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Detection of immunogenic cell death after oncolytic virotherapy of glioblastomas

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List of abbreviations

Ad-WT	wild-type adenovirus (subtype 5)
BBB	blood-brain barrier
CNS	central nervous system
DAMP	damage-associated molecular pattern
DAPI	4′,6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's Medium
DPBS	Dulbecco's phosphate-buffered saline
ELISA	enzyme-linked immuno-sorbent assay
EMA	European Medicines Agency
FCS	fetal calf serum
FDA	United States Food and Drug Administration
GBM	glioblastoma
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
HLA	human leukocyte antigen
HMGB1	high-mobility group box 1 protein
HSP70	heat shock protein 70
HSV	herpes simplex virus
ICD	immunogenic cell death
ICI	immune checkpoint inhibitor
IDH	isocitrate dehydrogenase
IF	immunofluorescence
IFU	infectious units
IN	intranasal
INA	intranasal application
IT	intratumoural
LN-229	Lausanne-229

MGMT	O(6)-methylguanine methyltransferase
MOI	multiplicity of infection
OAV	oncolytic adenovirus
OV	oncolytic virus
OVT	oncolytic virotherapy
P/S	penicillin-streptomycin
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PD-1	programmed cell death protein 1
PD-L1	programmed cell death ligand 1
PRR	pattern recognition receptor
RT	room temperature
SCID	severe combined immunodeficiency syndrome
TBS	tris-buffered saline
TBS-T	tris-buffered saline with TWEEN®
TGF-β	transforming growth factor β
TK	thymidine kinase
TME	tumour microenvironment
TMZ	temozolomide
TRIS	tris(hydroxymethyl)-aminomethane
T-Vec	Talimogene Laherparecvec
U87MG	Uppsala 87 malignant glioma
VEGF	vascular endothelial growth factor
WHO	World Health Organisation
YB-1	Y-box binding protein 1

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1 Introduction

1.1 Glioblastoma

Glioblastoma (GBM) is the most common primary malignant brain tumour in adults and known for its dismal prognosis and inevitable fatal outcome. The median survival time at diagnosis remains less than two years, and the 5-year survival rate is less than 10 %, despite immense efforts and multimodal therapeutic approaches (Stupp et al., 2009, Stupp et al., 2017). Due to its infiltrative properties, aggressive growth, and formation of necrosis, it is classified as grade 4, the highest grade in the World Health Organisation (WHO) classification of tumours of the central nervous system (CNS; Louis et al. (2021)).

GBM accounts for the majority of all gliomas (59,2 %) and occurs at an incidence of 3.26 per 100,000 based on registry data of tumours diagnosed in the United States from 2015 to 2019 (Leece et al., 2017, Ostrom et al., 2022). From a global perspective, incidences are highest in North America, Australia, and Northern and Western Europe. Incidence also varies by sex and race, with GBM being 1.60 times more common in men than in women and 1.95 times more common in Whites than in Blacks. GBM is primarily diagnosed in older age groups, with the median age at diagnosis being 65 years and the highest incidence rate in the 75-84 years age group (15.17 per 100,000).

Approximately 90 % of GBM develop rapidly *de novo*, meaning that there is no clinical or histological evidence of a malignant precursor lesion, and are therefore considered primary GBMs. In other words, these tumours are in the highest level of the WHO classification as soon as they arise (Ohgaki and Kleihues, 2007). Histopathologically to be distinguished from primary GBMs are secondary GBMs, which progress from diffuse astrocytoma WHO grade 2 or anaplastic astrocytoma WHO grade 3. These secondary tumours occur at a younger age with a median age at diagnosis of 45 years (Ohgaki and Kleihues, 2005), show no to comparatively little necrosis (Scherer, 1940) and provide a significantly better prognosis (Ohgaki et al., 2004a, Khan et al., 2017). However, it is important to note that this significantly longer survival of patients with secondary GBM is

largely attributable to the younger age of onset. In age-adjusted multivariate analyses, there was no longer a statistically significant difference in survival and young age was shown to be an independent prognostic factor for both primary and secondary GBM (Ohgaki et al., 2004b, Ohgaki and Kleihues, 2005).

In recent decades, molecular features have become increasingly important in the histopathological diagnosis of tumours, and since the 4th edition of the WHO classification of CNS tumours published in 2016, these have become firmly established in the assessment of brain tumours, leading to a re-organisation of the classification (Louis et al., 2016, Zhang et al., 2020). One of these main molecular features of secondary GBM are specific point mutations in the genes encoding isocitrate dehydrogenase (IDH), leading not only to decreased activity of the wild-type enzyme, that converts isocitrate to α-ketoglutarate, but also to altered levels of hypoxia-inducible factor 1α (HIF-1α), considered an important driver of tumour progression via the activation of angiogenesis and metabolic reprogramming (Zhao et al., 2009, Domènech et al., 2021). Primary GBM do not show these point mutations and are hence referred to as IDH-wildtype. Since the first description of IDH-mutations by Parsons et al. (2008), more and more molecular differences between primary and secondary GBM have been identified with respect to tumour biology and glioma genesis. Based on numerous studies, it can be assumed that primary and secondary GBM arise from different progenitor cells and along separate genetic pathways; IDH status therefore serves as a cell lineage marker in this context (Ohgaki and Kleihues, 2007, Ohgaki and Kleihues, 2013).

Another molecular marker that has been incorporated into routine clinical practice is the promoter methylation status of the gene for the DNA repair protein O(6)-methylguanine methyltransferase (MGMT). Active MGMT is associated with resistance to alkylating agents such as temozolomide (TMZ) by direct DNA repair (Fu et al., 2012). Vice versa, silencing or down-regulation of MGMT (e.g. by promoter methylation) is associated with improved therapeutic efficacy of TMZ and prolonged overall survival (OS) of patients (Hegi et al., 2005). In summary, both IDH-mutations as well as MGMT promoter methylation status have been shown to be independent favourable prognostic factors in several studies (Hegi

et al., 2005, Chen et al., 2016). Although the majority of these studies have shown this effect specifically after chemotherapy with TMZ, it is interesting to note that it is also true irrespective of treatment.

The current best standard of care consists of maximum reasonable surgical excision with preservation of neurological function, followed by adjuvant radio-chemotherapy with TMZ (marketed as Temodal in Europe) (Brown et al., 2016, Youngblood et al., 2021). The combination of TMZ and accompanying radiation is known as the "Stupp regimen" and, since Stupp et al. (2005) showed a significant survival benefit in their large clinical trial in 2005, has become an integral part of GBM therapy to this day. TMZ is an orally applied alkylating agent that acts as a non-selective cytotoxic chemotherapeutic agent by inducing DNA cross-linking, abnormal base pairing as well as DNA strand breaks, ultimately leading to proliferation stop or apoptosis (Roos et al., 2007). Furthermore, continuous administration of TMZ depletes cells of MGMT, a DNA repair enzyme responsible for mending damage induced by alkylating agents (Tolcher et al., 2003). In this specific chemotherapy regimen against GBM, daily doses of TMZ are combined with focal fractionated irradiation in 30 sessions of 2 Gy each, for a total dose of 60 Gy. The overall treatment is administered over several months and can be arduous and stressful for patients, both because of the side effects and because of the need to be in hospital almost every day.

In addition to this standardised radio-chemotherapy protocol for all patients with newly diagnosed GBM, other therapeutic agents are given on an experimental basis, mostly off-label, although there is little evidence of improvement in OS or quality of life. One of these agents is bevacizumab, an anti-angiogenic drug, that acts by inhibiting the vascular endothelial growth factor (VEGF). Bevacizumab was initially given credit to slow down tumour neo-angiogenesis, thus stemming one of the major hallmarks of cancer (Garcia et al., 2020). Although several phase II trials have shown a survival benefit in patients with newly diagnosed GBM (Vredenburgh et al., 2011, Omuro et al., 2014), this previously observed survival benefit was unfortunately not confirmed in large randomised trials such as the AVAglio trial, and toxicities were significantly more common in patients receiving bevacizumab (Chinot et al., 2014). As bevacizumab has potent

anti-edema effects and helps counteract the mass effect in non-resectable tumours by reducing capillary permeability, it is still in use as a supportive therapeutic agent and especially as a salvage therapy in recurrent GBM (Khasraw et al., 2014). This is just one of many examples how, despite great efforts and new drug developments, OS has barely improved since Stupp et al. set a new standard of care in 2005.

The barriers to a more effective therapy of intracerebral tumours are manifold, starting with a number of physiological constraints such as the blood-brain barrier (BBB). At its core, the BBB is formed by tight junctions of the cerebral capillary endothelium in collaboration with other cells such as pericytes, astrocytes or macroglia, which form a continuous lipid layer and restrict the influx of molecules from the bloodstream into the brain (Abbott et al., 2010, Liebner et al., 2018). Due to its lipophilic nature, TMZ can cross the BBB relatively freely and reach effective drug concentrations in the CNS, which sets it apart from the majority of chemotherapeutic agents (Ortiz et al., 2021). The BBB (or alternatively the blood-brain-tumour barrier, BBTB) not only passively impedes the transport of small molecules, but also carries efflux pumps such as the ATP-binding P-glycoprotein on the luminal side to actively remove foreign substances and prevent the influx of some lipophilic molecules, xenobiotics and drugs (Löscher and Potschka, 2005). This mechanism of multidrug resistance further complicates the challenge of ensuring an effective CNS permeation. In addition, cerebral endothelial cells exhibit a marked deficiency of pinocytic enzymes which are essential for cellular transcytosis and therefore further enhance the selectivity of transport across the BBB (Lesniak and Brem, 2004).

But that is not all: the enormous metabolic demand and rapid growth of high-grade gliomas create local areas of hypoxia and necrosis, which subsequently trigger the release of VEGF and the stabilisation of HIF-1α (Domènech et al., 2021). Together with other pro-angiogenic markers such as transforming growth factor β (TGF-β) secreted by glioma cells, this leads to the formation of abnormal blood vessels and a breakdown of the BBB, further contributing to the heterogeneous distribution of anti-cancer drugs (van Tellingen et al., 2015, Wirsik et al., 2021).

However, delivery is not the only challenge to effective GBM treatment. GBMs are known to establish an immuno-suppressive "cold" tumour microenvironment (TME) that favours glioma cell growth and further impedes a potent host immune response (for review, see DeCordova et al. (2020)). This immunosuppression is mediated by innumerable pathways (Himes et al., 2021), of which only a few are mentioned here as examples. For instance, GBM shows almost complete resistance to complement-mediated clearance, the very first line of defence of innate immunity, through the expression of complement regulators such as factor H on the surface of glioma cells, thus preventing the formation of C3 convertases (Junnikkala et al., 2000). Furthermore, macrophages infiltrating the TME are induced by anti-inflammatory cytokines such as interleukin-4 to polarise towards the immuno-suppressive M2-phenotype, thereby secreting anti-inflammatory cytokines and growth factors by themselves (Mantovani et al., 2002, DeCordova et al., 2020).

In addition, GBM exerts extensive control over the adaptive immunity, as evidenced by a large increase in $CD4^{+}CD25^{+}$ immuno-regulatory T cells which carry mostly immuno-suppressive characteristics in favour of the tumour (El Andaloussi and Lesniak, 2006). The control of immune cells by the tumour is also well illustrated by the PD-1/PD-L1 interaction. The latter is a well-characterised signalling pathway and an essential component in the maintenance of immunological homeostasis and protection against autoimmunity (Chen, 2004). However, overexpression of the programmed cell death ligand 1 (PD-L1) on the surface of GBM cells and microglia promotes the effective binding to the programmed cell death protein 1 (PD-1), which is mainly expressed on activated T cells, leading to negative regulation of the immune system and lymphocyte cytotoxic activity (Ishida et al., 1992, Yokosuka et al., 2012). These (co-)inhibitory interactions, which collectively influence the quality and magnitude of immunological responses and of which PD-1/PD-L1 is only one of many, are referred to as immune checkpoints, and the corresponding therapeutic agents targeting this site are called immune checkpoint inhibitors (ICIs; Korman et al. (2006)). However, not only are T cells restricted in their function, but in this pathological state they also have only a limited number of targets available, i.e.

free antigens or antigens presented on the surface of cells. Compared to other tumour entities, GBMs carry a low somatic mutational burden and therefore have fewer neo-antigens and a low intrinsic immunogenicity (Alexandrov et al., 2013).

1.2 Oncolytic virotherapy

Given the poor prognosis of GBM and the hurdles mentioned above, there is an urgent need for new, innovative treatment options. It is known that tumour cells in general, and glioma cells in particular, are *a priori* more susceptible to viral infection due to their tendency to engage in multiple mechanisms of immune evasion, such as downregulation of antigen presentation, resulting in reduced activation of the host adaptive immune system and consequently reduced clearance of foreign pathogens (Mittal and Roche, 2015, Razavi et al., 2016). This is the basic premise of an emerging and promising treatment modality called oncolytic virotherapy (OVT). In short, OVT involves administering oncolytic viruses (OVs) to the patient. Most OVs infect many cells, but selectively replicate only in tumour cells, lysing them as new virus particles are being released. In contrast, non-neoplastic cells will be left unaffected by oncolysis.

However, OVs are not a recent invention. In fact, the first case reports of viral infections interacting with malignant diseases date back to the turn of the 19th century. As early as 1896, the physician George Dock described the case of a 42-year-old woman with "myelogenous leukaemia" who, following what is now thought to have been an influenza infection, experienced a remarkable, albeit only transient, improvement in her disease that was strongly evident both clinically and in terms of laboratory chemistry (Dock, 1904). Over time, many similar case reports can be found, mostly in young, immuno-compromised patients with leukaemia or lymphoma (Pelner et al., 1958). It should be noted, however, that the remissions were only temporary, usually lasting between one and two months.

Attempts were soon made to artificially induce viral infections to mimic this previously observed immuno-stimulatory boost. Hoster et al. (1949) parenterally injected impurified human serum of donors with active hepatitis B to patients with

Hodgkin's disease. Apart from the fact, that 14 out of 22 patients developed hepatitis B, they achieved clinical improvement in regard to their primary disease in 7 patients. Numerous studies followed, testing a wide variety of viruses, including the use of "adenoidal-pharyngeal-conjunctival viruses", now known to be adenoviruses (Huebner et al., 1955). Although some therapeutic effect could be observed in clinical trials (e.g. necrosis in the majority of viral inoculations in an attempt to treat cervical cancer), infections were cleared too quickly and OS was not significantly prolonged (Smith et al., 1956). In the absence of significant survival benefit and durable remission, the interest in OVT soon waned (Kelly and Russell, 2007).

With the tremendous increase in our understanding of viruses, the complete sequencing of their genomes, and the new capabilities to modify and engineer viruses, there has been a resurgence of interest in the field over the last three decades. One of the first genetically engineered OVs was a thymidine kinase (tk) gene-deleted herpes simplex virus (HSV) developed by Martuza et al. in 1991. The tk gene is already known in the field of suicide gene therapy as a "suicide switch" as it renders cells sensitive to ganciclovir, allowing the administration of the antiviral drug ganciclovir to trigger the rapid and certain death of the host cells (Bonini et al., 1997, Straathof et al., 2003). In this context, however, the gene was completely deleted, resulting in viruses being able to replicate in dividing cells but not in non-dividing cells. The virus caused the death of several glioma cell lines *in vitro* and was able to slow down the growth of human xenografts in SCID (severe combined immunodeficiency syndrome) mice, resulting in an increased OS of these mice (Martuza et al., 1991).

Among all the new developments, one OV stands out for its so far unique success story: Talimogene laherparecvec (or T-Vec for short) is a modified type I HSV approved by the United States Food and Drug Administration (FDA) in October 2015 for the treatment of advanced and metastatic melanoma (Andtbacka et al., 2015, Conry et al., 2018). It carries three major modifications, most notably the coding for human granulocyte-macrophage colony-stimulating factor (GM-CSF), which enhances the recruitment of antigen-presenting cells to the TME and promotes cytotoxic T cell responses to tumour-associated antigens (Mach et al.,

2000). It also carries deletions in the ICP34.5 region to prevent neurovirulence as well as in the ICP47 region to enhance immunogenicity (Liu et al., 2003). In the registration trial including 436 randomly assigned patients, the T-Vec group had a significantly higher durable response rate of 16.3 % (defined as an objective response lasting continuously for at least 6 months) and a higher OS of 23.3 months compared to 2.1 % and 18.9 months, respectively, in the GM-CSF group (Andtbacka et al., 2015).

To date, T-Vec is the only OV approved for clinical use in Western countries, but numerous other clinical trials are underway, mostly testing and modifying herpes-, reo- or adenoviruses. The latter are of particular interest because of their wide permissive host cell range, the possibility of easy modification of their genomic DNA and their favourable clinical profile with few adverse events (AEs). In a summary of all clinical trials with adenoviruses as of March 2017, the most common AEs requiring clinical intervention (grade three AEs) were nausea (5.4 % of patients), injection site reactions (3.5 %), leukopenia (2.9 %) and asthenia (2.9 %), all of which are undesirable but clinically manageable (Matsuda et al., 2018).

One of the earliest, most extensively researched oncolytic adenovirus (OAV) is ONYX-015. This OAV harbours a deletion of the E1B-55kD gene, which is believed to make its replication selective for specific tumour cells. The end product of this gene, the E1B protein, typically inactivates p53, thereby allowing adenovirus replication (Bischoff et al., 1996), so deletion of this gene should restrict ONYX-015 replication to p53-deficient cells only. Deficiency of functional p53 is a feature found in almost half of all malignancies (Levine and Oren, 2009) and particularly in the majority of GBMs (England et al., 2013). Overall, the results of many studies with ONYX-015 indicate that it is safe but has limited therapeutic efficacy (Nemunaitis et al., 2001, Chiocca et al., 2004), with the most promising results seen in combination therapy with cisplatin and 5-fluorouracil in head and neck cancer (Khuri et al., 2000). As a result of this trial, ONYX-015 was licensed in China in 2005 with minor modifications and is now marketed as H101 or "Oncorine" (Liang, 2018). However, research outside of China has been suspended as several studies have contradicted the hypothesised

p53-dependent replication selectivity and no clear mechanism has yet been found (Goodrum and Ornelles, 1998, Turnell et al., 1999, Dix et al., 2001).

In general, several routes of administration are available for the treatment with OAVs. Systemic administration of adenoviruses is not a viable option among them because of the rapid complement-mediated neutralisation of the virus, especially in the liver. Furthermore, acute toxic and haemodynamic effects have been observed after systemic application of adenoviruses due to the interaction of viral particles with macrophages. For a review of the limitations of systemic delivery of adenoviruses, see Jönsson and Kreppel (2017). Therefore, direct intratumoural (IT) injection of the virus into the tumour lesion is one of the most common routes of application and was also used in part in this study. An advantage of this method is that large amounts of virus can be delivered directly to the tumour without first having to be transported through the bloodstream or to cross the BBB. This advantage is offset by the highly invasive nature of the procedure, which requires opening the skull and always carries some risk to the patient, such as bleeding or infection.

Therefore, we are also using another route of administration in our experiments, namely the intranasal administration (INA) of OAV-loaded shuttle cells. These shuttle cells are themselves infected with the OAV, migrate to the brain via the olfactory and trigeminal pathways, bypass the BBB, and release new viral particles in the brain when they are lysed (Dhuria et al., 2010, Jiang et al., 2015). With this alternative method, the OAV approaches the tumour from the outside and then has the potential to penetrate the tumour inwards. This is expected to have some advantages, such as improved access to dispersed infiltrating cells. In contrast to IT injection, INA of OAV-loaded shuttle cells is also non-invasive and can be repeated if and as often as required (Gadenstaetter et al., 2022). Moreover, cell-mediated transport provides camouflage to the OAVs from the immune system, preventing the premature inactivation by antibodies and immune cells.

The modified OAV XVir-N-31 used in our experiments carries a deletion in the CR3 region of E1A, which renders its replication dependent on the Y-box binding

protein 1 (YB-1) and thus virtually cancer-specific (Rognoni et al., 2009). YB-1 is a multifunctional protein that is involved in transcriptional and translational regulation, as well as drug resistance, and accomplishes its diverse functions primarily through its ability to shuttle between the nucleus and the cytoplasm (Wu et al., 2007). YB-1 is thought to play a causal role in oncogenesis, controlling the oncogenome by ultimately deciding whether oncogenic mRNA is translated (Evdokimova et al., 2006). However, it does not only intervene in fundamental biological processes at the cytoplasmic level. After translocation to the cell nucleus, it acts as a transcription factor and, by binding to promoters, can increase the gene expression of epidermal growth factor receptor (EGFR) and matrix metalloproteinase 2 (MMP2), among others, thereby supporting invasion and metastasis, as summarised by Holzmüller et al. (2011). Finally, it plays an essential role in adenoviral replication by binding to the adenoviral E2 late promoter and thus activating replication by controlling the E2 gene activity in the late phase of infection (Holm et al., 2002, Mantwill et al., 2006). In more practical terms, this means that XVir-N-31 can only selectively replicate in cells expressing YB-1 due to its modifications, such as the deletion of the CR3 transactivation domain of the adenoviral protein E1A13S. This is the case for GBM, the GBM cell lines used in this study, and even more so in recurrent GBM (Faury et al., 2007, Mantwill et al., 2013).

Once the applied OV has infected the first tumour cells and underwent a first cycle of replication, virus progeny spreads in all directions, thus infecting neighbouring cells. This chain reaction only continues as long as the neighbouring cells are susceptible to viral replication. In other words, they must be neoplastic themselves. Infiltrating tumour cells, a key feature of GBM, may therefore not be directly infected by the OV as they are separated from the main tumour by several rows of healthy cells. Given that these infiltrative growing cells are held responsible for the inevitable recurrence of GBM even after radio-chemotherapy and surgical resection (van Nifterik et al., 2006, Stupp et al., 2009, Kim et al., 2015), the limited spread of OVs to these cells is an important limitation of OVT and has several consequences.

Firstly, it is unlikely that an IT injection of the virus will be able to kill all tumour cells by oncolysis. Secondary effects of OVT such as immuno-inducing effects therefore play a very important role, as they can potentially reach those tumour cells that are not accessible to oncolysis. Secondly, there is also a need for the combination of OVT with other therapeutic modalities such as immune checkpoint inhibitors (ICIs). ICIs have revolutionised the treatment of many solid tumours in recent years (Pardoll, 2012, Jacob et al., 2021). However, for GBM, multiple phase III trials have so far failed to show a benefit of ICI monotherapy or the combination of ICIs with established therapies such as radiotherapy or TMZ (Reardon et al., 2020, Omuro et al., 2022, Lim et al., 2022). As this is thought to be presumably largely due to the low intrinsic immunogenicity of GBM, the combination of OVT and ICI is expected to have a particularly synergistic effect, as illustrated in the commentary by Naumann and Holm (2014).

To test this hypothesis, we chose the monoclonal anti-PD-1 antibody nivolumab, which blocks the "exhausting" receptor on T cells and is well established, for example, in the therapy of advanced malignant melanoma or non-small cell lung carcinoma, for the combination with XVir-N-31 in our study (Topalian et al., 2012, Robert et al., 2015). We are also using a derivative of XVir-N-31, called XVir-N-31-anti-PD-L1, which additionally expresses an antagonistic antibody against PD-L1. This strategy allows the antibody to be produced and released locally by the OAV, avoiding the need for systemic administration. The antibody expressed locally exerts the effect of an ICI on the cancer cells without causing the systemic side effects such as immuno-mediated toxicity to the cardiovascular system, skin, or liver (Naidoo et al., 2015, Geisler et al., 2020, Klawitter et al., 2022).

1.3 Immunogenic cell death

The first generation of anti-cancer therapeutics, whether conventional chemotherapy or radiotherapy, is limited in its understanding to direct cytotoxic effects. Accordingly, the primary mechanisms of action for most classical chemotherapeutic agents are direct damage to DNA (e.g. alkylating agents, platinum-based drugs), inhibition of mitosis (e.g. vinca alkaloids) or inhibition of

DNA production (e.g. antimetabolites) (Dasari and Tchounwou, 2014, Tilsed et al., 2022).

Over the last two decades, however, it has been increasingly recognised that other mechanisms may also contribute to anti-tumour efficacy, including the induction of a host immune response (Nowak et al., 2003a, Nowak et al., 2003b). This was first observed for radiotherapy as well as anthracycline-based chemotherapy (Fucikova et al., 2020, Vaes et al., 2021). A case in point is the study by Obeid et al. (2007), who showed that a single IT injection of the anthracycline mitoxantrone in a murine colon cancer model could induce permanent regression in some cases if the tumours were implanted in immuno-competent BALB/c mice. This was not the case if the tumours were established in immuno-deficient nu/nu mice, providing the basis for the hypothesis that the induction of a host immune response plays an essential role in the therapeutic success. This observation is particularly interesting given that chemotherapy has long been regarded as exclusively immuno-suppressive due to its leuko-depleting effect on the bone marrow (Rasmussen and Arvin, 1982).

It is now understood that this host immune response is triggered by signals from dying or otherwise compromised cells, a concept therefore referred to as immunogenic cell death (ICD). More precisely, ICD is characterised by a defined sequence of compositional changes: (i) the exposure of endoplasmic reticulum-derived calreticulin on the cell surface, which represents one of the biochemical correlates of ICD, a so-called "danger" or "eat-me" signal from dying cells (Gardai et al., 2005, Garg et al., 2012), and (ii) the release and presentation of specific alarm signals, called danger-associated molecular patterns (DAMPs), in response to certain types of cell stress, thereby triggering a tumour-specific immune response (Kroemer et al., 2013).

However, not every type of cell stress can elicit such a specific anti-tumour response (for review, see Fucikova et al. (2020)), and differences can be observed even within the same drug class: while oxaliplatin triggers ICD, cisplatin does not (Martins et al., 2011). This failure can be attributed to the inability of

cisplatin to trigger the translocation from calreticulin from the lumen of the endoplasmic reticulum to the cell surface (Obeid et al., 2007).

In recent years, more and more DAMPs have been discovered as it has become increasingly apparent that the dichotomic view of endogenous and exogenous (self and non-self) as described by Burnet (1959) does not represent the complexity of our immune system and that it is indeed capable of sensing danger within the "self". The "danger model" was first proposed by Matzinger (1994) and has gained wide acceptance, outlining an immune system that is concerned with harm rather than foreignness. The potential threat is detected by pattern recognition receptors (PRRs), receptors with the special ability to recognise and bind a chemically diverse set of molecules either exposed on the cell surface or released in the extracellular space (Takeuchi and Akira, 2010). In the case of infectious genesis, these recognised molecules have been termed "pathogen-associated molecular patterns" (PAMPs), which are characterised as highly conserved structures of microorganisms that enable recognition by the immune system and are thus of exogenous origin (Lim and Staudt, 2013). In the case of non-infectious genesis and thus endogenous origin, these molecules have been coined DAMPs, which are secreted, released or exposed to surfaces in dying, stressed or injured cells (Krysko et al., 2012). However, prior to release or exposure, most DAMPs have mainly non-immunological functions in the cell and are involved in basic biological processes such as transcription and translation in healthy cells (Garg et al., 2011).

A prototypical DAMP is the high-mobility group box 1 protein (HMGB1), which is ubiquitously expressed and determines chromosomal structure and function under physiological conditions by binding to transcription factors and the DNA itself (Di et al., 2019). Once released, it can interact as a potent pro-inflammatory cytokine (Wang et al., 1999) with various PRRs such as Toll-like receptor (TLR) 2 and 4 or the receptor for advanced glycation end products (RAGE), thus attracting various immune cells and supporting dendritic cell maturation (Messmer et al., 2004, Krysko et al., 2012). Both active secretion of HMGB1 by innate immune cells in response to pathogens as well as the passive release from

dying cells, the latter of which is of particular relevance to us, have been described (Andersson and Tracey, 2011).

Other DAMPs include, for example, the group of heat shock proteins (HSPs), of which the representatives HSP70 and HSP90 (named after their molecular weight in kDalton) have been specifically described in the context of ICD. HSPs are generally present at low levels in healthy cells, where they act as chaperones to ensure the correct folding of proteins (Lanneau et al., 2010). Characteristically, however, the transcription of HSPs can be dramatically increased under cellular stress such as heat, hypoxia or infection, in order to protect proteins from denaturation and aggregation (Lindquist, 1986). In the event of severe cell stress or in the process of apoptosis of the affected cell, HSP70 can also be actively released, mostly in the form of exosomes, more rarely also in its soluble or membrane-bound form (Théry et al., 2009). As HSP70 can also interact with TLR 2 and 4, among others, it shows partially overlapping effects with HMGB1 (Zitvogel et al., 2010). More specifically, HSP70 has been shown to stimulate the oncolytic and migratory capacity of NK cells (Gastpar et al., 2005), the activation of macrophages (Vega et al., 2008) and the maturation of antigen-presenting cells (Hagymasi et al., 2022). It should be noted that these immuno-stimulatory effects were only observed for extracellular HSP70, whereas intracellular HSP70 is cell-protective, has anti-apoptotic effects, is overexpressed in many tumours, and is associated with a poorer prognosis in these (Schmitt et al., 2007, Shevtsov and Multhoff, 2016).

The extent, but probably also the spatio-temporal sequence, in which DAMPs are exposed or released during cell death, as well as their interaction especially with PRRs, thus determines whether cell death is considered immunogenic, null (no effect on the immune system) or even tolerogenic (Gong et al., 2020, Fucikova et al., 2020). The induction of tolerance through apoptotic, tolerogenic cell death is essential for the body's homeostasis, as millions of cells die every second during physiological cell turnover (Galluzzi et al., 2017). At the same time, the induction of ICD is thought to be very beneficial in an oncological therapeutic setting, as a long-lasting, specific anti-tumour immune response can be generated by both the innate and adaptive immune system, potentially enhancing

the therapeutic efficacy of anti-cancer agents. This extended therapeutic benefit has been demonstrated in several studies in which ICD-inducing therapeutics have been more effective in immuno-competent mice than in immuno-deficient mice, whereas therapeutics that fail to induce ICD have shown the same efficacy in both experimental groups (Casares et al., 2005). To date, only a handful of therapeutics have been identified as *bona fide* ICD inducers. Understanding the exact circumstances and requirements for ICD induction remains a major challenge in the field of oncology.

OVs are potential and promising candidates as ICD inducers. This and the diverse mechanisms of action of OVs are the subject of current research and the focus of this thesis. In addition to the classical viral lysis of host cells, several other mechanisms of action have been described for OVs. Oncolysis is accompanied and complemented by multiple interactions, such as those with stromal cells of the TME, the vasculature and, most importantly, the immune system, as summarised by Martin and Bell (2018). It remains largely unresolved how these effects are weighted and to what extent lytic activity is a prerequisite for these complementary effects (Davola and Mossman, 2019).

Given these diverse mechanisms of action and the central role of the immune system, the choice of animal model is particularly important. Usually, immuno-deficient mice are used for xenograft models in order to prevent rejection of the human cancer cells by the hosts immune system and thus to allow tumour inoculation in the first place. A disadvantage of immunodeficiency, on the other hand, is that the impact of some secondary effects such as immune induction, which we hypothesise, cannot be observed. This study uses brain tissue from two mouse GBM models, one immunodeficient and one immunocompetent. In the immunodeficient mouse model, we can therefore only observe effects that are directly triggered by OVs. In the immunocompetent model, the effects triggered by potentially activated immune cells can also be observed, especially in tumour areas that are not in direct contact with OVs.

In 2019, Lichtenegger et al. published their data on the use of XVir-N-31 in bladder cancer. In an orthotopic mouse model, they demonstrated a more pronounced ICD after IT injection of XVir-N-31 compared to the application of a wild-type adenovirus (Lichtenegger et al., 2019). The brain is a uniquely immuno-privileged organ, and GBM in particular is an immensely immuno-suppressive tumour entity. The question therefore arises as to whether XVir-N-31 also induces ICD in GBM. To address this key question, this work will use immunofluorescence (IF) to examine the brain tissue of GBM-bearing mice for the presence of DAMPs after different treatment regimens to determine the induction of ICD.

2 Materials and methods

2.1 Histology and immunofluorescence

In this work, brain tissue obtained from two different mouse models of GBM was used for IF approaches. The animal work and all the tasks involved in the therapy, such as working with cell cultures and adenoviruses, were carried out by Ali El-Ayoubi (PhD) and Moritz Klawitter (PhD) from our research group, who kindly provided the brain tissue of GBM-bearing mice after different treatment regimens.

Cell line	Description	Organism	Further information	Source
LN-229	patient-derived GBM cells	human	Ishii et al. (1999)	ATCC, Manassas, USA
U87MG	patient-derived malignant glioma cells	human	Ishii et al. (1999)	ATCC, Manassas, USA
LX-2	hepatic stellate cells	human	Xu et al. (2005)	Scott Friedman (Division of Liver Diseases, Icahn School of Medicine at Mount Sinai, NY, USA)
LX-2^{FR}	migration-optimized, mCherry-labelled LX-2 cells	human	El-Ayoubi et al. (2023a)	lab-made

Table 1. Cell lines used in the experiments.

In the first mouse model (Model A), immuno-deficient NSG™ mice were equipped with a to the tumour HLA-A/B-matched humanised immune system and then orthotopically inoculated with U87MG cells (*Table 1*) in both striata to generate two separate GBMs. The rapidly developing, right-sided GBM was IT injected with either PBS (sham treatment) or with XVir-N-31, XVir-N-31-anti-PD-L1 or Ad-Wildtype (dl309) as previously described in Klawitter et al. (2022). For further details, please refer to *Table 2* and *Figure 1*. The left sided GBM remained untreated. Some mice were exclusively or additionally treated with intraperitoneal injections of nivolumab. All mice were sacrificed collectively at the same time

point at the first appearance of tumour-associated symptoms in the PBS control group (day 35) and before the onset of graft-versus-host-disease. This mouse model was established in our research group, work was carried out by Moritz Klawitter and a detailed treatment plan has been published in Klawitter et al. (2022).

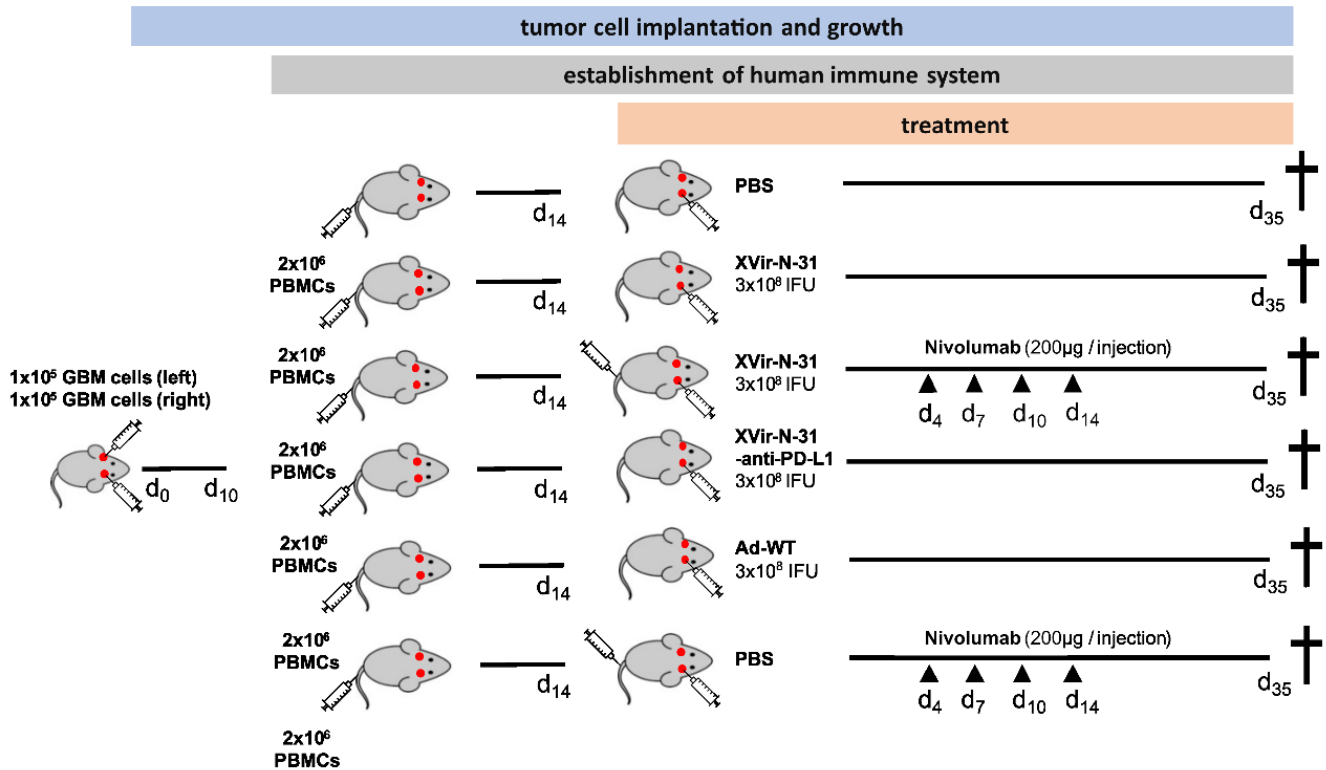

Figure 1. Treatment scheme of the combination therapy of OVT and ICI in the immuno-humanised GBM mouse model. *Adapted and reprinted with permission from Klawitter et al. (2022).*

In the second mouse model (Model B), immuno-deficient NSG™ mice were orthotopically inoculated with LN-229^{GFP} cells (*Table 1*) in the right striatum to generate a GBM. Mice were treated via intranasal application (INA) of mCherry-labelled shuttle cells (*Table 1*) that were either unloaded or loaded with XVir-N-31. Mice were sacrificed at different time points after INA. This mouse model was established in our research group, all animal work was carried out by Ali El-Ayoubi. Practicability and success of the INA of loaded shuttle cells was demonstrated in advance in our working group (El-Ayoubi et al., 2023b).

Model A: U87MG GBM-containing brain tissues were collected, fixed overnight in 4 % paraformaldehyde at room temperature (RT) and stored at -20 °C until further processing. Model B: LN-229^{GFP} tumour-bearing brains were snap frozen on dry ice and stored at -80 °C. All tissues were embedded in Tissue-Tek® O.C.T. Compound (Sakura Finetek, Alphen aan den Rijn, Netherlands). Brains were cryosectioned at 10 µm using a Leica cryomicrotome CM3050S (Leica Mikrosystems, Wetzlar, Germany) and mounted on SuperFrost Plus™ adhesive microscope slides (Langenbrinck, Emmendingen, Germany). Sections were stored at -20 °C until further use. The presence of tumours was confirmed by the intrinsic GFP fluorescence of LN-229^{GFP} cells or the high cell density of U87MG tumours detectable by DAPI staining using an Axiovert 200M fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Virus	Description	Host specificity	Further information
dl309	Ad-Wildtype derivate, subtype 5	human cells	Hibma et al. (2009)
XVir-N-31	oncolytic adenovirus	human cells	Rognoni et al. (2009)
XVir-N-31-anti-PD-L1	oncolytic adenovirus secreting a PD-L1 antagonistic antibody	human cells	Lichtenegger et al. (2019)

Table 2. Viruses used in the experiments.

Three variants of a common protocol, each with minor modifications, were used for IF stainings. This protocol is described in general terms below, the exact variations and areas of application will be described in more detail later.

Slides were washed in Dulbecco's phosphate-buffered saline (DPBS; Sigma-Aldrich) for 10 minutes in a Hellendahl staining trough. The plasma membrane was identified by staining with wheat germ agglutinin conjugated to Alexa Fluor™ 350 (#W11263, Thermo Fisher Scientific, Waltham, USA) at a concentration of 5.0 µg/ml for 10 minutes. Excess liquid was removed, and the sections were washed three times with Tris-buffered saline containing 0.05 % Tween-20 (TBS-T) for 5 minutes each. Due to the inherent photosensitivity of the antibodies, care should be taken to protect the sections from light as much as

possible from the time the first antibody was added, and throughout all subsequent steps and storage. Tissues were blocked with 3 % serum of the host species of the secondary antibody in PBS for 60 minutes, followed by washing the sections in TBS-T for 10 minutes. Excess fluid was again drained and blotted off before the primary antibody was added and incubated overnight at 4 °C in a humidified chamber.

No.	Target protein	Host species	Clonality	Dilution	Source	Catalog #
1	HMGB1	mouse	monoclonal	1:250	Thermo Scientific Fisher, Waltham, USA	MA5-17278
2	HMGB1	rabbit	monoclonal	1:200	Thermo Scientific Fisher, Waltham, USA	MA5-31967
3	HSP70	mouse	monoclonal	1:250	Thermo Scientific Fisher, Waltham, USA	MA3-007
4	HSP70	rabbit	monoclonal	1:400	Novus Biologicals, Littleton, USA	NBP2-89951
5	YB-1	mouse	monoclonal	1:200	Santa Cruz Biotechnology, Dallas, USA	sc-101198
6	YB-1	rabbit	monoclonal	1:50	Novus Biologicals, Littleton, USA	NBP2-67491
7	Hexon	mouse	monoclonal	1:450	Santa Cruz Biotechnology, Dallas, USA	sc-51748

Table 3. Primary antibodies used in the experiments.

The next day, slides were washed three times for 5 minutes each in TBS-T before the fluorochrome-conjugated secondary antibody was added at a dilution of 1:1,000 in PBS for 90 minutes in a humidified, light-proof chamber. The slides were again washed three times in TBS-T for 5 minutes each time. A few drops of mounting medium (Permount™, Thermo Scientific Fisher) were then added, and a coverslip placed on top. All the steps in this protocol were carried out at RT, unless otherwise stated. Stained sections were viewed in a timely manner, but no later than three to four weeks after initial staining, and were stored in a dark, dry box in the interim.

The first variation of the protocol was performed on a subset of the sections derived from mouse brains of Model B. Primary antibodies (nos. 1, 3, 5 or 7) are described in *Table 3*, as secondary antibody an Alexa Fluor™ Plus 680 conjugated donkey anti-mouse antibody (no. 1 in *Table 4*) was used. For blocking, donkey serum was added. Only one primary antibody was used at a time for each section.

No.	Species reactivity	Host species	Clonality	Isotype	Conjugate	Dilution	Source	Catalog #
1	mouse	donkey	polyclonal	IgG	Alexa Fluor™ Plus 680	1:1,000	Thermo Scientific Fisher, Waltham, USA	A32788
2	rabbit	goat	polyclonal	IgG	Alexa Fluor™ 594	1:1,000	Abcam, Cambridge, UK	ab 150080

Table 4. Secondary antibodies used in the experiments.

A second variation of the protocol was used for sections obtained from brain tissue of Model A. For these sections, the plasma membrane staining was omitted (the TBS-T wash step immediately followed the PBS wash step) and instead a DAPI-containing mounting medium (VECTASHIELD® PLUS, Vector Laboratories, Burlingame, USA) was used to identify cell nuclei. Apart from this modification, the same antibodies were used as in the previous version.

The final variation of this protocol was designed to allow the simultaneous detection of different proteins within the same tissue slice in order to better identify co-localisation. It was applied to a subset of sections obtained from Model A to determine adenovirus replication. Again, a murine antibody was used for Hexon (no. 7 in *Table 3*), but rabbit antibodies were used for the other target proteins (nos. 2, 4 or 6 in *Table 3*). Accordingly, two secondary antibodies from different host species were used (nos. 1 and 2 in *Table 4*), in our case donkey anti-mouse and goat anti-rabbit, to allow for separate amplification of the primary signals. The antibodies were applied simultaneously, taking into account the

correct dilution. Finally, both goat and donkey serum were added simultaneously in the blocking step to match the secondary antibodies.

2.2 Confocal laser scanning microscopy

Images were captured using a Zeiss LSM710 confocal laser scanning microscope (Carl Zeiss) at 5x, 10x, 40x, and 63x magnification as indicated in the figure legends. Up to four channels were observed simultaneously using different lasers at excitation wavelengths of 405, 488, 561 and 633 nm. Laser power, gain, and other settings were individually optimised for each channel according to an optimal visual representation and then left unchanged for all subsequent recordings.

3 Results

The induction of ICD is a prerequisite for a long-term anti-tumour immune response and thus can make an important contribution to the therapeutic effect of OVs. To determine ICD induction, we used IF staining for DAMPs to examine their presence in brain sections obtained from GBM-bearing mice following different OVT regimens. Since the replication of our OAVs is dependent on the presence of YB-1 and as YB-1 is a highly immunogenic protein if released from cells, we chose to stain for YB-1 as well as for two prototypical DAMPs well characterised in the literature, HMGB1 and HSP70 (Sims et al., 2010, Krysko et al., 2012). Details of our experiments on the presence of these immunogenic proteins as essential mediators of ICD under various treatment conditions are presented below.

3.1 OVT by IT injection of OAVs

3.1.1 XVir-N-31 potently induces ICD

Our first experiment was designed to determine whether *in vivo* XVir-N-31 shows superior effects in regard to ICD induction compared to dl309. To achieve this aim, we used tumour tissue from NSG™ mice bearing a U87MG GBM in both hemispheres, in which only the right-sided tumour was IT virus-injected, as described in Klawitter et al. (2022).

Almost no presence of HMGB1 and HSP70 was detected in the tumour region of OAV-injected GBMs in mice treated with an IT injection of dl309 (*Figure 2.B*), whereas mice that received IT injections of XVir-N-31 exhibited high levels of HMGB1 and HSP70 in the tumour core (*Figure 2.D*). At least with the use of our staining protocol, HMGB1 fluorescence was particularly intense, while HSP70 presented comparatively weaker and more scattered, occurring mainly in the cytosolic region and omitting the nucleus. A similar situation was observed for YB-1, but the overall signal was so weak that differentiation from the background signal was difficult. Thus, while we can confirm the induction of HMGB1 and HSP70 by XVir-N-31 in our mouse GBM model, we can neither confirm nor deny the induction of YB-1 at this stage.

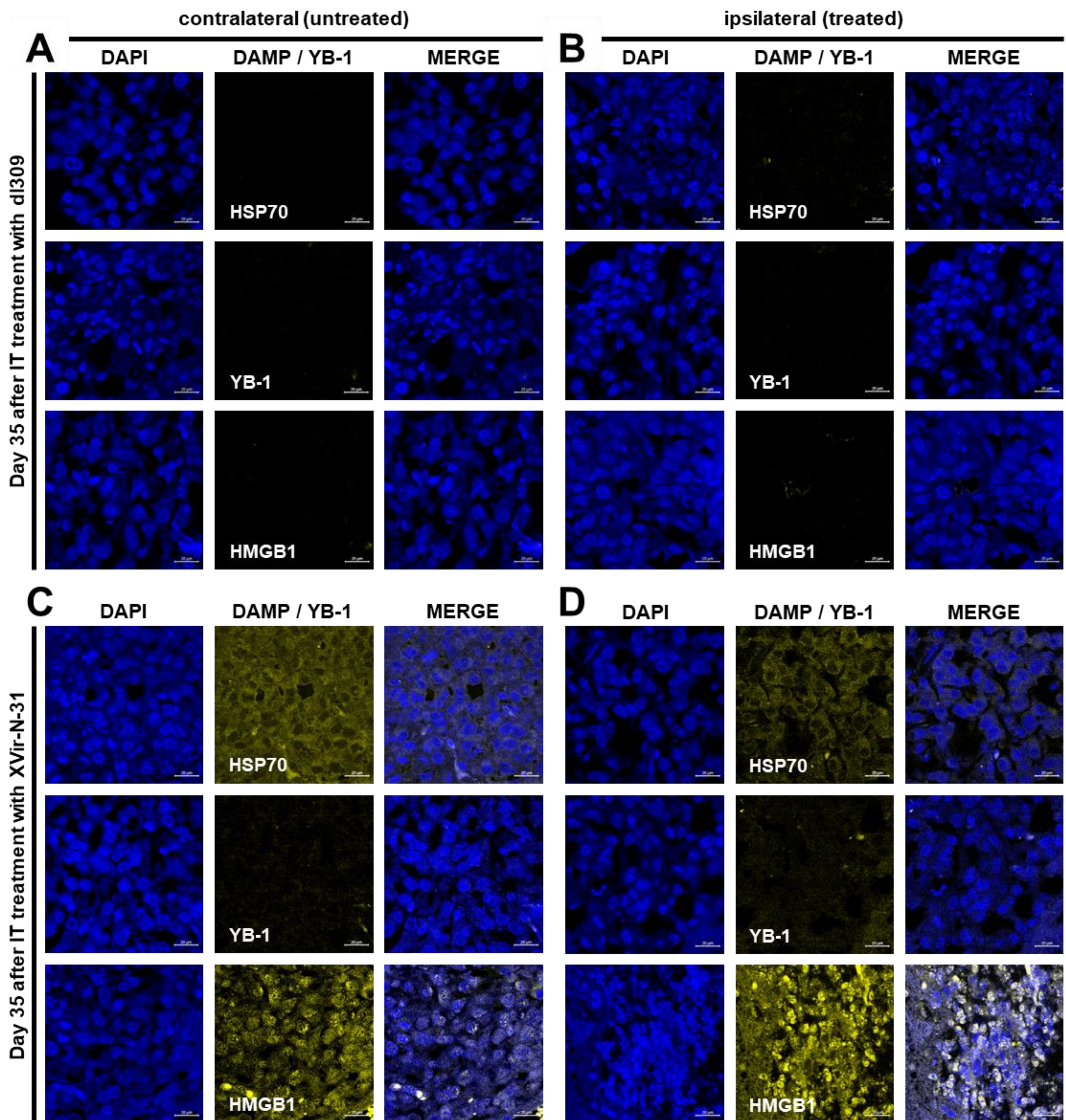

Figure 2. Identification of immunogenic proteins in IT virus-injected (ipsi-) and contralateral, untreated GBMs. *Immuno-humanised NSG™ mice bearing U87MG tumours were treated with an IT injection of 3 x 10^8 IFU of either dl309 (**A** and **B**) or XVir-N-31 (**C** and **D**) in the ipsilateral hemisphere only, while the tumour in the contralateral hemisphere was left untreated. On the day of onset of the first tumour-associated symptoms, all mice were sacrificed. Brains were prepared for IF and stainings for HSP70, YB-1 and HMGB1 were performed. Images were taken at the core of the tumour foci in both hemispheres (63x magnification, n=2 per group, one representative image is shown).*

We were also interested in the presence of DAMPs as well as YB-1 in the surrounding infiltration zone, where individual tumour cells are scattered in a predominantly healthy tissue without continuous contact with the tumour core. It can be assumed that the majority of these cells were not directly infected with the OAV, however, due to the complex three-dimensional structure of the tissue and our two-dimensional imaging technique, this cannot be ruled out with certainty. Here, we observed a strong display of HMGB1 staining, co-localised with the infiltrating neoplastic cells in XVir-N-31 treated mice (*Figure 3.B*). No HMGB1 could be detected under dl309 therapy, in agreement with the previous results (*Figure 3.A*). A similar but slightly weaker staining pattern was observed for HSP70, and YB-1 was again completely negative regardless of the virus chosen (data not shown).

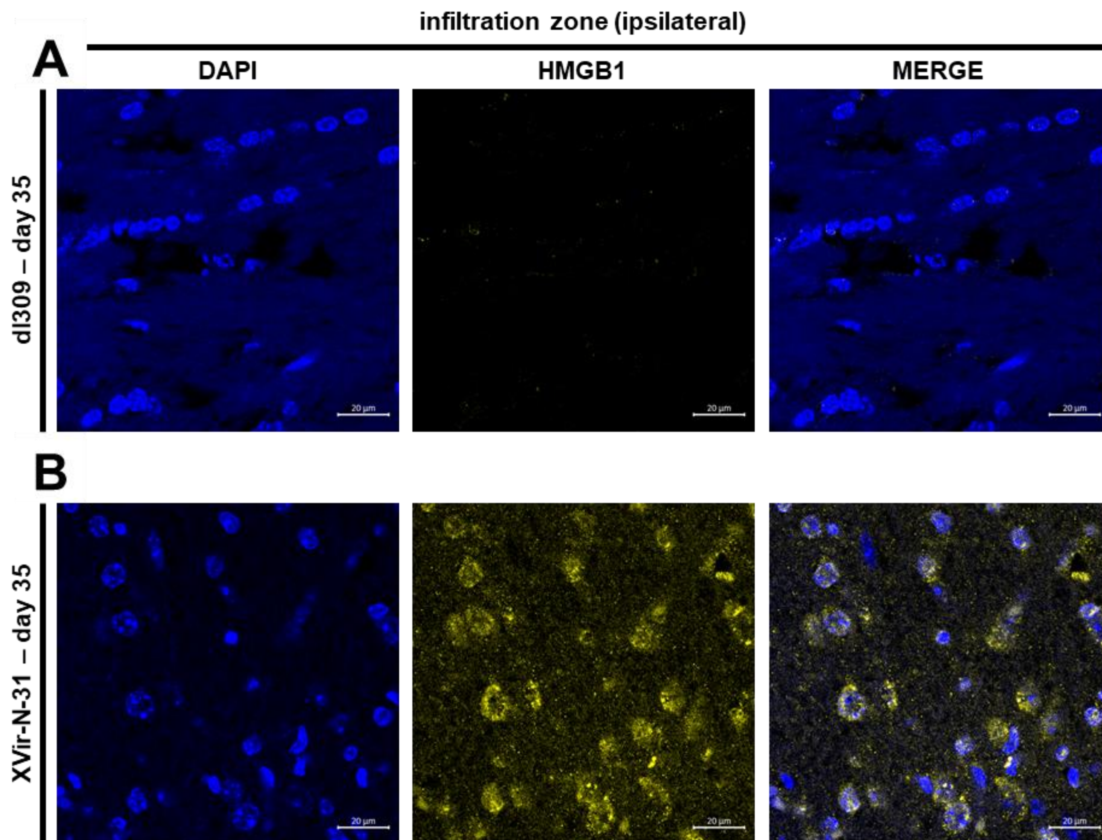

Figure 3. Identification of HMGB1 in the infiltration zone of GBM-bearing mice treated with an IT virus injection. *NSG™ mice bearing U87MG GBM in both striata were ipsilaterally IT injected with 3 x 10^8 IFU of either dl309 or XVir-N-31. Mice were sacrificed on the day the first mouse developed tumour-associated symptoms. Brains were prepared for IF and stained for HMGB1. Images were taken in the infiltration zone of the injected tumour (63x magnification, n=2 per group, one representative image is shown).*

So far, it can be concluded that specifically XVir-N-31, but not dl309, potently induces the presentation of the DAMP proteins HMGB1 and HSP70 in the tumour core as well as in the infiltration zone after a single IT virus injection. As DAMP staining was performed late stage after virus application (day 35), we assume a successful XVir-N-31-mediated induction of ICD.

3.1.2 ICD occurs in adjacent untreated tumours mimicking infiltrating tumour foci

We were then interested in the tumours in the untreated, contralateral hemisphere in the same mouse model. These tumours are separated from the initial virus injection site by numerous non-neoplastic cell layers, allowing us to evaluate the secondary effects of OVT under controlled conditions, without the risk of them being masked by viral oncolysis. The absence of viral particles in these contralateral tumour foci on day 35 after virus application was previously confirmed by Klawitter et al. (2022) using Hexon staining, whereas in the ipsilateral tumour foci, Hexon was detected at a late stage after virus application, indicating active virus replication.

In our experiment, both HMGB1 and HSP70 appeared clearly in the contralateral tumour foci of XVir-N-31 treated mice, with a slightly attenuated signal compared to the ipsilateral tumour site (*Figure 2.C*). In contrast, we did not observe any presentation of DAMPs on day 35 after treatment with dl309, neither in the treated nor in the untreated hemisphere (*Figure 2.A* and *2.B*). Finally, staining for YB-1 was negative in both hemispheres, irrespective of treatment. Therefore, as of now, we cannot answer whether OVT with XVir-N-31 induces YB-1 expression and presence.

In conclusion, based on these results, we can assume that after IT injection of XVir-N-31, ICD is induced not only in the OAV-treated, but also in the contralateral, untreated tumour, even if the latter did not have direct contact with the OAV.

3.1.3 Combination of OVT and ICI enhances ICD induction

The combinatorial therapy using OVT plus immune checkpoint inhibitors (ICIs) is particularly promising, as outlined in the introduction. In order to analyse the impact of this combination on the ICD induction, the same NSG™ mice bearing U87MG tumours in both hemispheres as previously described were used and divided into six groups receiving different treatment regimens. All but one group received an IT application of the respective virus and two groups were treated with systemic nivolumab either alone or as an addition to the virus injection (*Figure 1)*.

The first cohort of mice was given a control treatment consisting of only PBS and no detectable levels of DAMPs or YB-1 were found in either hemisphere. The second cohort received an IT injection of XVir-N-31 and subsequently showed, as previously described, a pronounced presence of HMGB1 in both tumours, ipsilaterally slightly stronger than on the contralateral side. The combination therapy of XVir-N-31 and multiple systemic applications of nivolumab in the third mouse cohort resulted in very prominent HMGB1 presentation, even more so than with XVir-N-31 monotherapy. The HMGB1 signal on the ipsilateral side was stronger than in any previous staining and locally very intense around the tumour cells, suggesting that combinatorial therapy does indeed have a positive effect on ICD induction through synergistic effects. A fourth group received XVir-N-31-anti-PD-L1, which additionally expresses an antagonistic antibody against PD-L1. The HMGB1 fluorescence signal was equally strong in our experiment with XVir-N-31-anti-PD-L1 as it was with the separate administration of XVir-N-31 and systemic nivolumab. The fifth group, which received the wild-type-like dl309, is shown here again for better comparison, with negative staining for all three proteins in both hemispheres. The last group of mice did not receive any virus, but only multiple systemic doses of nivolumab. Nivolumab alone resulted in only a very weak, equilateral fluorescence signal of HMGB1, which was considerably attenuated compared to the signal after OVT using XVir-N-31. A selection of representative images from both hemispheres displaying the presence of HMGB1 in brain sections of the different treatment groups is shown in *Figure 4*.

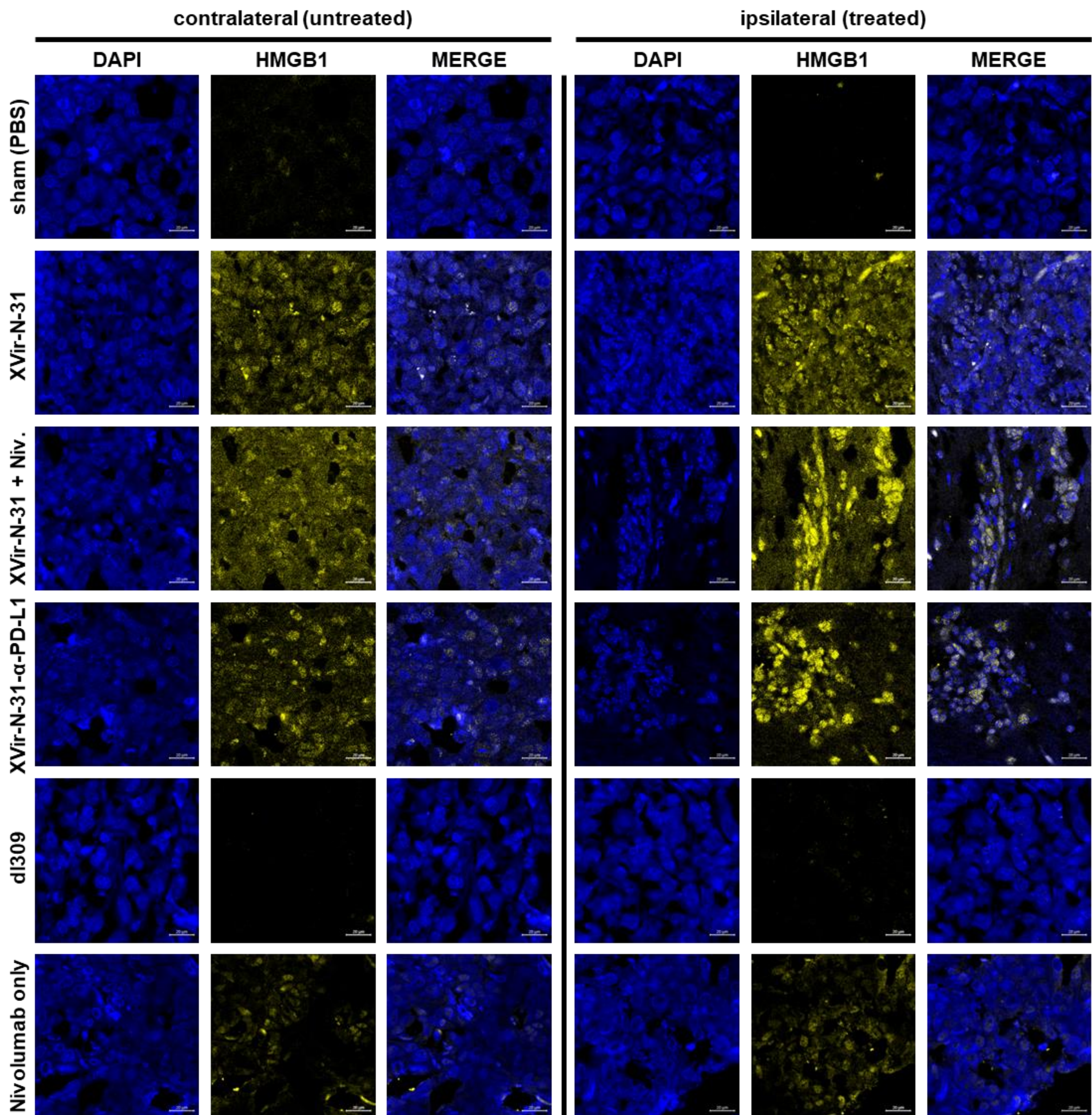

Figure 4. Detection of HMGB1 in GBM-bearing mice following different therapeutic regimens. *U87MG GBM-bearing NSG™ mice were ipsilaterally IT injected with either PBS (sham), 3 x 10^8 IFU of either dl309 or XVir-N-31 (alone or in combination with multiple systemic nivolumab applications), or XVir-N-31-anti-PD-L1. An additional group received only multiple systemic applications of nivolumab without any virus. Mice were sacrificed on the first day of onset of tumour-associated symptoms, brains were prepared for IF, and HMGB1 staining was carried out. Images were taken in the respective tumour core in both hemispheres (63x magnification, n=2 per group, representative images are shown).*

3.2 OVT by INA of OAV-loaded shuttle cells

The IT route of application used in the experiments to this point has significant limitations and drawbacks, such as the invasiveness of the intervention or the challenge of reaching infiltrating cells. Therefore, an alternative application method, namely the intranasal application (INA) of OAV-loaded shuttle cells, is being investigated in this second part of the experiments.

3.2.1 Our mouse model represents key features of GBM

In the following experiments, a second mouse model was used with LN-229^{GFP} GBM-bearing mice without the additional application of human PBMCs. The consistent expression of GFP by these tumour cells allows us to observe the tumour directly under a fluorescence microscope and differentiate it from healthy tissue without the need for additional staining.

In a sagittal whole-mount image it can be observed how the tumour has been orthotopically inoculated into the cerebral cortex, has already spread into the white matter tracts and has formed a tissue defect in the cortex due to its rapid growth (*Figure 5*). It can be appreciated how key features of the highly malignant GBM, such as infiltrative and aggressive growth, are well-represented in our GBM mouse model.

Before further analysing our IF stainings, we were able to verify that the tumour cell line had indeed been inoculated at the desired site and displayed GBM-typical growth with infiltration of individual cell groups early in the course of the disease (in this particular case, by day 10 after tumour inoculation).

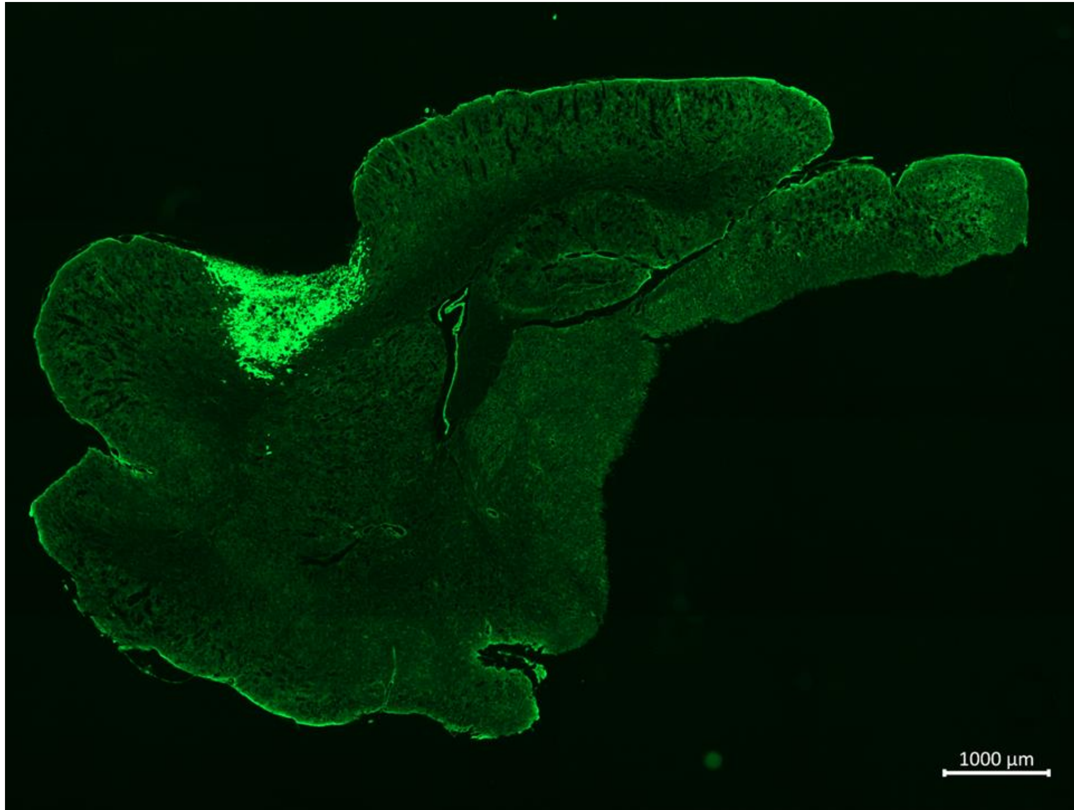

Figure 5. Whole-brain imaging of our GBM mouse model. *Orthotopic tumour inoculation was performed by injection of 1 × 10^5 LN-229^{GFP} patient-derived glioma cells in the right striatum of NSG™ mice. Mice were sacrificed according to the experimental protocol on day 10 post tumour inoculation, before the onset of tumour-related symptoms. Brains were prepared for subsequent IF observation and sectioned in the sagittal plane. Images were taken without additional staining at an excitation wavelength of 488nm (GFP) and reconstructed as whole-brain images (5x magnification, images reconstructed from 25 tiles, n=40, representative image is shown). Please note that the photograph was overexposed to demonstrate the brain structure in parallel to the tumour.*

3.2.2 Shuttle cells successfully transport OAVs

For the IN application in the following experiments, we use hepatic stellate cells, namely LX-2^{FR} cells, as shuttle cells for our OAV. Prior to their use as vectors, these cells were further selected for their high migratory capacity (LX-2^{FR}; fast running, "FR") and lentivirally transduced to express mCherry, which allows tracking under a fluorescence microscope. We were interested to confirm the successful transport of virus particles to tumour cells after INA of OAV-loaded shuttle cells. To this aim, we performed IF staining for the major adenoviral capsid protein Hexon, which lends itself to the reliable detection of adenoviruses in tissue due to its ubiquitous, consistently high expression.

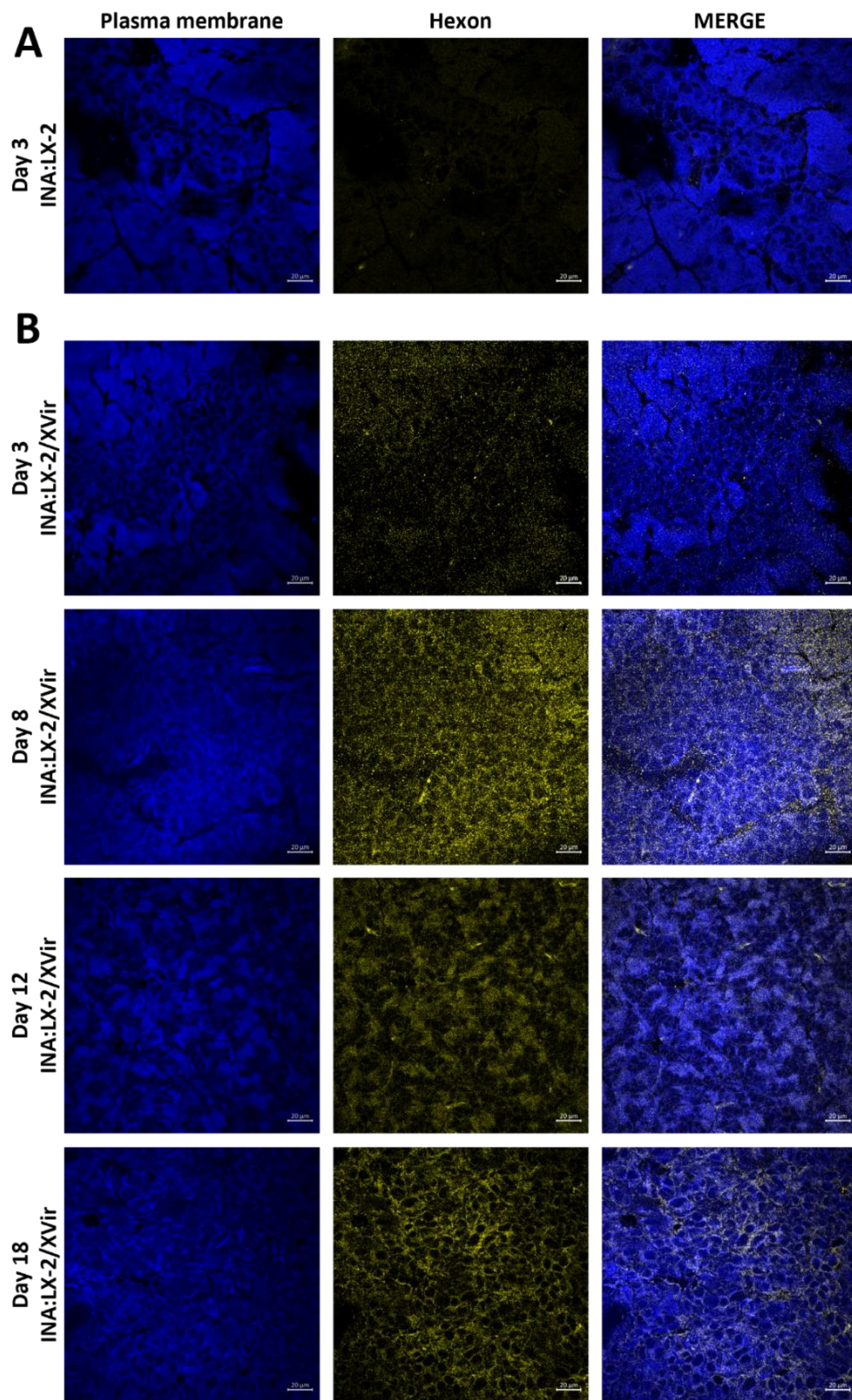

Figure 6. Detection of Hexon in GBM-bearing mice after INA of OAV-loaded shuttle cells. *LN-229^{GFP} GBM-bearing NSG™ mice received an INA of 4 x 10^6 LX-2^{FR} shuttle cells either (**A**) unloaded or (**B**) infected with 200 MOI of XVir-N-31 seven days post tumour cell inoculation. Mice were sacrificed on days 3, 8, 12 and 18 post INA before the onset of tumour-associated symptoms as indicated on the left. Brains were prepared for IF and staining for Hexon was performed (40x magnification, n=2, representative images are shown).*

In our experiment, Hexon was detectable by IF in and around tumour cells as early as day 3 after INA. In contrast, mice that received unloaded cells showed no Hexon on the same day (*Figure 6*). This suggests that virus-loaded shuttle cells had migrated from the site of application, i.e. the nasal mucosa, to the tumour within 3 days. Thereafter, the intensity of the Hexon signal increased considerably towards day 8 and remained at approximately the same high level until day 18, which was the end of the observation period. As it has been shown in previous experiments in our research group that the shuttle cells die after INA approximately after 3 to 5 days due to the viral lysis itself, any Hexon activity thereafter most likely indicates a successful infection of the neoplastic cells.

Our results indicate that the virus is being transported to tumour cells by OAV-loaded LX-2^{FR} cells after INA and even persists after the demise of the transport cells, suggesting that new virus particles are produced by neoplastic cells.

3.2.3 INA of OAV-loaded cells achieves comparable levels of DAMP induction as IT-injected OAV

The next experiment focused on the question of whether comparable levels of DAMPs are observed in the tumour area after INA of OAV-loaded shuttle cells as after IT injection of the OAV. To approach this question, LN-229^{GFP} GBM-bearing mice were treated with a single INA of XVir-N-31-loaded shuttle cells and sacrificed at different time points afterwards. As described before, the shuttle cells were labelled with the red fluorescent protein mCherry and can thus be visualised by fluorescence microscopy.

The shuttle cells were readily detectable in the early period after INA (*Figure 7*, day 3), but thereafter no mCherry signal was present as the cells themselves were lysed by the virus. In terms of DAMPs, no HMGB1 was detected in the negative control group, which had received virus-unloaded shuttle cells. In the brain sections of mice receiving OAV-loaded shuttle cells, however, a clear HMGB1 signal was seen. The intensity of this signal varied over time and, according to subjective assessment, reached its maximum around day 12, with the observation period ending after 18 days.

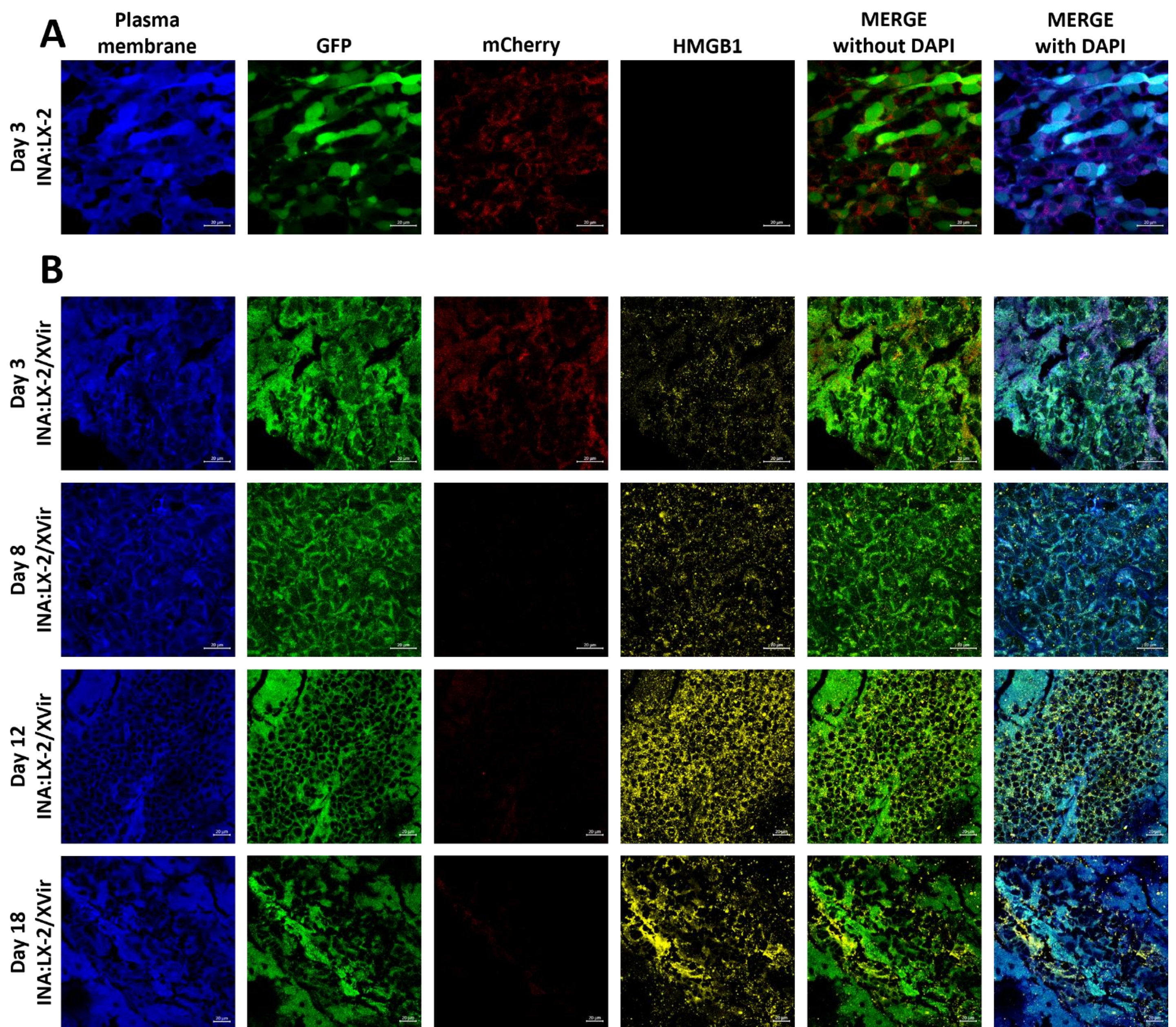

Figure 7. Identification of HMGB1 in the GBM tumour core after INA of mCherry-labelled, OAV-loaded shuttle cells. *LN-229^{GFP} GBM-bearing NSG™ mice were intranasally treated with 4 x 10^{6} LX-2^{FR} cells either (**A**) unloaded or (**B**) loaded with 200 MOI of XVir-N-31 7 days post tumour inoculation. Mice were sacrificed on the day indicated in the figure after INA. Brains were prepared for IF and staining for HMGB1 was performed. Images were taken in the core of the tumour (40x and 63x magnification, n=2, representative images are shown).*

We have carried out the same IF staining for the other DAMP of interest, HSP70, as well as the immunogenic protein YB-1. There was a defined presence of both proteins, as shown in *Figure 8* below for day 8, with a similar dynamic over the 18-day observation period as for HMGB1. Consistent with the results of the previous experiments using IT application, HMGB1 was also the most prominent DAMP, with HSP70 in second place. Interestingly, in this step we were able to detect YB-1 for the first time, albeit with the weakest signal, on day 8 after INA.

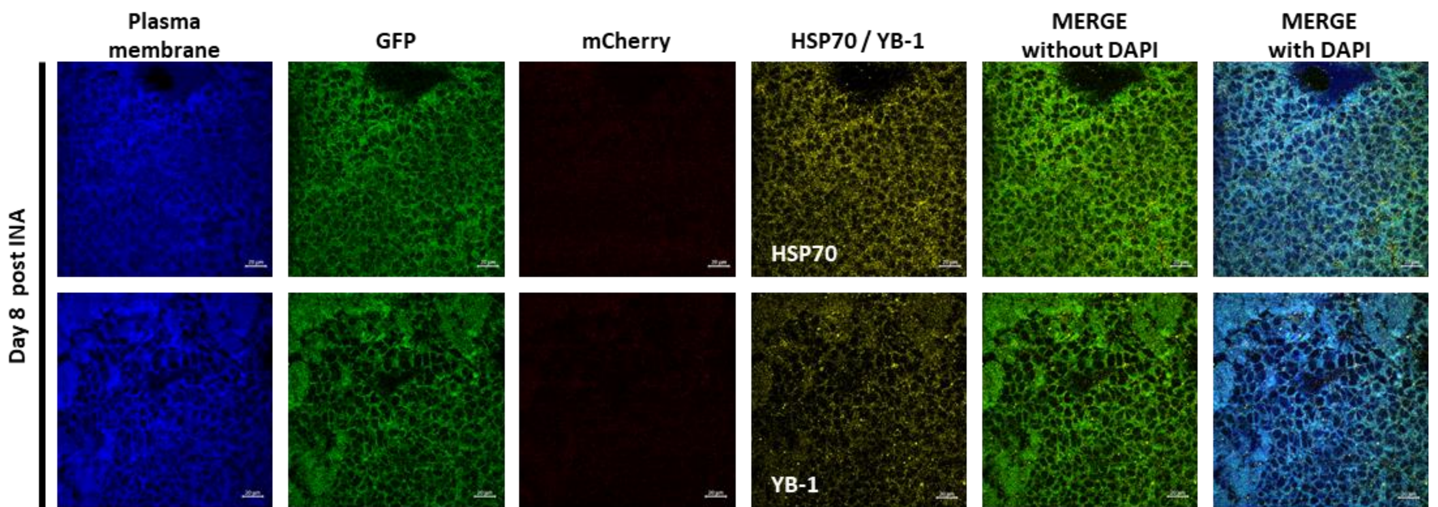

Figure 8. Identification of HSP70 and YB-1 in the GBM tumour core after INA of mCherry-labelled, OAV-loaded shuttle cells. *LN-229^{GFP} GBM-bearing NSG™ mice were sacrificed on day 8 after INA of 4 x 10^6 mCherry-labelled LX-2^{FR} cells infected with 200 MOI of XVir-N-31. Brains were prepared for IF and stained for the respective proteins, HSP70 and YB-1. Images were taken in the tumour core (63x magnification, n=2, representative images are shown).*

Thus, in addition to IT application of the virus, INA of OAV-loaded shuttle cells seems to be a suitable method to induce the presence of DAMPs as signals of ICD at a comparable level. Of particular note is the first successful detection of YB-1 after INA, which was not possible after IT application of the OAV.

3.2.4 Hexon co-localises with immunogenic proteins after INA of OAV-loaded shuttle cells

As the primary antibodies used so far have all been of murine origin, staining for multiple proteins of interest has only been possible within neighbouring brain sections, but not within the same section. To overcome this problem and to achieve more accurate co-localisation, in the next experiment LN-229^{GFP} GBM-bearing mice were sacrificed on day 18 after INA of OAV-loaded shuttle cells, and rabbit as well as mouse antibodies were used to analyse the tumour core for the presence of Hexon, DAMPs and YB-1.

Co-localisation of Hexon with the DAMPs and YB-1 was confirmed for all three proteins of interest (*Figure 9*). It is interesting to note that staining for YB-1 with the rabbit antibody gave a fairly clear signal, whereas with the previously used mouse antibody, as mentioned above, only weak detection could be achieved once (see *Figures 2*, *8* and *9* for comparison). As with the previous experiments, the HMGB1 signal was the strongest of the three proteins detected, while HSP70 was only slightly lower by comparison.

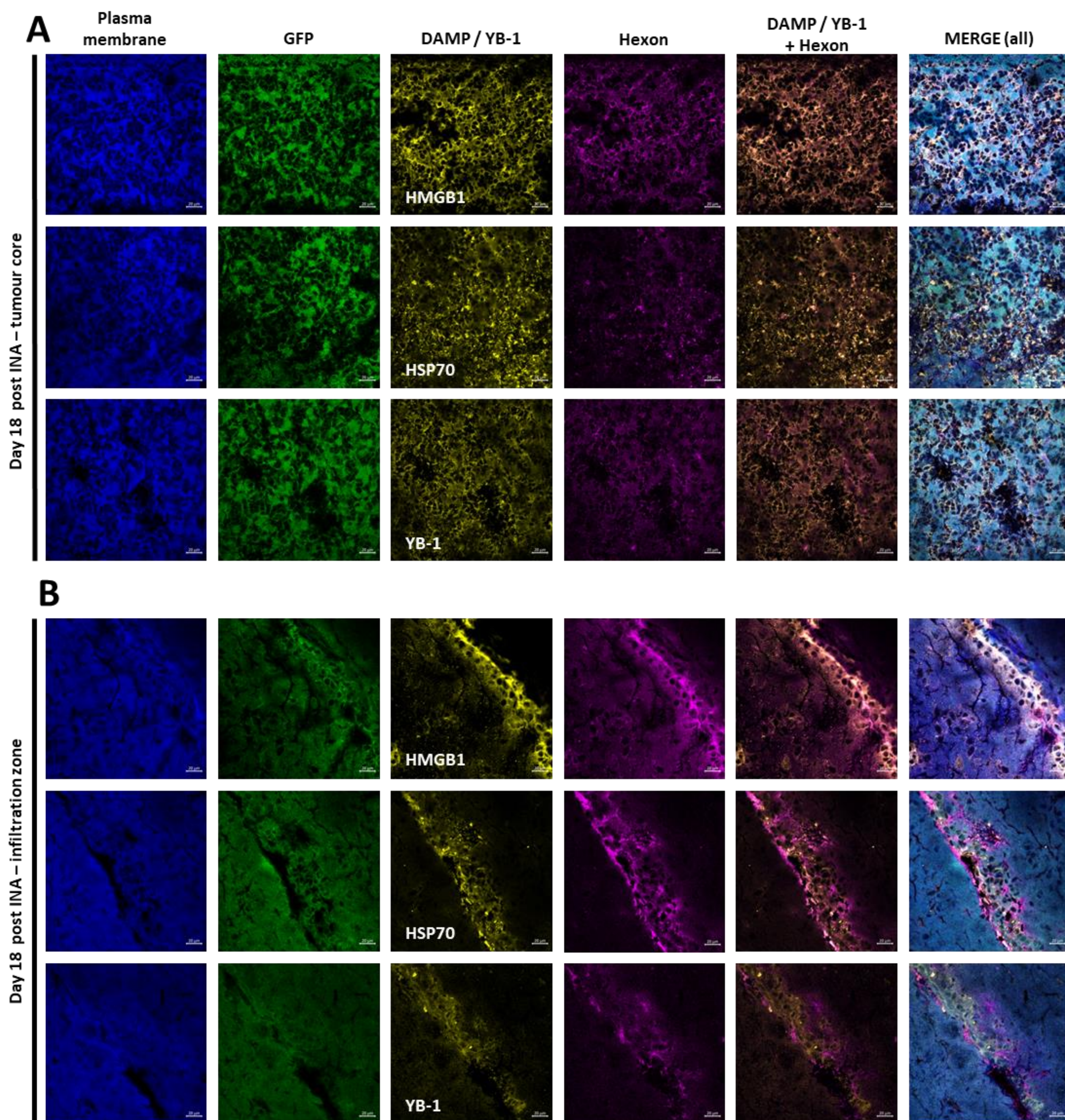

Figure 9. Co-detection of Hexon and immunogenic proteins in GBM-bearing mice treated with INA of OAV-loaded shuttle cells. *LN-229^{GFP} GBM-bearing mice were sacrificed on day 18 after INA of 4 x 10^{6} LX-2^{FR} cells infected with 200 MOI of XVir-N-31, prior to the onset of tumour-associated symptoms. Brains were prepared for IF and double staining for the respective protein as well as Hexon was performed. Images were acquired (**A**) in the tumour core and (**B**) in the infiltration zone (40x magnification, n=1, representative images are shown).*

Our interest was also to investigate whether the induction of DAMPs and YB-1 seen in the tumour core could also be observed in the infiltration zone. To achieve this goal, we analysed the brain sections of LN-229^{GFP} GBM-bearing mice on day 18 after INA of OAV-loaded shuttle cells, as described in the previous experiment.

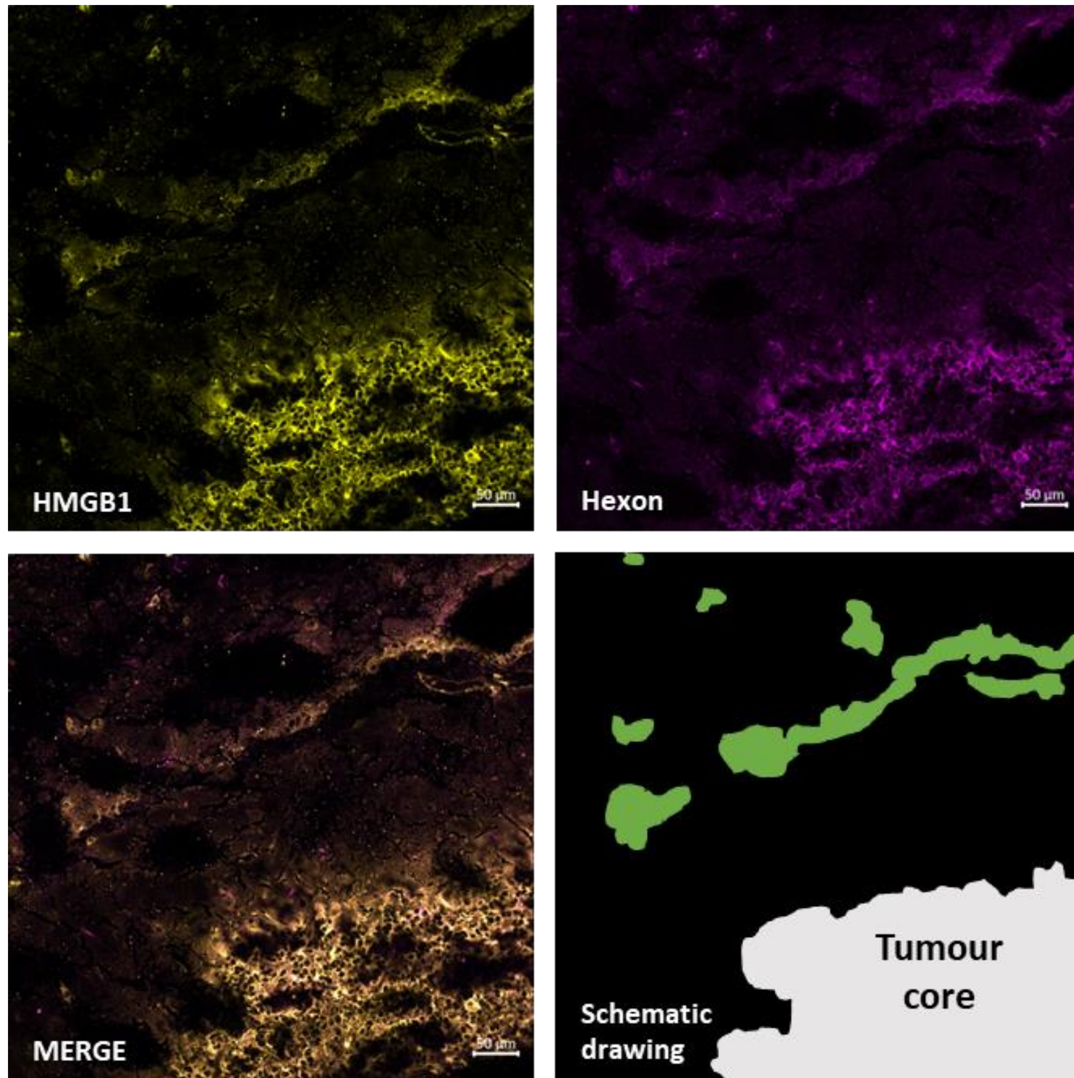

Figure 10. Co-detection of Hexon and HMGB1 in the infiltration zone in GBM-bearing mice treated with INA of OAV-loaded shuttle cells. *LN-229^{GFP} GBM-bearing mice were treated with an INA of 4 x 10^6 LX-2^{FR} cells infected with 200 MOI of XVir-N-31 and were sacrificed on day 18 according to the experimental schedule, before the onset of tumour-associated symptoms. Brains were prepared for IF and simultaneously stained for HMGB1 and Hexon. The schematic drawing shows the tumour core in the lower right corner (grey) and the infiltrating cells dispersed in the upper half of the image (green) (5x magnification, images reconstructed from 25 tiles, n=1, representative images are shown).*

A reconstructed overview presented in *Figure 10* illustrates the tumour core located in the lower right-hand corner of the image, and scattered infiltrating tumour pieces in the upper portion of the image, which are no longer in direct contact with the original tumour. Even on day 18 after the INA of OAV-loaded shuttle cells, a persistence of the OAV XVir-N-31 in infiltrating tumour parts could be observed. Furthermore, noticeable DAMP presentation could be observed in direct spatial proximity to these infiltrating tumour parts.

In summary, we can therefore say after this experiment that, firstly, the OAV persists in the tissue after INA until at least day 18 and, secondly, that the OAV co-localises with immunogenic proteins both in the tumour core and in infiltratively growing tumour cell groups.

4 Discussion

Over the last two decades, there has been a tremendous increase in interest and research on OVs, with numerous clinical trials currently in progress (for more information, refer to Mantwill et al. (2021) and Mondal et al. (2020)). As a result, the concept of OVT has transitioned from being mostly theoretical to being a tangible therapy that is already benefiting patients today. A pivotal breakthrough has been the 2015 approval by the FDA and EMA of the oncolytic herpesvirus T-Vec to treat advanced metastatic melanoma, making it the first OV in Western countries to receive approval (Andtbacka et al., 2015, Conry et al., 2018).

This first-in-class therapeutic is, of course, only the first step in a broad field of research, and current investigations are focused on several areas with the common goal of improving OVT and making it available to a greater number of cancer patients. More specifically, this may mean expanding the indications of already approved OAVs (e.g. ClinicalTrials.gov Identifier: NCT03458117), combining viruses with other therapeutic modalities such as radiotherapy (e.g. NCT05051696) or immunotherapy (e.g., NCT04330430), developing new viruses (e.g. Lang et al. (2018)) or exploring the underlying mechanisms of action for OVs (e.g. Kohlhapp and Kaufman (2016)).

This thesis is part of the latter group and aims to investigate the mechanisms of action of the OAV XVir-N-31, looking in detail at the extent to which an ICD is induced under a variety of therapeutic conditions and routes of administration. The results of our experiments will be placed in a broader context in the following discussion, with a particular focus on why OVT can be classified as a kind of immunotherapy of cancer.

4.1 Potent induction of ICD by XVir-N-31

In the used mouse model of GBM, we observed increased presentation of HMGB1 and HSP70 in XVir-N-31-treated tumours, which did not occur after treatment with the wild-type adenovirus (dl309). Of the DAMPs just mentioned, HMGB1 was the most prominent in our evaluations, while the immunofluorescent detection of HSP70 was in some cases weaker and more difficult to interpret.

Taken together, these are compelling findings that the OAV XVir-N-31 is a potent inducer of ICD in our mouse model, whereas dl309 failed to induce ICD. Even with nivolumab monotherapy, signs of ICD were only very marginally detectable. This illustrates that not every adenovirus-mediated cell lysis and not every cytoreductive therapy is capable of inducing an ICD, but that in our case, specifically XVir-N-31 is capable of doing so, even longer time after the IT injection of the OAV.

Interestingly, DAMPs were also detectable in foci of infiltrated tumour cells, although these had never been in direct contact with the OAV after the IT application of XVir-N-31. This suggests that human PBMCs are capable of independently targeting tumour cells after OVT, whereupon these in turn release more DAMPs, a concept we refer to as "secondary, T cell mediated ICD". In other words, therapy with XVir-N-31 is capable of changing the previously "cold" TME into an immunologically "hot" TME, this way directing cytotoxic T cells to distant tumour sites.

Staining for YB-1 was positive only after INA of XVir-N-31-loaded LX-2^{FR} shuttle cells, but not after IT administration of the OAV. Due to this inconsistency of results and the fact that different antibodies against YB-1 were used, no definitive conclusions can be drawn regarding the possible induction of YB-1 after OVT. The result of this inconclusive YB-1 staining leaves a number of possibilities open: it is possible that YB-1 was not present in the tissue in this particular section or at this particular time (day 18 after INA versus day 35 after IT application), or that there was a staining error or antibody failure. Finally, it is also conceivable that the way the virus is administered may have an effect on YB-1 induction, but this can only be speculated at this stage given the limited information available.

We acknowledge the inherent limitations of the techniques used, such as the difficulty in distinguishing between intra- and extracellular location of proteins detected by IF. This is of course due to the use of detergents such as Tween contained in PBS-T, which disrupts the membrane barrier, and the observation of multiple overlapping cell layers in a 10 μm section of the brain. However, this technique is suitable for our purposes as the behaviour of DAMPs such as

HMGB1 is well characterised and our observations are mainly derived from the comparison of different regimens. This was also the main reason for our decision not to further quantify the immunofluorescence signal. The latter is a complex process using image analysis software to separate the background signal from the actual "true" signal, which is also influenced by a variety of factors, including small but unavoidable differences in the staining process or natural variations in the laser power of the microscope. For quantitative data, we therefore refer to the work of Klawitter et al. (2022) from our research group, in which, among other things, the release of DAMP was measured *in vitro* using ELISA technology.

4.2 Advancing OVT through the combination of XVir-N-31 and ICI

Although OVT with XVir-N-31 has a pronounced therapeutic effect in our model and leads to a significant reduction in tumour size and to a significant prolongation of survival in a mouse GBM model using glioma stem cells (Mantwill et al., 2013), the therapeutic effect is still limited in the sense that neither INA nor IT application of XVir-N-31 per se is sufficient to eliminate entire tumours. One of the reasons for this is that the activation of the immune system by the OVT must outweigh the enormous intrinsic immunosuppression of GBM. In addition, GBM exhibits an extreme intratumoural heterogeneity and multiple escape mechanisms, and clinical experience over the past decades has shown that no monomodal therapy is likely to be successful as a stand-alone strategy (DeCordova et al., 2020, Prager et al., 2020). This is compounded by the inherent limitations of OVTs, including the aforementioned limitation that the viral infection cascade only continues if the tumour cells remain adjacent.

There is therefore a need to further develop OVT, for example by exploiting its therapeutic potential in combination with other treatment modalities. Among the many options, immune checkpoint inhibition (ICI) is a particularly suitable candidate for this purpose (for review, see Shi et al. (2020)). To date, only a small subset of GBM patients benefit from ICI monotherapy due to the poor immunogenicity of this tumour entity (Akintola and Reardon, 2021). While ICIs can take "the brakes off" the immune system, there are still very few antigens presented on cells that can activate T cells or other cellular immune defences.

This is where OVT comes into play, making the tumour more immunogenic by bringing not only virus-associated antigens but also tumour-associated antigens to the cell surface or, after cell lysis, into its environment. In a sense, OVT sensitises the tumour to ICI. But there is also a potential synergy in the other direction. By reversing immunosuppression, ICI facilitates viral spread and theoretically enhances the therapeutic effect of OVT. We would therefore expect and hypothesise that OVT and ICI would be ideal therapeutic companions (Naumann and Holm, 2014). Indeed, in our experiments we were able to show that mice treated with the combination of OVT and ICI showed much stronger ICD associated signals, suggesting a better therapeutic efficacy and a stronger anti-tumour response. Of particular interest, the ICD associated detection of DAMPs was as strong when the combination therapy was administered separately (XVir-N-31 intratumourally plus nivolumab intraperitoneally) as when XVir-N-31-anti-PD-L1, the derivative of XVir-N-31 that additionally expresses an antibody against PD-L1 locoregionally, was administered alone. This again suggests that the latter is a very elegant way to maximise the full effect of ICIs locally but hopefully avoid the systemic side effects.

Klawitter et al. (2022) have recently shown that the increased immunogenicity after OVT, indirectly observed in our experiments by means of increased DAMP presence, has a significant therapeutic consequence. In mice treated with a combination of XVir-N-31 and ICI, the authors observed an increased infiltration of activated T cells into the tumour tissue as well as significant, abscopal effects on infiltrating tumour foci. Of particular note is the fact that the wild-type adenovirus dl309 was also able to reduce the size of the main tumour, as viral lysis is the predominant mechanism of action in virus-infected cells, but not in distant tumour foci, as the wild-type virus lacks the immunogenic effect.

4.3 Advancing OVT through INA of OAV-loaded shuttle cells

In our experiments, the IN delivery of loaded shuttle cells proved to be an effective means of bypassing the BBB and delivering OAVs directly to tumour cells, particularly invasive and infiltrating GBM cells, as demonstrated by the co-localisation of the adenoviral capsid protein Hexon and tumour cells (see

Figures 9 and *10*). Although only a single INA of XVir-N-31 loaded shuttle cells was given in our experiments, the setup itself allows for simple, repeatable, and non-invasive application. Thus, INA is an exciting future approach for the therapy of GBM and potentially other CNS diseases, including Alzheimer's disease (Zhang et al., 2021). In these diseases, for example, cerebroactive drugs can be pharmacologically formulated as lipid nanoparticles and administered intranasally, which can then easily cross the BBB and thus avoid the problem of efflux transporters within the BBB (such as P-glycoprotein) when administered systemically (Arora et al., 2020).

We experimentally observed that after INA of XVir-N-31-loaded shuttle cells, GBM-bearing mice showed a strong presentation of DAMPs in the tumour tissue, which reached its maximum around day 12 within the observation period of 18 days. This was true for the main tumour as well as for isolated tumour foci, highlighting the control of infiltrating tumour foci as one of the major strengths of the INA of virus-loaded shuttle cells. Of course, a potential disadvantage of this technique compared to IT virus delivery is the large amount of virus that needs to be delivered intranasally, as a large proportion of the applied cells are expected to be degraded in the nasal mucosa. Translated into everyday clinical practice, this could mean higher production effort and cost.

In our experiments, the IT and IN applications were relatively equivalent in terms of ICD induction. However, both routes of application are also comparable in terms of therapeutic efficacy, as recently shown in our research group: the survival of mice was significantly prolonged after both IT administration of XVir-N-31 and INA of XVir-N-31-loaded LX-2^{FR} shuttle cells, with no significant difference between the two treatment groups. Notably, mice treated with a combination of the two regimens had an even longer survival time and two out of eight mice in this group had no detectable tumours at the end of the experiment (El-Ayoubi et al., 2023b). These results support our hypothesis that the combination therapy using both routes of administration is particularly promising, as IT injection has a potent cytoreductive effect on the tumour core, while INA has an excellent ability to reach infiltrating cells.

4.4 Challenges and chances in OVT

OVs are a diverse group of biological therapeutics that share the ability to lyse tumour cells as well as the ability to induce anti-tumour activity (for review, see Lawler et al. (2017)). Their attractiveness as cancer therapeutics is based on their tumour cell-specific effects, the multiple ways in which they can be manipulated and modified and, most importantly, their potential to induce long-lasting immunity that extends beyond the actual presence of the therapeutic in the body. The exact way in which the different mechanisms interact and the extent to which they contribute to the overall effect is not yet fully understood (Zhang and Cheng, 2020).

However, there are still a number of obstacles that stand in the way of effective cancer treatment. For example, an important unanswered question is how long the virus can persist in the tissue and to what extent a constant presence of the virus is necessary to maintain high levels of anti-tumour immunity. In our experiments, for example, we observed the death of shuttle cells around day 3-5 after INA, while the levels of Hexon continued to increase, suggesting that neoplastic cells had been infected by shuttle cell released OAVs and now subsequently produce new virus particles that further on infect and replicate in adjacent tumour cells. Although this process is theoretically infinite and the virus should continue to infect cell after cell until there are no more adjacent neoplastic cells, we were only able to demonstrate this experimentally up to day 35. Therefore, the present work will be followed by another experiment in our group using a slow growing GBM mouse model, that will be examined months after INA to answer this question. Looking a little further into the future, this experiment may help us to determine whether multiple, consecutive IN applications of the virus could be beneficial, and if so, at which intervals.

Of course, there is also the challenge of choosing the right time in the course of treatment for OVT and integrating it into the existing treatment regimen. At present, immunotherapies are generally used relatively late in the course of the disease, usually at the time of relapse or after failure of conventional methods. This is partly because there are very few established treatment options available

at the time of relapse, which opens the door to experimental therapies and trials and encourages patients to seek innovative treatments. This is likely to change soon, and immunotherapies are likely to be used earlier, possibly as first-line therapies, as knowledge and trials in this area increase. This is particularly true for OVs, which have the potential to mediate both tumour debulking and residual disease eradication due to their theoretically exponential proliferation. At present, however, XVir-N-31 still needs to take the first steps in clinical trials to confirm its tolerability, safety and later its therapeutic potential in humans. A first-in-human Phase I trial, named XVIR-01, in which a convection-enhanced delivery of XVir-N-31 into the tumour core is administered to GBM patients at the time of relapse, is registered to start at the end of 2022 (EudraCT number 2016-000292-25), results are still pending at the time of publication.

4.5 Conclusion

In summary, this work, combined with the current state of research, underlines once again why OVT is now rightly classified as a kind of immunotherapy of cancer. To summarise briefly, it could be said that the immune system per se is capable of recognising and fighting cancer cells even prior to treatment, but tumours that grow and become clinically manifest have shown that they outweigh the capacities of the immune system. OVT therefore joins the ranks of immunotherapies as it has the same main goal, namely to reverse the pathological imbalance between tumour and immune system. Since ICD induction is a fundamental requirement for this immune activation and the development of a strong, durable anti-tumour response, it should therefore be a critical part of the evaluation of new OVs.

Initial practical trials with XVir-N-31 in animal models have confirmed this potential to activate the immune system, but also revealed the limitations that have so far prevented a resounding success in the fight against GBM. We are therefore pleased to have addressed two important approaches in this work that may help to further advance OVT and overcome the existing hurdles.

Firstly, IN delivery is an efficient, non-invasive and easily repeatable method of delivering therapeutics to the brain. It further broadens the clinically available toolbox for the treatment of various CNS disorders. The ability to target even dispersed neoplastic cells appears to be a particular advantage of this technique in the context of GBM treatment.

Secondly, the combination of OVT and ICI appears to induce an even stronger ICD compared to OVT monotherapy. This may also be associated with improved, long-lasting anti-tumour immunity, which is necessary for effective GBM therapy. As a result, this is a particularly promising area for future OVT research, and it is with cautious hope that we can look forward to research results over the next few years.

5 Abstract

GBM is the most common primary malignant brain tumour in adults. With a median survival time of less than 20 months, it remains incurable, mostly due to its highly aggressive, infiltrative growth. OVT is an approach to cancer treatment in which patients receive OVs that selectively replicate in and lyse neoplastic cells. Of particular interest is the induction of ICD, mediated mainly by the release and presentation of DAMPs. ICD can contribute to a long-lasting anti-tumour response, allowing the patient's immune system to attack not only the main tumour but also infiltrating GBM cells that were not directly infected and killed by IT applied OVs. Thus, the clinical relevance of ICD induction is enormous.

The aim of this thesis was to test whether the OAV XVir-N-31 induces ICD in GBM. Since adenoviruses replicate in human, but not in murine cells, immuno-deficient NSG™ mice were equipped with a to the tumour HLA-A/B-matched humanised immune system and inoculated with human GBM cells in both striata. The right sided rapidly developing GBM was then IT injected with either PBS (sham treatment), XVir-N-31, XVir-N-31-anti-PD-L1 (secreting a PD-L1 binding antagonistic antibody) or dl309 (an Ad-Wildtype derivate), while the contralaterally located tumour was left untreated. All mice were sacrificed collectively at the onset of tumour-associated symptoms in the first mouse of any group. The detailed treatment has been published in Klawitter et al. (2022). Immunofluorescence was used to identify ICD by the detection of DAMPs in the tumour and surrounding tissue.

We identified key DAMPs such as HMGB1 and HSP70 in the tumour tissue after OVT with XVir-N-31 that were absent after virotherapy using dl309, suggesting that XVir-N-31 specifically induces ICD. In following experiments, we observed that the combination of XVir-N-31 with an ICI such as nivolumab enhances this effect. Furthermore, XVir-N-31 delivered to GBM by the INA of XVir-N-31-loaded shuttle cells also induced ICD in the tumour. In conclusion, our data show that XVir-N-31 is a potent inducer of ICD, at least in our mouse models of GBM. This is a prerequisite for a successful clinical application of XVir-N-31 in GBM patients in the future.

6 Zusammenfassung

Das GBM ist der häufigste primäre maligne Hirntumor des Erwachsenen. Gekennzeichnet durch hochaggressives, infiltratives Wachstum, geht es mit einem obligat letalem Verlauf bei einem medianen Überleben von weniger als 20 Monaten einher. Die OVT ist ein therapeutischer Ansatz, bei dem GBM-Patienten OVs verabreicht werden, die sich selektiv in neoplastischen Zellen replizieren und diese bei Freisetzung des Virus lysieren. Von besonderem Interesse ist dabei die Induktion des ICD, der maßgeblich durch die Freisetzung und Präsentation bestimmter Alarmsignale, sog. DAMPs, vermittelt wird. Da das patienteneigene Immunsystem dadurch den Primärtumor, aber insbesondere auch infiltrativ wachsende, nicht direkt vom Virus erreichbare Tumorzellen nachhaltig angreifen kann, ist die klinische Relevanz des ICD enorm.

In dieser Arbeit sollte die Induktion von ICD im GBM durch das onkolytische Adenovirus XVir-N-31 untersucht werden. Da sich Adenoviren nur in humanen, nicht aber in murinen Zellen replizieren, wurden immundefiziente NSG™-Mäuse mit einem Tumorzell-HLA-gematchten humanen Immunsystem ausgestattet und in beide Striata humane GBM-Zellen appliziert, die im Gehirn zum GBM heranwachsen. Nach Tumorgeneration erfolgte in den rechtsseitigen Tumor eine intratumorale Injektion von PBS (Scheinbehandlung), XVir-N-31, XVir-N-31-anti-PD-L1 (sezerniert einen PD-L1 antagonisierenden Antikörper) oder dl309 (Ad-Wildtyp Derivat), während der kontralateral lokalisierte Tumor unbehandelt blieb. Alle Mäuse wurden beim Auftreten von Tumor-assoziierten Symptomen in der ersten Maus kollektiv geopfert. Die detaillierte Behandlung wurde in Klawitter et al. (2022) veröffentlicht. Mittels IF wurde ICD durch den Nachweis von DAMPs im Tumor und im umliegenden Gewebe identifiziert.

Nach OVT des GBM mit XVir-N-31 waren bedeutende DAMPs (HMGB1 und HSP70) detektierbar, die nach Injektion von Ad-WT (dl309) nicht nachweisbar waren. Spezifisch XVir-N-31 induziert demnach einen ICD, ein Effekt, der durch die Kombination der OVT mit Immun-Checkpoint-Inhibitoren wie z.B. Nivolumab noch verstärkt wurde. ICD wurde ebenfalls induziert, wenn das OV mittels intranasaler Applikation OV-beladener Shuttle-Zellen zum Tumor transportiert

wurde. Zusammenfassend liefern unsere Ergebnisse den prinzipiellen Nachweis, dass XVir-N-31 ein potenter Induktor des ICD ist, zumindest in den hier verwendeten Maus-GBM-Modellen. Dies ist eine wesentliche Erkenntnis für die zukünftige erfolgreiche klinische Anwendung von XVir-N-31, vor allem in Kombination mit weiteren Immun-induzierenden Therapieansätzen.

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8 Erklärung zum Eigenanteil

Die Arbeit wurde im Hertie-Institut für Klinische Hirnforschung, Tübingen, unter Betreuung von Prof. Dr. rer. nat. Ulrike Naumann durchgeführt.

Die Konzeption der Studie erfolgte durch Prof. Dr. rer. nat. Ulrike Naumann (Arbeitsgruppenleiterin), Ali El-Ayoubi (PhD Doktorand) und Moritz Klawitter (PhD Doktorand).

Die Arbeit mit den Zellen und Viren sowie jegliche Tierarbeit wurden von Ali El-Ayoubi and Moritz Klawitter durchgeführt. Die Gehirne der behandelten Mäuse wurden von diesen freundlicherweise zur weiteren Aufarbeitung zur Verfügung gestellt.

Die histologische Aufarbeitung, die Immunfluoreszenzfärbung sowie die Arbeit am konfokalen Lasermikroskop wurde nach Einarbeitung durch die Labormitglieder Prof. Dr. rer. nat. Ulrike Naumann, Ali El-Ayoubi und Moritz Klawitter eigenständig durchgeführt.

Ich versichere, das Manuskript selbstständig verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.

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9 Veröffentlichungen

EL-AYOUBI, A., KLAWITTER, M., RÜTTINGER, J., WELLHÄUSSER, G., HOLM, P. S., DANIELYAN, L. & NAUMANN, U. 2023b. Intranasal Delivery of Oncolytic Adenovirus XVir-N-31 via Optimized Shuttle Cells Significantly Extends Survival of Glioblastoma-Bearing Mice. *Cancers (Basel),* 15.

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