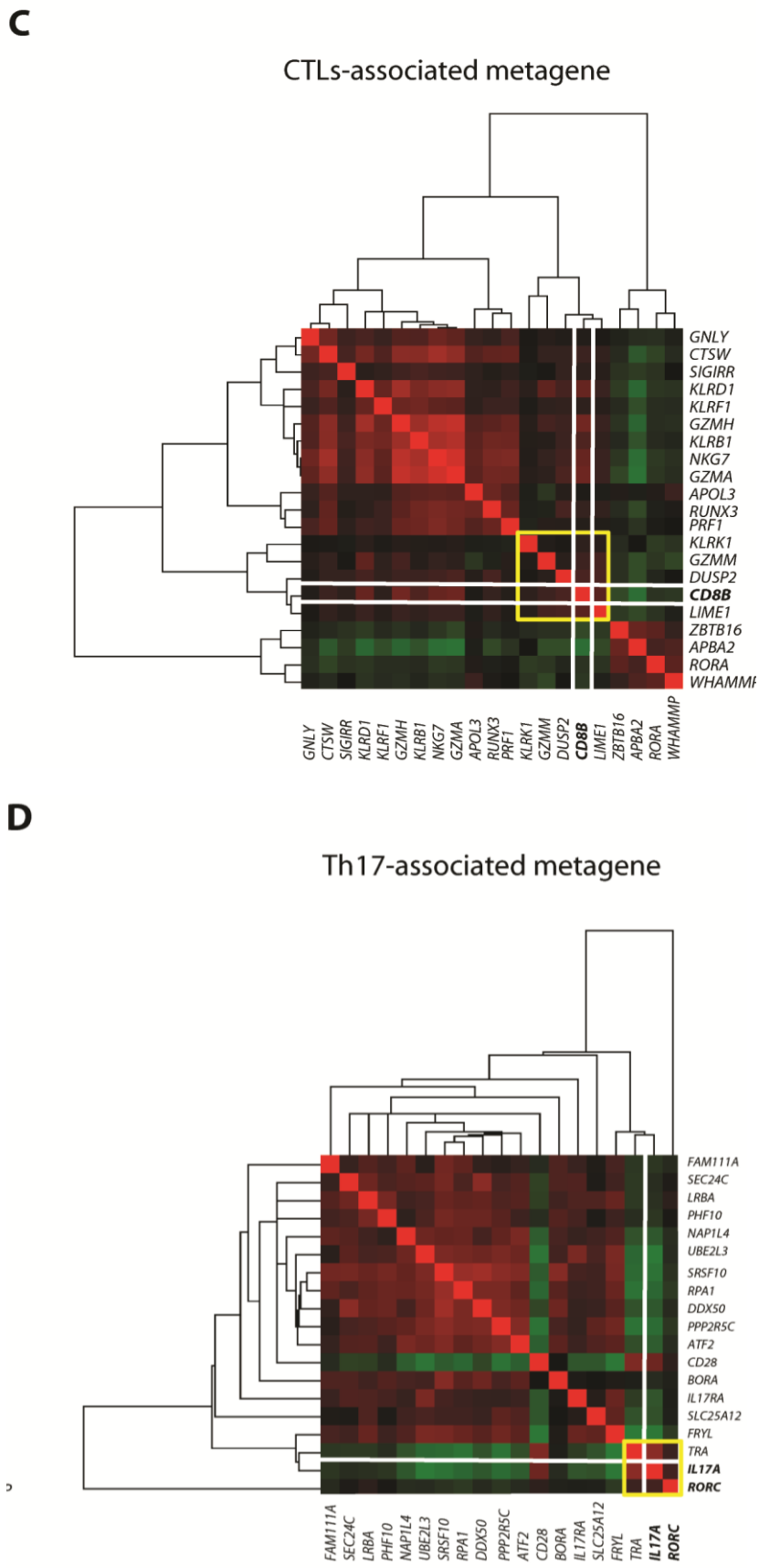
Supplementary Figure S10. Long-term survivors immunized previously by ICD-based DC vaccines tend to significantly reject re-challenge with orthotopic HGG. Long-term surviving mice of the TMZ+Hyp-PDT-based DC vacc and TMZ+Hyp-PDT+F/T-necrosis based DC vacc conditions (Fig 6) were re-challenged with intra-axial inoculation with GL261 (along with age-matched, untreated CNTR mice). Kaplan-Meier curve depicts survival of three "cohorts" (CNTR, n=10; TMZ+Hyp-PDT-based DC vacc, n=6; TMZ+Hyp-PDT+F/T-necrosis based DC vac, n=5; Log-rank (Mantel-Cox) test; *$p<0.05$ vs. CNTR).



Supplementary Figure S11. Increased tumoral expression of CD8⁺ T cell-associated metagenes, but not CD4⁺ T cell-associated metagene, associate with prolonged overall survival in glioblastoma (GBM) patients. (A-B) Metagenes associated with specific T cell-types were established by generating gene co-expression profiles (from TCGA GBM patient data-sets, n=541 (1, 2)) for independently established T cells-associated gene patterns (3, 4). Pearson's correlation coefficient (indicated in color-code legend) was used to analyze tendency of gene co-expression; and metagenes were characterized by centering the profiles (indicated by yellow line delineation) to specific T cell markers e.g. *uncentered* (for CD4⁺ T cells, **A**) or *CD8A/CD8B* (for CD8⁺ T cells, **B**). **(C-D)** Publicly-available TCGA cohort of GBM patients (n=541) was stratified (at 75th percentile for CD4⁺ T cells, or, median for CD8⁺ T cells, of gene expression) into "high expression" (red; n=136 for CD4⁺ T cells or n=271 for CD8⁺ T cells) or "low expression" (black; n=405 for CD4⁺ T cells or n=270 for CD8⁺ T cells) groups for each metagene; followed by Kaplan-Meier plotting of patient's overall survival (OS; Y-axis) versus follow-up duration in days (X-axis). This was done for metagene expressions associated with CD4⁺ T cells (**C**) and CD8⁺ T cells (**D**). In all **C-D** graphs, respective log-rank (Mantel-Cox) test p-values and hazard ratios (HR; with its 95% confidence interval in parenthesis) are displayed. Two dotted lines represent approximate point of 2 and 5 year survival (y.s.) windows.

Supplemental Figure S12:





Supplemental Figure S12. Enlargements of the metagenes associated with specific T cell-types as presented in main figure 8 A, B, C and D.

Supplementary Table S1. Literature meta-analysis of, prognostic impact of, intra-tumoral T cell infiltration in high-grade glioma or glioblastoma (GBM) patients.

	Patient Prognosis					
	Untreated/Surgery or Chemo/Radio-therapy-based set-up			DC vaccination-based set-up		
T cells ($CD3^+$ cells)	Good[5-7]	Poor[8]	None[9-11]	-	-	-
$CD4^+$ T cells	Good[7, 12-14]	Poor[15]	None[5]	Good[16]	-	-
$CD8^+$ T cells	Good [5-7, 13, 15, 17, 18]	-	None[11, 19]	Good[16]	-	-
IFN-γ^+CD4^+ T cells	-	-	-	Good[16]	-	-
IFN-γ^+CD8^+ T cells	-	-	-	Good[16]	-	-
$Foxp3^+CD4^+$ T cells (Tregs)	-	Poor[5, 8, 19-21]	None[10, 13, 15, 22]	-	Poor[23]	-
$IL17A^+CD4^+$ T cells (Th17 cells)	Good[24]	-	-	-	-	-

Following studies were utilized for this meta-analysis:

Sayour EJ, McLendon P, McLendon R, De Leon G, Reynolds R, et al. 2015. *Cancer Immunol Immunother* DOI: 10.1007/s00262-014-1651-7

Kmiecik J, Poli A, Brons NH, Waha A, Eide GE, et al. 2013. *J Neuroimmunol* 264: 71-83

Wong ET, Lok E, Gautam S, Swanson KD. 2015. *Br J Cancer* 113: 232-41

Wiencke JK, Accomando WP, Zheng S, Patoka J, Dou X, et al. 2012. *Epigenetics* 7: 1391-402

Han S, Liu Y, Li Q, Li Z, Hou H, Wu A. 2015. *BMC Cancer* 15: 617

Thomas AA, Fisher JL, Rahme GJ, Hampton TH, Baron U, et al. 2015. *Neuro Oncol* 17: 801-9

Berghoff AS, Kiesel B, Widhalm G, Rajky O, Ricken G, et al. 2015. *Neuro Oncol* 17: 1064-75

Gousias K, Voulgaris S, Vartholomatos G, Voulgari P, Kyritsis AP, Markou M. 2014. *Surg Neurol Int* 5: 89

Lohr J, Ratliff T, Huppertz A, Ge Y, Dictus C, et al. 2011. *Clin Cancer Res* 17: 4296-308

Grossman SA, Ye X, Lesser G, Sloan A, Carraway H, et al. 2011. *Clin Cancer Res* 17: 5473-80

Han S, Zhang C, Li Q, Dong J, Liu Y, et al. 2014. *Br J Cancer* 110: 2560-8

Fadul CE, Fisher JL, Hampton TH, Lallana EC, Li Z, et al. 2011. *J Immunother* 34: 382-9

Kim YH, Jung TY, Jung S, Jang WY, Moon KS, et al. 2012. *Br J Neurosurg* 26: 21-7

Yang I, Tihan T, Han SJ, Wrensch MR, Wiencke J, et al. 2010. *J Clin Neurosci* 17: 1381-5

Yue Q, Zhang X, Ye HX, Wang Y, Du ZG, et al. 2014. *J Neurooncol* 116: 251-9

El Andaloussi A, Lesniak MS. 2007. *J Neurooncol* 83: 145-52

Jacobs JF, Idema AJ, Bol KF, Grotenhuis JA, de Vries IJ, et al. 2010. *J Neuroimmunol* 225: 195-9

Heimberger AB, Abou-Ghazal M, Reina-Ortiz C, Yang DS, Sun W, et al. 2008. *Clin Cancer Res* 14: 5166-72

Fong B, Jin R, Wang X, Safaee M, Lisiero DN, et al. 2012. *PLoS One* 7: e32614

Cui X, Xu Z, Zhao Z, Sui D, Ren X, et al. 2013. *Int J Biol Sci* 9: 134-41

Supplementary Methods:

Protein Carbonylation Analysis: The detection of protein carbonyls was performed published previously (25). In brief, 50 µl of GL261 cell lysates, corresponding to a total protein concentration of 5-10 mg/ml were incubated with an equal volume of 200 µM fluorescein-5-thiosemicarbazide (FTC, Sigma-Aldrich) overnight (at room temperature and in dark); 24 h later, proteins were precipitated, centrifuged and the supernatant was discarded. Consequently the protein precipitates were washed with acetone. The acetone supernatant was discarded followed by air drying of the precipitates. These were then solubilized with 50 µl of guanidine hydrochloride (GuHCl, 6M) and diluted with 450 µl of NaH_2PO_4 (pH 7.0). Protein concentrations were then measured via the bicinchoninic (BCA) protein assay kit (Thermo Scientific) and 50 µL of these samples were aliquoted in a black microtiter plate and fluorescence was measured with a FlexStation 3 microplate reader (Molecular Devices, Berkshire, UK). A standard curve prepared using pure FTC allowed for the calculation of the nanomoles of FTC-reacted carbonyls. These values were divided by the protein concentrations to derive the amount of protein carbonyls (expressed as, nmol/mg protein).

Cell death, Cell survival and Clonogenic survival analysis: Cell death analysis was carried out *via* flow cytometry by scoring for cells staining positive for Sytox Green alone or in combination with Annexin-V-APC (BD Biosciences; as applicable), as described previously (26); while cell survival analysis was done via MTS assay (Promega), as per the manufacturer's instructions. Clonogenic survival analysis was carried out as described elsewhere (27). Starting plating efficacy was set at 500 cells per plate. Wherever applicable, Tauroursodeoxycholic acid (TUDCA) was purchased from Merck-Millipore (Darmstadt, Germany) while Z-Val-Ala-Asp(OMe)-fmk (zVAD-fmk) was purchased from Bachem (Weil am Rhein, Germany).

5-ALA-based PDT treatment: GL261 cells were pre-incubated with 0.5 mM Gliolan® or 5-aminolevulinic acid (Medac GmbH, Hamburg, Germany) (for 2 h in serum-free media) followed by light irradiation (2.13 J/cm^2) performed as described previously (28) and recovered 24 h post-treatment.

Analysis of DAMPs: After treatment, cells were collected with TrypLE Express (Life Technologies, Belgium), washed, incubated for 1 hr at 4°C with anti-CRT (Abcam, Cambridge, UK) or anti-HSP90 (Stressgen) or anti-HSP70 (Santa Cruz Antibodies) antibodies; followed by another wash and subsequent incubation for 1 hr at 4°C with goat anti-rabbit or anti-mouse antibodies conjugated with Alexa Fluor®647 (Invitrogen, Belgium) or DyLight 680/DyLight800 (Thermo Scientific), as applicable. After final washes cells were incubated in FACS (Flow Cytometry) buffer (2% FBS, 1% BSA in PBS) including 1 µM Sytox Green (Life Technologies, S7020) for 15 min and analyzed on Attune Flow Cytometer (Life Technologies). The permeabilised cells (i.e. Sytox Green positive cells) were excluded from the analysis, and the mean fluorescence intensity (MFIs) for ecto-DAMPs was analyzed. In another case, after treatment, extracellular ATP was measured in the conditioned media via ATP Bioluminescent assay kit (Sigma, St. Louis, MO, USA) as described previously (29). For analysis of passively released DAMPs, the 'conditioned' culture media (5–8 ml), were collected and concentrated to manageable volumes (200–500 µl) *via* centrifugation (2000 × g for 5 min) using Pierce Concentrator 7ml/9K filters (Pierce), according to the manufacturer's instructions. They were then analyzed by immunoblotting.

Analysis of MHC-I and MHC-II expression levels: GL261 and CT-2A cells were treated (or not) with 200 U/mL recombinant murine IFN-γ (Roche) for 48 h. Thereafter the cells were collected, washed and incubated for 30 minutes at 4°C with either anti-MHC-I (H2Kb) or anti-MHC-II (I-A/I-E) antibodies conjugated with PE-cyanine 7 (eBioscience). Data acquisition was performed on a LSRFortessa flow cytometer (BD Biosciences); and the mean fluorescence intensities (MFI) were analyzed by the FlowJo software.

Immunoblotting: Immunoblotting was performed as described previously (30, 31). Densitometric analyses were generated using Image J. Anti-CHOP, anti-P-eIF2α, anti-Total eIF2α, anti-caspase 3, anti-HSP90 and anti-Bip/GRP78 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibody against actin was purchased from Sigma (St. Louis, MO, USA) while anti-HMGB1 antibody was purchased from Abcam (Cambridge, UK). Secondary antibodies conjugated to horseradish peroxidase were purchased from Cell Signaling Technology (Danvers, MA, USA) or Abcam (Cambridge, UK). Also, the following secondary antibodies were used: goat anti-mouse-DyLight680 (Thermo Scientific, Belgium) and goat anti-rabbit-DyLight800 (Thermo Scientific, Belgium). Chemiluminescence detection was done using the Odyssey infrared-imaging system (Li-Cor Biosciences, Lincoln, NE, USA) or Chemidoc™ MP system (Bio-Rad, Nazareth Eke, Belgium), as applicable.

Splenocytes analysis: Spleens were isolated and single cell suspensions were prepared by passaging through a cell strainer (BD Biosciences). Next, nylon wool fiber (Polysciences, Germany) was used for enriching T cell fraction. These splenocytic T cells were co-cultured with mouse T-activator CD3/CD28 Dynabeads® (positive control; ThermoFisher Scientific) or DCs (5:1 ratio) pulsed with naïve or untreated GL261 lysate (prepared via GentleMACS® system) at 200 µg protein/10^6 DCs, for 5 days. At the end of this co-incubation, the respective supernatants were harvested and analyzed for secreted IFN-γ levels using the CBA kit (BD Biosciences), as per the manufacturer's instructions.

Literature meta-analysis for prognostic impact of immune-infiltrates in glioblastoma patients: Electronic databases including PubMed, Scopus and Web of Knowledge were searched for relevant studies conducted in humans, until 25th November 2015. The following search keywords were used: ("glioblastoma" OR "glioma") AND ("T lymphocytes"), ("T cells"), ("CD3"), ("CD4"), ("IL17A"), ("interferon gamma"), ("Foxp3") AND ("patient"). To further recognize potentially pertinent studies, the list of articles identified in the early search, were also scanned manually. Studies or specific results within them were considered eligible if they met all of the following criteria: (1) explore the association or correlation between immunoinfiltrates and long-term clinical survival parameters like progression-free survival, overall survival or disease free survival and (2) explore the association or correlation between immunoinfiltrates and higher glioma/glioblastoma grade. Studies or specific results were excluded based on any of the following reasons: (1) not sufficient data reported, (2) letters, reviews, commentary, perspectives, case reports, conference abstracts, editorials or expert opinion, (3) studies where correlation was done with short-term clinical response determinants like clinical responders (complete or partial response) vs. non-responders (progressive or stable disease).

Construction of immune-infiltrate associated metagenes and their prognostic impact in TCGA glioblastoma patient data-set: The metagene sets (32) associated with specific immune cell types were taken from either Bindea et al. *Immunity*, 2013 (3) ($CD4^+$ T cells, $CD8^+$ T cells, Th1 cells, Th17 cells, CTLs) or Gentles et al. *Nature Medicine*, 2015 (4) (Tregs). The co-expression of the respective genes in each metagene set was re-analysed for the glioblastoma (GBM) patients in order to derive GBM-specific T cells-associated metagene sets. Here, The Cancer Genome Atlas (TCGA) (33) gene expression dataset (transcriptome data produced by U133 microarray technology) of GBM patients (n=541) (1, 2) was utilized to generate correlation submatrix of respective gene's expression levels by estimating the respective Pearson's correlation coefficients, using the cBioPortal (34). These coefficient values were then used to carry out hierarchical clustering (32) through the Cluster 3.0 software (35) and the metagene was visualized as a heatmap through TreeView (36). The statistical parameters utilized to derive GBM-specific T cells-associated metagene were based on specific centering of highly co-expressing gene clusters to *bona fide* T cell markers wherever applicable (as described previously (3, 37)) e.g. Th1 cell-associated metagene was centred on *IFNG* gene, Th17 cell-associated metagene was centred on *IL17A*/*RORC* genes, CTLs/$CD8^+$ T cells-associated metagenes were centred on *CD8A/CD8B* genes, Treg cell-associated metagene was centred on *CD3D/CD3G/CD2* gene, while $CD4^+$ T cell-associated metagene didn't require specific centering due to highly homogeneous expression patterns.

Thereafter, the respective metagene expression levels in TCGA GBM patient data-set (n=541) and associated clinical survival information (overall survival or OS) (1, 2) was analysed through the PROGgeneV2 platform (38). This platform stratified the respective patients on the basis of the 75th percentile of overall gene expression profile into two risk-groups i.e. high risk or low risk, with respect to the mean of the collective expression of multiple genes/transcripts composing a metagene (32). The respective patient risk groups were plotted with respect to OS of the patients to produce respective Kaplan-Meier curves using the Graphpad Prism software. Hazard ratio (and its 95% confidence intervals) and log-rank (Mantel-Cox) P values were calculated (statistical significance threshold set at $p<0.05$) (3232). Patients surviving beyond the follow-up thresholds were censored.

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5. FINAL DISCUSSION AND PERSPECTIVES

"Learn from yesterday, life for today, hope for tomorrow. The important thing is not to stop questioning."

- Albert Einstein

5.1. Oxidation in cancer cells as driver of potent DC vaccines

Over the past five years researchers have realized that besides therapeutically exploiting innate or adaptive immune cells directly (e.g. through DC vaccines or adoptive T cell transfer) and/or improving the effector function of T cells (through check-point blockade), cancer cells themselves need to be made immunogenic either *in vivo* or *ex vivo* for application in cancer vaccines (179). Our results, as well as increasing data from other groups, point towards a critical involvement of oxidative modifications or OAMPs in the immunogenicity of cancer cells (116, 130, 131, 216). In this project, we evaluated two different physicochemical treatments that are known to induce oxidative stress in cancer cells (i.e. x-ray irradiation and Hyp-PDT), for application in DC vaccines. While the former instigates oxidative stress over the entire cell, the latter specifically induces it in the ER (177, 178). Our data indirectly suggest that ER-directed oxidative stress induced by Hyp-PDT-treatment of the GL261 cells might be more effective in the context of DC vaccines. Indeed, Hyp-PDT-based DC vaccines yielded higher levels of tumor rejection and a stronger median survival advantage as compared to (FT+)IR-DC vaccines or IR+FT-DC vaccines in prophylactic vaccination setups. This might be attributed to our observation that Hyp-PDT-treatment of GL261 cells can potently stimulate ICD, in addition to its ability to generate OAMPs like carbonylated proteins. This enables oxidation- and danger signal-mediated immunogenic signaling dependent CTL-, Th1- and Th17-driven glioma rejection (Figure 1). Irradiation- or H_20_2-based oxidation of signaling-incompetent freeze-thawed GL261 cells, on the other hand, lacks this ICD component. Here, the oxidation-induced OAMPs associate with the vaccines' capacity to reject tumors in a presumably Th1- and CTL-dependent manner (Figure 2).

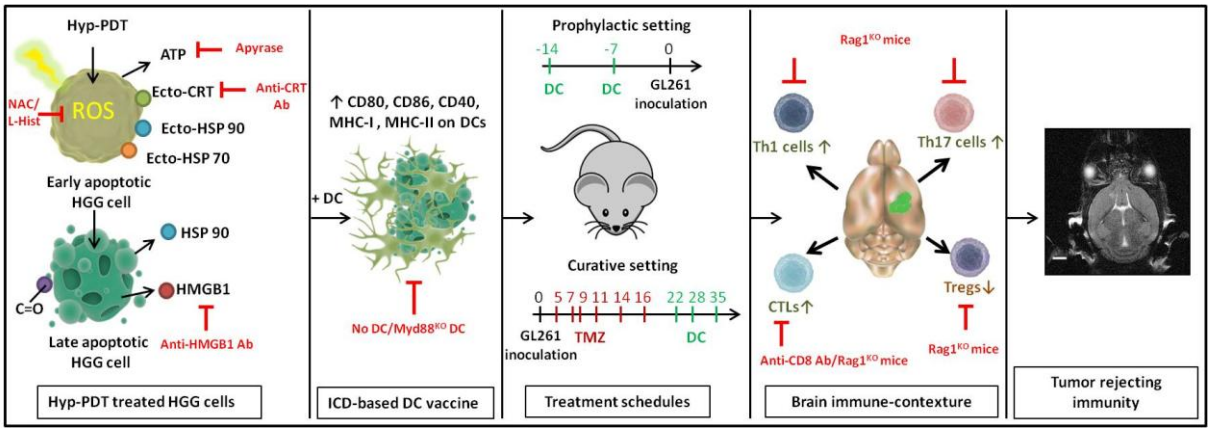

Figure 1: *DC vaccines based on Hyp-PDT elicit strong ROS- and DAMPs-dependent Th1/Th17/CTL-driven antitumor immunity against orthotopic HGG.*

As can be derived from chapters 3 and 4 and figures 1 and 2, we have put the biggest effort in the preclinical evaluation of the Hyp-PDT-based DC vaccines. In fact, FT+IR-DC vaccines are already regularly implemented in clinical practice (32, 81, 82, 114, 158-163), making extensive preclinical evaluation otiose. We simply wished to address their possible superior immunogenicity as compared to standard FT-DC vaccines, given their ability to induce oxidative modifications.

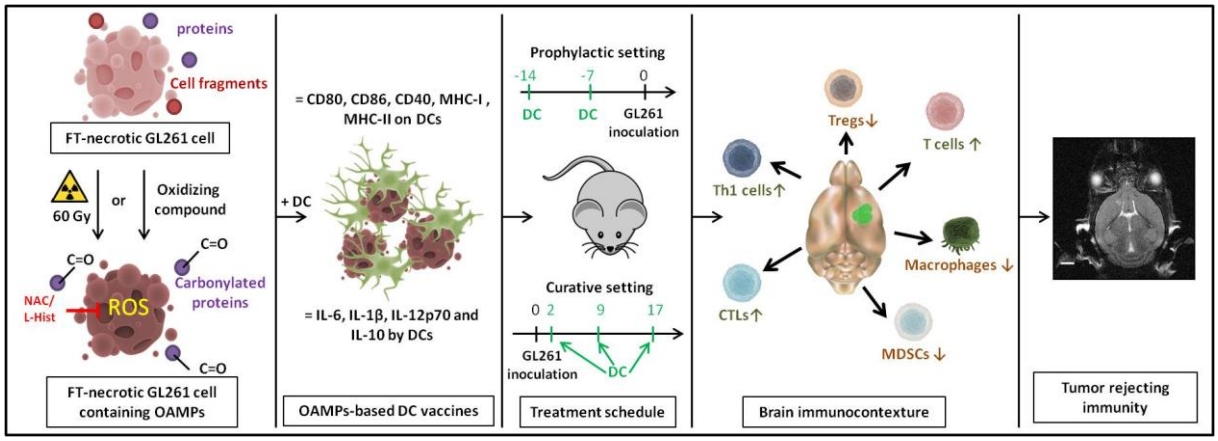

Figure 2: *OAMPs-based DC vaccines provide protective immunity associated with a favorable brain immune-contexture against intracranial HGG*

5.2. Are Th1/Th17-driving DC vaccines the next generation glioma vaccines?

The data presented in this thesis highlight the importance of eliciting anti-tumor T cell responses for effective DC vaccination. In the setting of Hyp-PDT-based DC vaccines, the antitumor effects were completely abrogated in $CD8^+$ T cell-depleted mice and $Rag1^{KO}$ mice, suggesting the critical importance of $CD8^+$ effector T cells. These results impose minimal contribution of innate immune cells to the antitumor effect of Hyp-PDT-based DC vaccines.

Our flowcytometric data confirmed these results, with increased infiltration of intrabrain Th1/CTL/Th17 cells relative to reduction in Tregs in prophylactic as well as curative Hyp-PDT-based vaccination settings. Sayour and colleagues have already demonstrated the superior informative value of the effective balances of $CD4^+$ and $CD8^+$ T cells to Tregs for predicting clinical outcomes in GBM patients (26). Of note, TMZ treatment or combined chemo-immunotherapy did not affect the NK cell frequencies in our model (data not shown). To evaluate the clinical relevance of the T cell subpopulations contributing to the immunogenicity of the Hyp-PDT-based DC vaccines in our preclinical model, we performed a retrospective analysis based on lymphocyte subpopulation-specific immune signatures in 541 newly diagnosed GBM patients (available in the TCGA database) (217, 218). Our results show a significant survival advantage in GBM patients having a strong prevalence of intratumoral Th1/Th17/CTL-linked genetic signatures (i.e., metagenes). Hence, this clinical analysis confirms the relevance of the favorable brain immune-contexture we observed in our DC vaccinated mice. Interestingly, the patient analysis uncovered the Th17 population (i.e. its corresponding molecular signatures) as being the main T cell subpopulation affecting patient survival. Th17 cells are known to play both protumorigenic (e.g. by inducing angiogenesis or exerting direct immunosuppressive effects) and antitumorigenic roles (e.g. by inducing the activation of macrophages and DCs and by stimulating the cytotoxic activity of CTLs and NK cells) in different cancer settings (219). This suggests that Th17 cells might be considered a double-edged sword depending on multiple factors like tumor type and tumor stage. The contribution of Th17 cells to antiglioma immunity is poorly characterized until now, with only one study linking IL-17 expression to survival in the HGG patients (33). In line with our data, this study designated high intratumoral IL-17A expression levels as an independent positive prognostic factor affecting the overall survival of 541 GBM patients. In order to ratify these findings, in future, detailed studies should be carried out to evaluate the anti-HGG immunological effect of Th17 cells and its possible mediators, especially in (ICD-based) DC vaccination settings.

Together, our preclinical and clinical brain immune-contexture analyses argue that next-generation (DC)-vaccines should ideally elicit potent combined Th1 and Th17 responses able to orchestrate effective cytolytic CTL responses, especially in HGG settings. In addition, our findings suggest that HGG patients representing with a pre-existing Th1/CTL/Th17 tumor immune-contexture might benefit most from (ICD-based) DC vaccination. To confirm this hypothesis it would be of great value to (re)analyze the immune infiltrates in FT(+IR)-DC vaccinated HGG patients. In the case of Hyp-PDT-based DC vaccines, new trials will have to be initiated. However, considering the fact that Hyp-PDT constitutes a FDA-approved therapy and given the notion that Hypericin has proven to be safe when used for fluorescence-guided resection in HGG patients, clinical trials testing Hyp-PDT-based DC vaccines in HGG patients can be envisaged in the near future.

5.3. DAMPs as molecular regulators of potent ICD-based DC vaccines

Our preclinical study is the first demonstration of a causal association between ICD-associated DAMPs and the efficacy of ICD-based (DC) vaccines in an orthotopic tumor model, especially for HGG. Here, while all three DAMPs contributed to the immunogenicity of the vaccine (extracellular-HMGB1>extracellular-ATP>surface-CRT), the HMGB1-MyD88 axis can be considered the predominant molecular determinant. Moreover, our data confirm the necessity of DCs as a cellular adjuvant in this glioma model. While some studies suggested the dispensability of cellular adjuvants like DCs in the context of ICD (122), the severe HGG-induced immune suppression, including the low immunogenic/tolerant state of endogenously circulating APCs (20), might make them indispensible in the setting of HGG. Interestingly, through concurrent blockade of all three major ICD-associated DAMPs, we showed that these three DAMPs account for most of ICD's *in vivo* potential, leaving little (but not null) space for contribution of other ICD-related DAMPs (like HSPs) or yet uncharacterized danger signals.

Hence, with respect to ICD-based DC vaccines, intratumoral levels of ICD-related DAMPs might also constitute promising prognostic and predictive biomarkers (either as single DAMPs or as a cluster). Retrospective analysis of data retrieved from published ICD-based DC vaccination trials or prospective analysis of new trials (as applicable for Hyp-PDT) should corroborate this hypothesis. High serum or intratumoral HMGB1 levels have for instance been shown to correlate with improved patient survival following radiotherapy, anthracycline-based chemotherapy and treatment with oncolytic adenoviruses (179). Interestingly, Di Nicola *et al.* showed that B cell lymphoma patients respond better to DC vaccines loaded with autologous cancer cells exposed to a multimodal ICD regimen (consisting of heat shock, γ-ray and UVC ray) if the neoplastic cancer cells emitted high amounts of CRT and HSP90 following the cell death protocol (193).

5.4. How to reduce the chance of clinical failure of promising preclinical DC vaccines?

It needs to be highlighted that more attention should be paid to some clinically-driven considerations when evaluating DC vaccines in preclinical experiments. Indeed, the rigid clinically-relevant operational parameters are often not tested in the early stages of anti-cancer vaccine development. Eventually these parameters create a stiff "bottleneck" that causes failure of several anti-cancer vaccines that pre-clinically showed great translational promise. First, one should consider the requirement of 100% mortality of the tumor cells before *in vivo* application. Subcutaneous injection of irradiated tumor cells has already induced subcutaneous tumor growth in one GBM patient (79). In general, most single treatment modalities (except for non-immunogenic freeze-thawing) cannot meet this requirement, postulating their combination with other (potentially less immunogenic) cell killing

modalities. A second clinical consideration (especially in multi-center clinical trials) is the fact that most tumor specimens arrive in the lab in a frozen state. This implies that a significant number of cells have already undergone non-immunogenic necrosis before the experimental cell killing strategies can be applied. In case of ICD inducers, this could potentially hamper the immunogenicity of the tumor cells as these modalities mainly rely on active danger signaling pathways. We have taken these two considerations into account for both the OAMPs-based DC vaccines and the Hyp-PDT-based DC vaccines. Finally, for a more clinically relevant evaluation of the effect of immunogenic DC vaccines on tumor cell stromal interactions, orthotopic tumor inoculation should be preferred. As can be deduced from table 3, heterotopic models are most commonly used in DC vaccine settings. However, these models suffer from several caveats like the inability to recapitulate the early interaction between transformed cells and the immune system and the incompatibility between the cancer type and the site of transplantation. Therefore, subcutaneous non-orthotopic models could be questioned as the Achilles' heel of DC vaccination research. For this reason and others (see box 1), we opted for the orthotopic GL261 and CT2A models to conduct our preclinical experiments. Nevertheless, it has to be admitted that no rodent model can perfectly represent all immunological parameters.

5.5. Towards the clinical implementation of Hyp-PDT-based DC vaccines

We believe that on the basis of the results described in chapter 4, pending final preclinical checks (relating to safety and regulatory parameters) and final human *ex vivo* experiments (with autologous patient material), Hyp-PDT-based anti-glioma vaccines can be translated towards the clinical in the near future. Beyond the application in glioma setting, our group is currently also testing Hyp-PDT-based DC vaccines in ovarian cancer.

To allow straightforward application of Hyp-PDT-based DC vaccines in the clinical setting (including in multicenter clinical trials), we envisage to apply Hyp-PDT-treatment *ex vivo* on a homogenized single cell suspension derived from a snap-frozen HGG tumor specimen. In first instance, phase I and II clinical trials of Hyp-PDT-based DC vaccines should focus on safety and immune monitoring parameters. With regard to the latter, it will be imperative to study the basal immune contexture in the resected HGG tumor tissue (by means of flowcytometry/immunohistochemistry/DNA-microarrays). These studies will allow us to delineate the single or combined immune cell populations (e.g. formatted as an immunoscore) that may favorably impact patient outcome. Moreover, evaluation of pre- and postvaccination frequencies of systemic immune cell subtypes can be of interest for characterizing the immune populations mainly affected by the vaccine. In addition, it would be very informative to evaluate several ICD-related parameters in the *ex vivo* Hyp-PDT tumor cell homogenate. Hyp-PDT-treatment should be able to induce sufficient apoptosis, ER stress and significant upregulation of pivotal ICD-related DAMPs (i.e. CRT, HSP70/90, HMGGB1 and ATP).

Correlating these data with patient survival or immune monitoring data could allow us to determine specific cut-off values for predictive purposes. Besides this quality control at the level of the tumor cells, the DCs will also be routinely monitored for upregulation of maturation markers and for their cytokine profile.

With the clinical application of Hyp-PDT-based DC vaccines in the offing, one should also start envisaging how to implement this immunotherapy in the standard of care treatment regimen of HGG patients. Based on our therapeutic preclinical experiments, applying ICD-based DC vaccines subsequent to a cytoreductive and lymphodepleting TMZ regimen might constitute a promising strategy. Indeed, several groups have already uncovered an inverse association between tumor burden and immunotherapy effectiveness (40, 81). Moreover, Sampson *et al.* have recently demonstrated that adjuvant TMZ regimens that induce greater degrees of lymphopenia (dose-intensified regimen, 100 mg/m^2 for 21 days during each 28-day cycle) can boost antitumor T cell responses induced by EGFRvIII peptide vaccines to a higher extend as compared to TMZ regimens that induce less lymphopenia (standard regimen, 200 mg/m^2 for 5 days during each 28-day cycle) (220). Here, lymphodepletion, might lower the threshold for T cell activation and might trigger homeostatic T cell proliferation in a thymic-independent, yet antigen-dependent manner. Additionally, TMZ-induced apoptosis can release an immense pool of tumor antigens and has been shown to enhance the priming of CD4$^+$ and CD8$^+$ T cells following DC vaccination in the GL26 glioma model (221).

Unfortunately, even the most potent active immunotherapeutic strategies like (ICD-based) DC vaccines will be hampered by the presence of immunomodulatory checkpoint molecules (like PD-1 and CTLA-4) that can inhibit cytotoxic immune responses or even induce immune tolerance. The development of drugs that can unleash these inhibitory molecules has become one of the most active areas in oncology. This provides the opportunity to combine checkpoiunt inhibitors with DC-based immunotherapy. In the light of our findings, we believe that HGG patients representing with an unfavorable (Th1/Th17/CTL-low) tumor immune-contexture might benefit most from this combination immunotherapy. The synergistic action of CTLA-4 blocking Abs in combination with DC therapy has already been demonstrated in advanced melanoma patients and several other trials evaluating this approach are on the horizon (40, 222, 223). In August 2015, a phase I clinical trial was set-up to evaluate the safety profile of combined nivolumab (a fully human anti-PD-1 antibody) and DC vaccine treatment in recurrent HGG patients (NCT02529072). Hence, it would be interesting to test the combination of Hyp-PDT-based DC vaccines and checkpoint blockade in our preclinical HGG models.

Abstract

HGG represents a highly aggressive CNS malignancy with mortality rates matching incidence rates. DC vaccines aiming to stimulate the endogenous antiglioma immune response constitute a safe and promising treatment modality. Thus far, the efficacy of DC vaccines is considered suboptimal, in part owing to a lack of attention towards the immunogenicity of the tumor lysate used as an antigen source for loading the DC vaccines. Given the recent evidence that oxidation and the resultant oxidation-associated molecular patterns (OAMPs) can impact tumor cell immunogenicity, we applied two physico-chemical oxidizing therapies to obtain a more immunogenic tumor cell cargo: x-ray irradiation and hypericin-based photodynamic therapy (Hyp-PDT).

DC vaccine immunogenicity was investigated in the orthotopic, syngeneic GL261 HGG model. Mice prophylactically immunized with DC vaccines loaded with x-ray irradiated freeze-thawed lysates survived significantly longer as compared to mice vaccinated with DCs loaded with freeze-thaw lysate only. This was associated, both in prophylactic and curative vaccination setups, with increased intrabrain infiltration of Th1 cells/CTLs and reduced infiltration of Tregs, MDSCs and TAMs. Through further application of anti-oxidants and hydrogenperoxide, we found a striking correlation between the amount of lysate-associated protein carbonylation/OAMPs and the capacity of the DC vaccines to mediate glioma rejection. This result fostered the preclinical investigation of other strong oxidizers like Hyp-PDT, a potent type II inducer of ICD, in our HGG model. Hyp-PDT induced prototypical ICD in the GL261 cell line. In prophylactic as well as curative vaccination settings, both biologically and clinically-relevant versions of Hyp-PDT-based ICD-based DC vaccines provided strong anti-HGG survival benefit. The ability of DC vaccines to elicit HGG rejection was significantly blunted if cancer cell-associated ROS and emanating danger signals were blocked (either singly or concomitantly; showing hierarchical effect on immunogenicity i.e. extracellular-HMGB1>extracellular-ATP>ecto-calreticulin) or if DCs, the DCs-associated MyD88 signal or the adaptive immune system (especially $CD8^+$ T cells) were depleted. In a curative setting, ICD-based DC vaccines synergized with standard-of-care chemotherapy (temozolomide) to increase survival of HGG-bearing mice by ~300% resulting in ~50% long-term survivors. Additionally, DC vaccines also induced an immunostimulatory shift in the brain immune-contexture from Tregs to Th1/cytotoxic T lymphocytes/Th17 cells. Analysis of the TCGA glioblastoma-cohort confirmed that increased intra-tumor prevalence of Th1/cytotoxic T lymphocytes/Th17 cells-linked genetic signatures associated with good patient prognosis.

Our research demonstrates that combining DC vaccination with oxidizing treatment modalities can induce Th1-driven antitumor immune responses, enabling CTL-mediated

glioma rejection. ICD-based DC vaccines exploiting ER-directed oxidative stress might be considered next generation DC vaccines.

Summary

In this project, we investigated two approaches to enhance the immunogenicity of tumor lysate preparations used for loading dendritic cell (DC) vaccines as a treatment strategy for malignant brain tumors (i.e. high-grade gliomas). DC vaccines aim to stimulate the endogenous antitumor immune response. This therapy is safe and has been shown to elicit antitumor T cell responses and survival benefit in glioma patients. Nevertheless, its efficacy is still considered suboptimal, owing to the severe tumor-induced immunosuppression and, amongst other DC vaccine parameters, a lack of attention towards the immunogenicity (i.e. the ability to provoke an immune response) of the tumor lysate used as an antigen source for loading DC vaccines.

Given the recent evidence that oxidation and the related oxidation-associated molecular patterns (OAMPs) can impact tumor cell immunogenicity, we applied two physico-chemical oxidizing therapies to obtain an immunogenic tumor cell cargo: x-ray irradiation and hypericin-based photodynamic therapy (Hyp-PDT). While the former instigates oxidative stress over the entire cancer cell, the latter specifically induces it in the endoplasmatic reticulum (ER), this way triggering prototypical immunogenic cell death (ICD). ICD is a cell death subroutine which is apoptotic in nature, but associated with enhanced immunogenicity, owing to the emission of certain immune activating factors, also called damage-associated molecular patterns (DAMPs).

During my PhD research we have shown that irradiation can enhance the *in vivo* immunogenicity of freeze-thawed necrotic cancer cells used as an antigen source for loading DCs. This was demonstrated in an orthotopic mouse glioma model. This survival advantage was associated with increased infiltration of antitumor immune cells (i.e., Th1 cells and cytotoxic T lymphocytes) in the tumor environment of vaccinated mice (both in preventive/prophylactic as well as therapeutic/curative vaccination settings). At the same time, the infiltration of certain immunosuppressive immune cells (like Tregs and myeloid-derived suppressor cells) in the brain was reduced. The increased immunogenicity of DC vaccines loaded with irradiated freeze-thaw lysate was, at least in part, mediated by irradiation-induced OAMPs like protein carbonylation. Indeed, through further application of antioxidants and hydrogenperoxide (a strong oxidizer), we found a striking correlation between the amount of lysate-associated protein carbonylation/OAMPs and the capacity of the DC vaccines to mediate glioma rejection.

Previous research has demonstrated that ER-directed oxidative stress, as elicited by Hyp-PDT, might be more immunogenic in nature than collateral ER stress, this way inducing more potent ICD. Hence, ICD triggered by Hyp-PDT might constitute a very attractive treatment modality to increase the efficacy of DC vaccines. In this project, we have shown that Hyp-PDT can induce prototypical ICD in glioma cell lines. In our mouse glioma model (involving

prophylactic as well as curative settings) we have shown that both biologically and clinically-relevant versions of ICD-based DC vaccines provided a strong antiglioma survival benefit. Interestingly, curative treatment with the ICD-based DC vaccines synergized with standard-of-care chemotherapy (temozolomide), resulting in 50% tumor rejection.

This ability of the ICD-based DC vaccines to elicit glioma rejection was blunted if cancer cell-associated oxidation or emanating danger signals were blocked. Additionally, HGG rejection was dependent on an intact adaptive immune system and upon a productive (MyD88-signalling dependent) cancer cell-DC interface. Additionally, our ICD-based DC vaccines induced infiltration of immune effector T cells (i.e. Th1 cells, cytotoxic T lymphocytes and Th17 cells) in the tumor environment, while at the same time reducing the accumulation of immunosuppressive Tregs. Analysis of patient data, collected in the TCGA (The Cancer Genome Atlas) glioblastoma cohort, confirmed the clinical relevance of this immunostimulatory shift in brain immune contexture. This patient analysis revealed that an increased intratumor prevalence of genetic signatures associated with Th1/cytotoxic T lymphocytes/Th17 cells correlated with good patient prognosis.

This research highlights the importance of oxidation-driven tumor cell immunogenicity in the context of DC vaccines. This finding has important socio-economic impact as it should incite researchers to step away from standard non-immunogenic freeze-thaw lysates. At the same time it may stimulate researchers to test other strong oxidizers or ICD-inducers in combination with DC vaccines. Moreover, the potent Th1/Th17-driven antitumor immunity induced by Hyp-PDT-based DC vaccines warrants urgent clinical investigation of this therapy in glioma patients, but also in other cancer settings. Given the favorable safety profile of Hypericin and its approval by the Food and Drug Administration (in the United States), this can be envisaged for the near future.

Samenvatting

In dit project hebben we twee benaderingen bestudeerd om de immunogeniciteit van tumorlysaat-bereidingen te verbeteren. Dit tumorlysaat wordt vervolgens gebruikt om dendritische cel (DC) vaccins te beladen als behandelingsstrategie voor kwaadaardige hersentumoren (i.e. hooggradige gliomen). DC-vaccins beogen het stimuleren van een endogene immuunrespons tegen de tumor. Deze therapie is veilig bevonden en kan T-cel-reacties en een verbeterde overleving bewerkstelligen in gliomapatiënten. Toch wordt de werkzaamheid van DC-vaccins nog steeds ondermaats bevonden. Dit is te wijten aan de ernstige immuunsuppressie die wordt geïnduceerd door het glioom, alsook aan een gebrek aan aandacht voor de immunogeniciteit (i.e. de mogelijkheid om een immuunrespons uit te lokken) van het tumorlysaat dat gebruikt wordt als bron van antigenen om de DCs te beladen.

Gezien het recente bewijs dat oxidatie en de gerelateerde oxidatie-geassocieerde moleculaire patronen (OAMPs) de immunogeniciteit van tumorcellen kunnen beïnvloeden, hebben we twee fysico-chemische therapieën toegepast om een meer immunogeen tumorlysaat te bekomen: bestraling met x-stralen en fotodynamische therapie met hypericine (Hyp-PDT). Terwijl de eerste therapie oxidatieve stress induceert over de gehele cel, induceert Hyp-PDT dit specifiek in het endoplasmatisch reticulum (ER). Op deze manier veroorzaakt Hyp-PDT een immunogene celdood (ICD). Deze vorm van celdood, die apoptotisch is van aard, gaat samen met een versterkte immunogeniciteit en dit door een verhoogde vrijzetting van factoren die het immuunsysteem activeren, ook wel DAMPs genoemd.

Tijdens mijn doctoraatsproject hebben we aangetoond dat het bestralen van gevries-dooide necrotische kankercellen (die gebruikt worden voor het beladen van de DC vaccins) de immunogeniciteit van de DC-vaccins kan verhogen. Dit werd onderzocht in een orthotoop muismodel voor glioma. Het overlevingsvoordeel van de muizen ging gepaard met een verhoogde infiltratie van antitumorale immuuncellen (i.e. Th1 cellen en cytotoxische T cellen) in de omgeving van de tumor van gevaccineerde muizen. Gelijktijdig leidde dit tot een verminderde infiltratie van immuunsuppressieve cellen (zoals regulatoire T cellen en tumor-geassocieerde macrofagen) in de hersenen. De verhoogde immunogeniciteit van de DC-vaccins beladen met bestraald vries-dooi lysaat werd, tenminste deels, gemedieerd door de door bestraling geïnduceerde OAMPs zoals gecarbonyleerde proteïnen. Door verdere toepassing van antioxidanten en waterstofperoxide (een sterke oxidator) konden we een sterke correlatie aantonen tussen de hoeveelheid gecarbonyleerde proteïnen in het tumorlysaat en de capaciteit van de DC-vaccins om de gliomen af te weren.

Voorgaand onderzoek heeft aangetoond dat oxidatieve stress gericht op het ER, zoals uitgelokt wordt door Hyp-PDT, sterker immunogeen kan zijn dan collaterale stress in het ER. Oxidatieve stress gericht tot het ER zou ook een krachtige vorm van ICD kunnen uitlokken. Vandaar dat ICD, in dit geval veroorzaakt door Hyp-PDT, een zeer aantrekkelijke behandelingsmethode zou kunnen zijn om de werkzaamheid van DC-vaccins te verhogen. In dit project hebben we aangetoond dat Hyp-PDT een prototypische vorm van ICD kan uitlokken in de gliomacellijnen. We hebben in onze muis gliomamodellen aangetoond dat zowel biologisch als klinisch relevante versies van de op ICD-gebaseerde DC vaccins een

sterk overlevingsvoordeel opleverden in de glioma-dragende muizen. Meer nog, curatieve behandeling met deze Hyp-PDT DC-vaccins leidde tot synergie in combinatie met het chemotherapeuticum temozolomide. Deze combinatietherapie kon 50% van de muizen genezen van glioma.

De mogelijkheid van ICD-gebaseerde DC vaccins om gliomen af te weren kon onderdrukt worden door de oxidatie in de kankercellen of de vrijgestelde DAMPs te blokkeren. Bovendien was de glioma-afweer afhankelijk van een intact adaptief immuunsysteem en een productieve interfase tussen de kankercel en de DCs (op een MyD88-afhankelijke manier). Verder induceerden de op ICD-gebaseerde DC-vaccins een verhoogde infiltratie van antitumorale T-cellen (Th1 cellen, cytotoxische T cellen en Th17 cellen) in de omgeving van de tumor. Gelijktijdig accumuleerder er mincer immuunsuppressieve regulatoire T-cellen. Een analyse van patiëntendata, verzameld in een TCGA glioblastoma-databank van de TCGA (The Cancer Genome Atlas), bevestigde de relevantie van deze immuunstimulerende shift in de hersenen. Deze patiëntenanalyse onthulde dat een verhoogde prevalentie van Th1/cytotoxische T cellen/Th17 genetische handtekeningen in de tumor associeert met een betere prognose van gliomapatiënten.

Dit onderzoek belicht het belang van een door oxidatie gedreven immunogeniciteit van de tumorcellen in de context van DC vaccins. Deze bevindingen hebben een aanzienlijke socio-economische impact, aangezien ze wetenschappers zouden moeten aanzetten om af te stappen van het gebruik van standaard (niet immunogeen) vries-dooi lysaat. Tegelijkertijd zou het wetenschappers moeten overtuigen om andere sterk oxiderende behandelingen of uitlokkers van ICD te testen in combinatie met DC vaccins. Bovendien vraagt de krachtige Th1/Th17-gedreven antitumorale immuunrespons, die we konden uitlokken met de op Hyp-PDT-gebaseerde DC vaccins, om een dringende klinische evaluatie van deze therapie. Gezien het gunstige veiligheidsprofiel van hypericine en de goedkeuring van dit geneesmiddel door de Food and Drug Administration (een overheidsorgaan in de Verenigde Staten), kan dit voorzien worden voor de nabije toekomst.

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melanoma. Clinical cancer research : an official journal of the American Association for Cancer Research. 2009;15(19):6267-76.
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Curriculum Vitae

Lien Vandenberk was born in Lommel (Belgium) on January 25th, in 1987. She graduated from secondary school in 2005 at the Wico Campus Sint-Jozef in Lommel with a major in Latin and Science. In 2005 she started her studies in Biomedical Sciences at the KU Leuven. During this study she performed internships at the Stem Cell Institute Leuven (SCIL) and in the laboratory of Pediatric Immunology. In 2009 she started her master thesis work under supervision of Prof. Dr. Van Gool and Dr. Tina Verschuere in the laboratory of Pediatric Immunology. In 2010 she graduated magna cum laude as Master in Biomedical Sciences with a major in Biomedical Research and a minor in Management and Communication in Biomedical Sciences. Subsequently, she started her PhD in the laboratory of Pediatric Immunology under supervision of Prof. Dr. Stefaan Van Gool, Dr. Abhishek D. Garg and Prof. Dr. Adrian Liston.

Bibliography and awards

❖ Publications in Peer-reviewed journals

1. **Vandenberk L**, Van Gool SW. Treg infiltration in glioma: a hurdle for antiglioma immunotherapy. Immunotherapy. 2012;4(7):675-8.
2. Koks CA, Garg AD, Ehrhardt M, Riva M, **Vandenberk L**, Boon L et al. Newcastle disease virotherapy induces long-term survival and tumor-specific immune memory in orthotopic glioma through the induction of immunogenic cell death. International Journal of Cancer. 2015;136(5):E313-25.
3. **Vandenberk L**, Garg AD, Verschuere T, Koks CA, Belmans J, Beullens M et al. Irradiation of necrotic cancer cells, employed for pulsing dendritic cells (DCs), potentiates DC vaccine-induced antitumor immunity against high-grade glioma. OncoImmunology. http://dx.doi.org/10.1080/2162402X.2015.1083669
4. Garg AD, Galluzzi L, Apetoh L, **Vandenberk L**, Zitvogel L, Agostinis P et al. Molecular and Translational Classifications of DAMPs in Immunogenic cell Death. Frontiers in Immunology. 2015;588(6).
5. **Vandenberk L**, Belmans J, Van Woensel M, Riva M, Van Gool SW. Exploiting the immunogenic potential of cancer cells for improved dendritic cell vaccines. Frontiers in Immunology. 2016; 663(6)
6. **Vandenberk L***, Garg AD*, Koks CA, Verschuere T, Agostinis P, Van Gool SW et al. Dendritic cell vaccines based on immunogenic cell death elicit danger signals and T cells-driven rejection of high-grade glioma. Science Translational Medicine *(Revised accepted).* ***Shared first authorship**
7. Garg AD, **Vandenberk L**, Feng S, Van Eygen S, Maes J, Fasche T, de Witte P, Van Gool S, Petri S, Agostinis P. Pathogen response like chemokines-based neutrophil recruitment: a novel cell death-associated danger signaling pathway. *(Manuscript in preparation)*

❖ Grants and Awards

1. Recipient of the Strategic Basic Research grant from the Agency for Innovation by Science and Technology in Flanders (IWT Vlaanderen) (2011-2015)
2. Belgian Brain Tumor Support (BBTS) prize for the results obtained in my PhD project (December 2013)

❖ Poster presentations at scientific meetings

1. Oncoforum, Leuven (Belgium), May 2014
   Title: Irradiation of necrotic cancer cells, employed for pulsing dendritic cells (DCs), potentiates DC vaccine-induced antitumor immunity against high-grade glioma
2. Immunology Summer School, Leuven (Belgium), September 2014
   Title: Irradiation of necrotic cancer cells, employed for pulsing dendritic cells (DCs), potentiates DC vaccine-induced antitumor immunity against high-grade glioma
3. SITC 2014 annual meeting, National Harbor, Maryland (US), November 2014,
   Title: Irradiation of necrotic cancer cells, employed for pulsing dendritic cells (DCs), potentiates DC vaccine-induced antitumor immunity against high-grade glioma
4. Cancer Immunotherapy (CIMT) meeting, Mainz (Germany), May 2015
   Title: Irradiation of necrotic cancer cells, employed for pulsing dendritic cells (DCs), potentiates DC vaccine-induced antitumor immunity against high-grade glioma

❖ Oral presentations at scientific meetings

1. Experimental Neuro-oncology Meeting, Minden (Germany), April 2011
   Title: Immunotherapy for malignant glioma: preclinical models to improve efficacy of the immune attack
2. HGG-Immuno meeting, Leuven (Belgium), December 2011
   Title: Modulation of tumor-infiltrating lymphocytes as a tool to improve efficacy of immunotherapy for high-grade glioma
3. HGG-Immuno meeting, Leuven (Belgium), December 2012
   Title: Inhibition of indoleamine 2,3-dioxygenase as an adjuvant treatment strategy for high-grade glioma to improve the chemo-immunotherapeutic efficacy
4. Rostock symposium for tumor immunology in pediatrics, Rostock (Germany), February 2013
   Title: Inhibition of indoleamine 2,3-dioxygenase as an adjuvant treatment strategy for high-grade glioma to improve the efficacy of immunotherapy
5. Experimental Neuro-oncology meeting, Minden (Germany), April 2014
   Title: Irradiation of freeze-thaw lysate used to pulse dendritic cells further increases anti-tumor immunity in a mouse model for high-grade glioma
6. Annual HGG-Immuno meeting, Würzburg (Germany), November 2014
   Title: Improving the immunogenicity of tumor lysate preparations to enhance the efficacy of DC-based immunotherapy in glioma

7. 13th symposium of the Belgian association for neuro-oncology, Brussels (Belgium), December 2014
   Title: Improving the immunogenicity of tumor lysate preparations to enhance the efficacy of DC-based immunotherapy in glioma
8. Rostock symposium for Tumor Immunology and Brain Tumor Research in Pediatrics, Rostock (Germany), February 2015
   Title: Improving the immunogenicity of tumor lysate preparations to enhance the efficacy of DC-based immunotherapy in glioma