KU Leuven

Biomedical Sciences Group

Faculty of Medicine

Department of Microbiology, Immunology and Transplantation

Laboratory of Pediatric Immunology

Immunotherapy with subcutaneous immunogenic autologous tumor lysate against glioblastoma

Jochen BELMANS

Jury:

Promotor: Prof. Dr. Dominique Bullens
Co-promotor: Prof. Dr. Stefaan Van Gool
Co-promotor: Prof. Dr. Jean-Pierre Locquet
Jury members: Prof. Dr. Matthias Eyerich
Prof. Dr. Jacques Piette
Prof. Dr. Patrick Matthys
Prof. Dr. Paul Clement

Dissertation presented in partial fulfilment of the requirements for the degree of Doctor in Biomedical Sciences

Leuven, October 2019

This work was mainly supported by the Olivia Hendrickx Research Fund vzw

www.olivia.be

Acknowledgements

Twee en een half jaar nadat ik de laatste experimenten van mijn doctoraatsonderzoek heb uitgevoerd, sta ik hier eindelijk voor mijn publieke verdediging. De laatste horde van een project van vallen en opstaan dat iets meer dan acht jaar geleden begon. Uiteraard hebben vele mensen mij geholpen om tot dit punt te komen en daarom verdienen zij allemaal een vermelding in dit dankwoord.

Wetenschappelijk onderzoek is een kostelijke zaak en vereist veel inbreng alvorens er een beperkte oogst is. Het onderzoek dat ik heb uitgevoerd was enkel en alleen mogelijk dankzij de financiële steun van het Olivia Fund (www.olivia.be). Mevrouw Ilse De Reze, ik wil u en uw organisatie van harte bedanken voor de continue steun en het vertrouwen in dit project. Zonder u was er nooit sprake geweest van mijn docoraatsonderzoek.

Als volgende wil ik zowel mijn promotoren, Stefaan Van Gool en Dominique Bullens, alsook mijn copromotor Jean-Pierre Locquet bedanken. Stefaan, we zijn samen aan een uitdagend project begonnen waarbij we het voor ons onbekende veld van nanotechnologie hebben trachten te introduceren in het veld van immuuntherapie bij hersentumoren. De eerste resultaten bleven lang uit, maar geleidelijk aan kwam er toch schot in de zaak. Dankzij jou geloof in immuuntherapie, jou inzet voor je patiënten en je constante zoektocht naar onderzoeksgeld, geraakte ik telkens opnieuw gemotiveerd om het onderzoek verder te zetten. Bedankt om in je drukke agenda telkens een plaatsje te maken om mijn nieuwste resultaten te bespreken. Toch blijft het mij een raadsel hoe iemand die dagelijks met zo veel miserie in contact komt, zo positief door het leven kan blijven gaan. Je hebt me geleerd om negatieve resultaten vanuit een ander perspectief te bekijken en er de positieve conclusies uit te trekken (iets wat we vaak hebben moeten doen in dit project). Ik heb enorm veel respect voor jou als arts, als onderzoeker en als familieman. Dominique, bedankt om de begeleiding van mijn doctoraatsonderzoek over te nemen. Ook al had je de vooruitgang al enkele jaren opgevolgd tijdens mijn datasessies, het zal niet evident geweest zijn om als specialist in allergie een doctoraat omtrent immuuntherapie bij hersentumoren te helpen finaliseren. Het is mede dankzij jou herhalende vraag “wanneer zal het doctoraat afgerond zijn?”, dat ik hier nu sta. Bedankt voor jou wetenschappelijke input alsook taalkundige tips bij het schrijven van mijn artikel en thesis. Merci om meermaals jou vrije woensdagavond op te offeren om mijn resultaten (samen met Stefaan) te bespreken of het manuscript van mijn artikel en mijn thesis door te nemen. Beste Prof. Locquet, hoewel wij weinig rechtstreeks contact hebben gehad en de focus van mijn onderzoek gericht was op de biologische aspecten, heeft u mij mede wegwijs gemaakt in de wereld van nanotechnologie. Graag wil ik u bedanken voor de mogelijkheden die u en uw onderzoeksgroep mij geboden heeft. Onze samenwerking is uitgegroeid tot het doctoraatsproject van Stephanie, een project waarin nanotechnologie terecht meer focus van het onderzoek heeft gekregen.

I would also like to thank the members of the jury for their careful review of this manuscript. Your comments and suggestions helped me further optimizing this work. Moreover, I would like to thank them for being here today. Voorts wil ik de professoren van het labo Klinische Immunologie bedanken: Prof. emeritus Ceuppens, Prof. De Vleeschouwer, Prof. Hellings en Prof. Schrijvers. Vanaf de start met de uitdagende IWT oefensessies, en voorts met de datasessies op vrijdagochtend, hebben jullie voor een constante evaluatie van mijn project gezorgd. In het bijzonder wil ik Prof. emeritus Jan Ceuppens en Prof. Rik Schrijvers bedanken dat ze mij hebben toegelaten om mijn experimenteel werk uit te voeren in hun labo.

Het labo Klinische Immunologie was voor mij nieuw op het moment dat ik aan mijn doctoraat begon en dus heb ik de kans gehad om vele nieuwe mensen te leren kennen. Matthias, Jeroen, Brecht S, Brecht C, Joost, Matteo, Sven, Kasran, Jonathan, Lieve, Ellen, Lien VDB, Tina, Carolien, Liana, Leen, Isabel, Valerie, Sonja, Christine, Laura, Ina, Inge, Emilie, Sofie, Anaïs, Femke, Kelly, Kim, Goedele, Danny, Lien S, Thaïs, An, An-Charlotte, Simone, Irina, Isabelle, Zhe, Abhishek, Omer, Caroline, Stephanie, Bert en vele anderen: bedankt voor de toffe momenten, de hulp met experimenten en de leuke labo-uitjes.

Enkele collega's wil ik toch apart bedanken. Allereerst mijn 'partners in crime' Matthias, Jeroen en Brecht. Ik herinner mij nog dat Jeroen zeer blij was dat hij en Sven niet meer de enige jongens waren onder de doctoraatstudenten toen ik begon. Een Jaar later zouden we nog een keer verdubbelen in aantal met de komst van Matthias en Brecht. Het klikte van in het begin en al snel werden er plannen gesmeed om extracurriculaire activiteiten te organiseren: van de labodrinks op vrijdagnamiddag en de droppings tot de shortski. Ik ben blij dat ik in jullie team zat! Matthias, mijn kempenbroeder, bedankt om me mee over de streep te trekken. De gesprekken tijdens onze eindeloze autoritten van het verre Westerlo naar Leuven en terug hebben mij heel vaak deugd gedaan. Maar daarnaast wil ik jou ook bedanken voor de experimentele hulp, jou kennis en expertise. Brecht, ik vraag me af wanneer we nog eens een keer op drakenjacht gaan. Het was me een waar genoegen om naast jou te kunnen zitten aan de bureau, ook al betekende dat wel dat mijn kasten vol met verrassende prentjes kwamen te hangen. Jeroen, de sportman van ons labo, proficiat voor al die keren dat je de pronostiek van sportevenementen hebt gewonnen. Ik denk niet dat ik 1 keer beter heb gescoord dan jou. Als je volgend seizoen nog een keer naar de voetbal in Westerlo wilt komen kijken, de kans bestaat dat ze tegen Anderlecht spelen in 1B. Kortom, mannen bedankt voor alle hulp en de aangename werksfeer die er was, het was vaak een plezier om te komen werken. Ik ben blij dat we elkaar nog geregeld proberen te ontmoeten 'In den Rozenkrans'.

Daarnaast wil ik ook Lieve, Ellen en Jonathan bedanken om de onmisbare, drijvende krachten te zijn achter het labo. Ik wil jullie ook alle drie bedanken voor de uren die we samen aan de flow cytometer hebben doorgebracht. Brecht C. en Stephanie, jullie hebben beide een aandeel gehad in mijn onderzoek toen jullie als masterthesisstudenten in ons labo hebben meegedraaid. Ondertussen zijn jullie beiden aan het einde van jullie doctoraatsonderzoek gekomen. Ik wens jullie beide enorm veel succes met de laatste loodjes! Karin, merci om mijn

muizen zo goed te verzorgen, soms te goed hebben we nog gelachen. De gezellige babbels met jou en Griet waren vaak een welgekomen ontspanningsmoment in een drukke werkdag.

Beste BMW vrienden, tijdens onze studies hebben we samen bloed zweet en tranen gedeeld, maar daarna zijn we elk onze weg gegaan. De meesten van ons hebben hun doctoraatstitel al een tijdje geleden behaald en als laatste kan ik mij daar eindelijk ook bij voegen. Ook al heb ik vaak moeten afzeggen voor onze bijeenkomsten, het doet me telkens opnieuw deugd om jullie allemaal terug te zien. Ik wens jullie allemaal nog zeer veel succes met jullie toekomstige carrières. Tot de volgende!

Ook mijn huidige collega's bij Cypress Diagnostics wil ik bedanken voor de aangename werksfeer, de deugddoende babbels en de 'je kan altijd op ons rekenen' mentaliteit. Beste Herman en Thomas, bedankt voor jullie begrip betreffende mijn doctoraat, de kans die jullie mij gegeven hebben om te mogen starten en voor de doorgroeimogelijkheden binnen het bedrijf. Beau, een speciale dank je wel om mij wegwijs te maken in de wereld van de *in vitro* diagnostiek en voor alle tips die je gegeven hebt om het schrijven van mijn thesis tot een goed einde te brengen. Zonder jou weet ik niet of ik hier 'nu al' had gestaan.

Voorts wil ik alle mensen die ik via Chiro Oevel heb leren kennen bedanken. Inspanning is enkel mogelijk mits voldoende ontspanning en de Chiro was altijd mijn uitlaatklep. Vele vrienden voor het leven heb ik hier aan overgehouden. Voornamelijk de 'Grote Jongens' wil ik bedanken om zelfs na onze leidingscarrière te zorgen dat we nog geregeld een avondje inplannen met een frisse pint in de hand of op weekend gaan. Merci om me er altijd aan te herinneren dat mijn doctoraat nog niet afgelopen was met woorden als: "zedde daar nu nog altijd mee bezig?", "Zedde nu al doctor?" of "hebde kanker al genezen?". Weet dat ik bij deze weer een goede reden heb om iedereen een keer samen te brengen, want deze dag moeten we vieren!

Familie komt bij mij steeds op de eerste plaats, no matter what. Moeke en vokke, merci om mij altijd gesteund te hebben in alles wat ik wilde doen. Van de kleinste beslissing tot de hele grote, jullie zijn altijd meegegaan in mijn verhaal. Ik kan jullie niet genoeg bedanken voor de kansen die ik allemaal heb gekregen. Ik hoop dat ik naar jullie voorbeeld mijn eigen kinderen kan grootbrengen. Moemoe, op zondagavond bij jou en vava samenkomen was het familiemoment bij uitstek. Samen met mijn neven en nichten smullen van het heerlijks dat jij had klaargemaakt en dan bijpraten. Bedankt om onze familie zo hecht te houden ook al heb je dit jaar wel zware momenten gekend! Bovendien wil ik ook mijn schoonfamilie bedanken voor de interesse die jullie altijd hebben getoond.

Tenslotte, Annick, wil ik jou bedanken voor je onvoorwaardelijke steun, je opofferingen en je liefde. Vorige week zei je nog tegen mij: "Wist je dat ik al meer dan de helft van mijn leven samen ben met jou?". Er zijn geen woorden om te zeggen hoe blij ik daarmee ben. Jij voelt me altijd perfect aan en weet exact wat ik nodig heb. De laatste jaren zijn voor ons enorm druk geweest van bouwen en touwen tot het krijgen van twee prachtige kinderen en dan kwam

die doctoraatsthesis daar nog bij. Bedankt om me de kans te hebben gegeven om dit project volledig af te ronden. Het heeft veel van onze tijd samen weggenomen, maar nu ben ik er volledig voor jou en ons gezinnetje. Emmelien en Mil, jullie weten me altijd op te beuren als het een beetje moeilijk gaat. Voor jullie glimlach, jullie eerste woordjes/zinnetjes of gebrabbel, jullie knuffels en kusjes, bedankt! Weet dat nu dit doctoraat gepasseerd is, papa nog meer tijd voor jullie zal maken!

Eigenlijk zou ik iedereen nog veel uitgebreider moeten bedanken dan hier neergeschreven, maar iedereen weet dat ik een man ben van weinig woorden. Laten we bij pot en pint nog een keer afspreken, zoals we wel weten kan alcohol de tong wat meer losmaken en wie weet welke mooie woorden volgen er dan nog.

Peace out,

Jochen

Table of contents

Acknowledgements i

Table of contents v

List of abbreviations ix

1 General introduction 1

1.1 Glioblastoma: the nemesis amongst tumors of the central nervous system 1

1.1.1 An introduction to glioblastoma 1

1.1.2 Diagnosis, standard of care and adjuvant targeted therapies 2

1.2 Cancer and the immune system 3

1.2.1 Cancer immunoediting – with a focus on glioblastoma 4

1.2.2 Immunotherapy 7

1.3 Dendritic cells and their application in immunotherapy 9

1.3.1 General characteristics of dendritic cells 9

1.3.2 Antigen uptake, processing and (cross-)presentation by dendritic cells 10

1.3.3 Dendritic cell vaccination 16

1.3.4 Active immunotherapy through *in vivo* antigen delivery to dendritic cells 21

1.4 Nanoparticles to further optimize antigen delivery to dendritic cells 25

1.4.1 Nanoparticles in cancer: diagnosis and treatment 26

1.4.2 Nanoparticle based approaches to elicit antitumor immunity 29

1.5 Murine glioblastoma models to study immunotherapy 38

2 Research objectives 41

3 Methodology and materials 43

3.1 Glioma cell lines and cell culture 43

3.1.1 Tumor lysate generation and FITC labelling 43

3.2 Generation of NP-lysate conjugates 43

3.3 DC generation and culturing 44

3.3.1 Analysis of lysate uptake by dendritic cells and presentation in MHC class I molecules 45

3.3.2 MTT assay 45

3.4 Orthotopic glioma mouse models 45
3.5 Brain immune cell isolation and characterisation 47
3.6 Lymph node biodistribution of lysate, NPs and nanovaccine 48
3.7 Functional testing – systemic T cell proliferation 48
3.8 Spleen, bone marrow, skin cell isolation 48
3.9 Statistical analysis 49
4 Results 51
4.1 Immunotherapy with subcutaneous immunogenic autologous tumor lysate increases murine glioblastoma survival 51
4.1.1 Uptake of lysate and presentation of lysate fragments by DCs *in vitro* 52
4.1.2 Pre-tumor lysate treatment improves survival in a murine glioma model 54
4.1.3 Early after tumor inoculation, lysate treatment induced a T cell influx combined with diminished immune suppression in the brain 57
4.1.4 Pre-tumor lysate treatment induces a long-lasting immunological memory 60
4.1.5 Subcutaneous, pre-tumor peptide vaccination results in comparable treatment efficacy as compared to peptide-loaded DC therapy in the GL261 glioma model 62
4.1.6 Combining temozolomide and lysate in a therapeutic treatment model improves median survival of glioma-bearing mice 63
4.2 Production of whole tumor lysate-loaded nanoparticles as an anti-cancer nanovaccine 67
4.2.1 Gel filtration of pre-activated nanoparticles is required to create nanoparticle-lysate complexes within the preconceived size range of 200 to 500 nm 68
4.2.2 Fragments of lysate are stably coupled to nanoparticles 69
4.2.3 Reduced toxicity of nanoparticle-lysate complexes for dendritic cells when gel filtration is implemented in the conjugation protocol 71
4.2.4 Uptake of nanoparticle-lysate complexes and presentation of lysate fragments by dendritic cells *in vitro* 73
4.2.5 Pre-tumor nanovaccine therapy improves survival in two different mouse glioma models 76
4.2.6 Pre-tumor treatment with nanovaccines and autologous tumor lysate results in equivalent survival benefit for glioma inoculated mice 78
4.2.7 Absence of an inherent adjuvant function for polystyrene nanoparticles in the nanovaccines 79

4.2.8 Nanovaccine treatment introduces influx of effector T cells in combination with reduced immunosuppression in the brain 81

4.2.9 Nanoparticle, lysate as well as nanoparticle-lysate conjugate injection develop a depot at the injection site and partly drain to local lymph nodes via dendritic cells 86

4.2.10 Long-lasting immunological memory following pre-tumoral nanovaccine administration 88

4.2.11 Peptide-loaded nanoparticles are able to treat 90

4.2.12 Therapeutic treatment by combining temozolomide and nanovaccine injections improves survival outcome in glioma-bearing mice 90

5 Discussion & Conclusion 93

6 Global conclusion and future perspectives 106

Addendum – immunotherapeutic treatment in glioma clinical trials 107

Addendum – flow cytometry information 108

Abstract 113

Summary 115

Nederlandstalige samenvatting 117

List of references 119

Acknowledgements, personal contributions and conflicts of interest 139

Curriculum Vitae 141

Bibliography and awards 143

List of abbreviations

APC	Antigen Presenting Cell
BBB	Blood Brain Barrier
BM	Bone Marrow
CAR	Chimeric Antigen Receptor
cDC	Conventional Dendritic Cell
CLR	C-type Lectin Receptor
CNS	Central Nervous System
CpG ODN	CpG Oligodeoxynucleotide
CLIP	Class II-associated Invariant chain Peptide
CSF	Cerebrospinal Fluid
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T Lymphocyte Associated protein 4
DAMP	Damage Associated Molecular Pattern
DC	Dendritic Cell
DLS	Dynamic Light Scattering
EDAC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EGFR	Epidermal Growth Factor Receptor
EGFRvIII	Epidermal Growth Factor Receptor variant III
EMA	European Medical Agency
EPR	Enhanced Permeability and Retention
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum-associated Protein Degradation
FDA	Food and Drugs Agency
GBM	Glioblastoma (Multiforme)
GF	Gel Filtration
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
Gp100	Glycoprotein 100
HGG	High Grade Glioma
HLA	Human Leukocyte Antigen
HNSCC	Head and Neck Squamous Cell Carcinoma
HSP	Heath Shock Protein
Hyp-PDT	Hypericin induced Photodynamic Therapy
IDH	Isocytrate Dehydrogenase
IFN-γ	Interferon gamma
Ii	Invariant Chain
IL	Interleukin
ICD	Immunogenic Cell Death
iDC	Immature Dendritic Cell
i.p.	Intraperitoneal(ly)
ISO	International Organisation for Standardization
LLC	Lewis Lung Carcinoma
LN	Lymph node
LPS	Lipopolysaccharide
mAb	Molecular Antibody
mDC	Mature Dendritic cell
MDSC	Myeloid Derived Suppressor Cell
MGMT	O^6-Methylguanine DNA Methyltransferase

MHC	Major Histocompatibility Complex
MIIC	MHC class II Compartment
MRI	Magnetic Resonance Imaging
NHS	N-hydroxysuccimide
NK cell	Natural Killer cell
NP	Nanoparticle
OAMP	Oxidation Associated Molecular Pattern
OS	Overall Survival
OVA	Ovalbumin
PAMP	Pathogen Associated Molecular Pattern
pDC	Plasmacytoid Dendritic Cell
PDT	Photodynamic Therapy
PD-1	Programmed Death-1
PEG	Polyethylene Glycol
PFS	Progression Free Survival
PHA	Phytohaemagglutinin
PLGA	poly(D,L-lactic-co-glycolic acid)
Poly(I:C)	Polyinosine-polycytidylic acid
Pre-DC	Precursor Dendritic Cell
PRR	Pathogen Recognition Receptor
ROS	Reactive Oxygen Species
s.c.	Subcutaneous(ly)
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
siRNA	Small Interfering RNA
SPION	Super Paramagnetic Iron Oxide Nanoparticle
TAA	Tumor-Associated Antigen
TAM	Tumor Associated Macrophage
TAP	Transporter associated with Antigen Processing
TCR	T Cell Receptor
TDLN	Tumor Draining Lymph Node
TDSF	Tumor Derived Soluble Factors
TGF-β	Transforming Growth Factor-beta
T_h cell	T helper cell
TIL	Tumor Infiltrating Lymphocyte
TLR	Toll-like Receptor
TME	Tumor Microenvironment
Treg cell	Regulatory T cell
TRP-2	Tyrosine Related Protein 2
TSA	Tumor-Specific Antigen
TMZ	Temozolomide
VCAM-1	Vascular Cell Adhesion Molecule 1
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organisation
γPGA	Poly(gamma-glutamic acid)

1 General introduction

Worldwide, cancer remains a leading cause of morbidity and mortality. In 2012 approximately 14 million new cases and 8.2 million cancer related deaths were described. The amount of annually new cases is expected to rise to 22 million, a rise of 70%, within the next two decades (1). Although brain tumors together with central nervous tumors only account for 2% of the worldwide cases, they have a marked impact on cancer morbidity and mortality (1). Expressed in the value 'years of life lost', brain tumors score higher than any other malignancies (2, 3).

1.1 Glioblastoma: the nemesis amongst tumors of the central nervous system

1.1.1 An introduction to glioblastoma

Gliomas are by far the most common primary brain tumors, representing over 80% of all malignant brain tumors with an incidence of 4 to 5 per 100,000 persons per year (4). They can be subdivided according to the originating cell type in astrocytic, oligodendrocytic and ependymal. On the other hand, the World Health Organization (WHO) scores gliomas using a grading system, grade I to IV, with grade IV being the most malignant tumors (5). This grading is based on histopathological criteria such as proliferative potential, infiltrative nature and the presence of necrosis. Grade III anaplastic astrocytoma together with glioblastoma (GBM), a grade IV malignancy, are considered high grade gliomas (HGG). Of all glioma cases, GBM is the most aggressive, but unfortunately also the most common type of glioma.

In the latest years, classifying gliomas is not merely based on histology, instead molecular parameters in addition to this histology are used to define different tumor entities (6). For glioblastoma, isocitrate dehydrogenase (IDH) genes are an example of these molecular parameters. The absence or presence of IDH mutations are used to distinct between primary and secondary glioblastoma as two disease subtypes. The first group consists of patients with newly developed disease, while the latter group is a consequence of transformation or evolution from a lower grade to a higher grade malignancy. IDH-wildtype glioblastoma is detected in about 90% of the disease cases and nearly always corresponds to "primary glioblastoma". In comparison, IDH-mutant glioblastoma, corresponding to "secondary glioblastoma", only represent about 10% of cases and is found in a younger population (median age of 44 years as compared to 62 years) (7). Primary glioblastomas go through rapid *de novo* development without histological or clinical evidence of a precursor lesion. They are more likely to develop from a loss of heterozygosity in certain regions of chromosome 10, amplification of epidermal growth factor receptor (*EGFR*) gene or *PTEN* mutations (8). As stated earlier, *IDH* genetic alterations can be used as diagnostic molecular markers for secondary glioblastomas (7). The final result of *IDH1* and *IDH2* mutations is the inhibition of DNA demethylation.

1.1.2 Diagnosis, standard of care and adjuvant targeted therapies

GBM diagnosis can be considered a multidisciplinary task ranging from observation of neurological symptoms to imaging and even genetics. Cognitive dysfunction, headaches and motor and sensibility deficits are possible symptoms that can be experienced by the patient. In case a brain tumor is suspected, a detailed neurological examination including imaging techniques such as magnetic resonance imaging (MRI) is performed. For histological confirmation of the diagnosis, stereotactic biopsies are obtained. Even though patients receive maximum therapy, the median survival upon diagnosis is only 14.6 months, with only 10% of patients surviving 5 years after diagnosis (9, 10). Relapse is universal and in this case the patient's prognosis is even worse, with a median survival expectancy of only 9 months and nearly all patients die within 18 months (11). Treatment failure is attributed to remaining therapy-resistant tumor cells often infiltrating into the surrounding healthy brain tissue. As a result, recurrent tumor growth in 80% of the cases develops very close to the resection cavity.

The standard of care for GBM consists already for more than a decade of a multidisciplinary treatment based on the 'Stupp protocol' (9, 10). If possible, the first treatment step is debulking of the neurological tumor. This is facilitated/optimized by preoperative imaging, which makes image-guided surgery possible, and often by using fluorescence-guided surgery for example with 5-aminolevulinic acid (12). Importantly, maximal safe surgical resection seems to be an important determinant in the disease outcome. Next, the patients receive radiotherapy plus concomitant and adjuvant temozolomide (TMZ) administration. Fractionated radiotherapy to a total dose of 60 Gy is given together with a daily dose of 75 mg/m² TMZ. Afterwards, adjuvant TMZ (150-200 mg/m²) is administered for five days a month with a maximum of six months. In their publications, Stupp *et al.* have shown an advantage of this combined radio- and chemotherapy over radiotherapy alone in the primary end point of overall survival (OS) (9, 10). With nearly 300 newly diagnosed patients in each group a significant benefit in median OS was shown for combined treatment in comparison to radiotherapy alone, respectively 14.6 and 12.1 months survival.

For glioma treatment, TMZ is therefore the most commonly used chemotherapeutic agent. An important feature of the drug is its capability to pass the blood-brain barrier (BBB), attributed to the correct lipophilicity in combination with a low molecular weight. As an alkylating agent its mechanism of action mainly works through methylation of the O^6-position of guanine in the DNA, creating O^6-methylguanine (13). These methylated nucleotides will mispair with thymidine during a next cell replication cycle which will ultimately lead to apoptosis via double strand breaks. However, cells possess DNA mismatch repair mechanisms such as the O^6-methylguanine-DNA methyltransferase (MGMT). This enzyme is able to remove alkyl groups from the DNA, preventing DNA mismatches. MGMT promotor methylation positively correlates with better outcome for patients treated with alkylating agents such as TMZ (14). Methylation of this promotor inhibits the biological activity of

MGMT and therefore MGMT promotor methylation is considered a predictive biomarker for TMZ response (15).

Next to standard of care the scientific community is in search for other therapies which can serve as an adjuvant therapy to further improve survival outcome of glioma patients. Different treatment options target important features of cancer cells for example: growth factor dependency and angiogenesis. Bevacizumab, a humanized IgG1 monoclonal antibody (mAb) directed against vascular endothelial growth factor variant A (VEGF-A), is the most widely used angiogenesis inhibitor in cancer treatment (16). For GBM, the drug has been approved by the US Food and Drug Administration (FDA) for treatment of recurrent glioblastoma, but on the other hand, the European Medicines Agency (EMA) did not (yet) approve it. Based on encouraging results of bevacizumab in combination with standard GBM treatment, two placebo-controlled phase III clinical trials were initiated (NCT00884741 and NCT00943826). Although both trials showed benefit in progression free survival (PFS) for combining bevacizumab with standard radio-chemotherapy in comparison to standard treatment alone, neither study demonstrated an OS benefit (17, 18). Another group of targeted therapies houses inhibitors of growth factors, their receptors and their intracellular pathways. However, up till now none of these therapeutics have been successfully tested in phase III clinical trials for GBM patients (19). Next to these targeted treatment strategies, other treatment options like immunotherapy and immunomodulatory anti-cancer agents have been studied. Further elaboration on both treatment options will be provided in later sections of this introduction. As a conclusion, during the past decades a lot of effort is put in further improving the outcome of GBM patients without any major results. As stated by many authors, the answer for successful GBM therapy might lay in the combination of different treatment options (19-21).

1.2 Cancer and the immune system

What makes a healthy cell become a cancer cell? This is summarized in two articles of Hanahan and Weinberg in which multiple characteristics are proposed which are necessary to develop tumor cells (22, 23). Originally, these so-called hallmarks of cancer comprised of resistance to cell death, evade growth suppression, sustain proliferative signaling, obtain replicative immortality, induce angiogenesis and activation of invasion and metastasis. Due to scientific progress in the last decade, two emerging hallmarks were suggested: evasion of the immune system and reprogramming of the energy metabolism. More and more it has become clear that an important interaction between cancer cells and the immune system occurs. For example immunosuppressed transplantation patients have greater risk for certain types of cancer due to an impaired immune system which allows more tumor growth. Another example is the presence of tumor infiltrating lymphocytes (TILs) which is correlated with better survival.

1.2.1 Cancer immunoediting – with a focus on glioblastoma

Immune editing is a process in which tumors develop escape mechanisms to outsmart immune system control. The concept was first proposed by Dunn *et al.* who described the process to consist of three distinct phases: elimination, equilibrium and escape (24). Together these phases are designated the 'three E's' of cancer immunoediting. Originally the concept arose from another theory, namely immune surveillance. In mid-20th century it was stated that the immune system constantly scans our body for transformed (cancerous) cells and eliminates these cells (25). By the beginning of the 21st century it had become clear that immune system-tumor interactions form a dynamic process with protection against cancer development on the one hand and shaping the characteristics of emerging tumors on the other hand. In the next three paragraphs the concept of cancer immunoediting will be explained in depth with a particular focus on glioma (26). **Figure 1** gives a general overview of the concept and its main effectors as discussed next.

1.2.1.1 Elimination

During the protective phase of cancer immunoediting, innate and adaptive immunity join forces to respond to arising tumor cells (27). Innate immune cells like natural killer (NK), NKT and γδ T cells, form the first line of defense by recognizing tumor cells, leading to limited killing and an inflammatory environment with presence of interferon-γ (IFN-γ). As a consequence tumor associated antigens (TAAs) are released and taken up by dendritic cells (DCs) that are able to mature due to the pro-inflammatory environment. In the tumor draining lymph node (TDLN) antigen-specific T cells are generated due to migrated DCs which present the TAAs to naïve T cells. Finally, antigen-specific $CD4^+$ and $CD8^+$ T cells, representing the adaptive immune component, home to the primary tumor site and eliminate the remaining tumor cells.

For glioma and other brain tumors, the BBB creates an important obstacle that needs to be overcome before immune cells can access the tumor cells. Due to this barrier, for long, the brain was considered to be an immune privileged organ. Researchers have abandoned this idea because of discovering ventricles and perivascular spaces to lack BBB (28). Moreover, the immune sentinels, the DCs, are found in perivascular spaces of the brain as well as at the cerebrospinal fluid (CSF)-blood barrier. With the central nervous system (CNS) lacking conventional lymphatics, antigen-loaded DCs are able to migrate to cervical lymph nodes via a lymph-like perivascular drainage system (29). Of course when T cells are activated, they need to get to the region of inflammation and thus must pass the BBB. Upon inflammation, transendothelial migration of T cells can occur due to the CXCL12 ligand, expressed on T cells, and its receptor, CXCR4, on endothelial cells (30). Next to this chemokine and its receptor, T cell homing depends on the expression of CNS homing-specific α4β1-integrin, which will interact with vascular cell adhesion molecule 1 (VCAM-1) on cerebral vascular endothelium (31). Importantly, activated T cells express the required receptors to pass the

BBB, while resting T cells do not. Hence the term "immune privileged organ" can perhaps be replaced by "very strictly immune regulated organ".

1.2.1.2 Equilibrium

Due to the elimination process, a selection of tumor cells with a reduced immunogenicity can be established within the tumor. The equilibrium phase is actually a Darwinian selection period in which continuous sculpting of tumor cells occurs. Mutations in combination with genetic instability and the selective pressure elicited by the immune system select for less immunogenic tumor cells (32).

1.2.1.3 Escape

The equilibrium phase can result in two outcomes: 1) the immune system might take over and completely eliminate the tumor cells, or 2) the tumor cells might outsmart the immune system, escape immune surveillance and start growing. Many different tumor escape mechanisms exist such as increased resistance to apoptosis, reduced immune recognition and development of an immunosuppressive tumor microenvironment (TME) (27, 33). As already stated earlier, tumor localization in the brain can be a factor interfering with immune surveillance. The highly regulated immune presence in the brain can be advantageous for tumor cell growth under the immunological radar. Along the same lines, lies the downregulation of adhesion molecules (α4β1-integrin and VCAM-1) on activated lymphocytes by transforming growth factor β (TGF-β) causing failure of tumor homing (34). Next, attention will be drawn to three examples which are, amongst other mechanisms, observed/studied in glioma.

(a) To inhibit immune function, immunosuppressive factors like VEGF, interleukin 10 (IL-10), TGF-β, prostaglandin E and indoleamine 2,3-dioxygenase (IDO) are released by tumor cells (27). Together these molecules are part of a group called tumor derived soluble factors (TDSF) which aim to create an immunosuppressive TME. Of these, TGF-β has been considered the most potent immunosuppressive cytokine in glioma. Its functions range from affecting proliferation of immune cells to induction of apoptosis of T and B cells. Glioma cells are able to secrete IL-10, which allows the tumor to become insensitive to cytotoxic T lymphocyte (CTL)-mediated lysis (35). Another possible source of these immunosuppressive mechanisms are regulatory T cells (Tregs). Next to working through soluble factors, these cells can also work via direct cell-cell contact (e.g. CTLA-4), which will be discussed later. In the group of gliomas, a greater amount of Tregs is detected in GBM and other HGG in comparison to lower graded gliomas (36). Moreover, Treg tumor infiltration is inversely correlated with clinical outcome and associated with higher risk for recurrence.

(b) Also within the myeloid cell fraction immune suppressive cells can be detected, namely myeloid derived suppressor cells (MDSCs). These cells are considered a group of immature myeloid cell types that ended their differentiation due to overexpression of growth factors like

granulocyte macrophage colony stimulating factor (GM-CSF) and cytokines such as VEGF (37). MDSCs can execute their immunosuppressive function on T cells through multiple mechanisms including production of arginase and reactive oxygen species (ROS), and even expansion of Tregs. Both granulocytic and monocytic MDSCs, detected in glioma patients' blood and tumor tissue, are correlated with worse prognosis (37, 38).

(c) Other immune evasion techniques are T cell receptor dysfunction and even escape from apoptosis induced by immune cells. Tumor cells and the TME are capable of influencing the activity of effector T cells through T cell receptor (TCR) impairment (39). Under impulse of the increased levels of TGF-β and IL-10, and the downregulation of IFN-γ in the TME, TILs can have loss of the signal transducer CD3-ζ (27). Normally, the CD3-ζ chain forms a complex with the TCR, in which the CD3-ζ functions as a signal transducer important for the functional integrity of the immune cells. Under normal circumstances cytotoxic T cells are able to kill tumor cells through Fas/Fas ligand (FasL) interaction. Glioma cells have been shown to express FasL, normally expressed on activated T cells, making it possible to drive TILs into Fas-mediated apoptosis (40, 41).

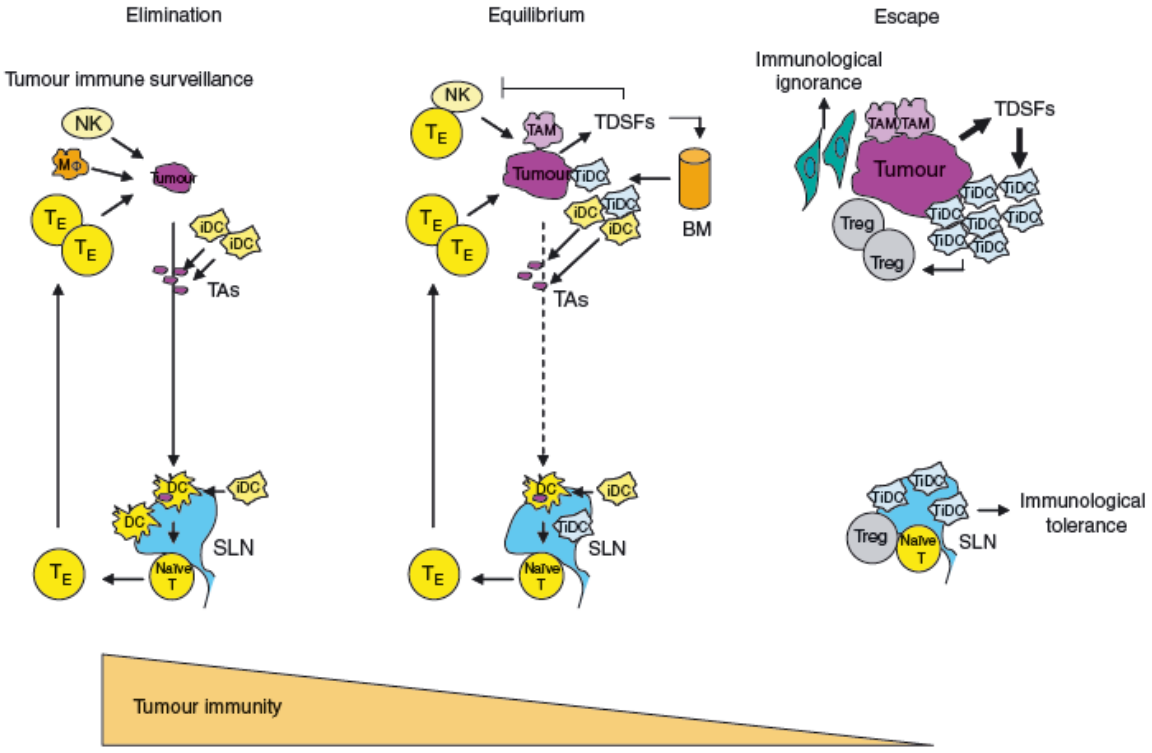


Figure 1. Cancer immunoediting. A schematic presentation of immunoediting from immune surveillance to escape. When early tumor cells are generated, these cells can easily be eradicated by innate and adaptive immunity. During tumor growth, tumor cells require angiogenesis and stromal remodelling, leading to tumor cell variants that have low immunogenicity and are resistant to immune attack. Consequently, tumor cells proceed to the equilibrium phase even though the elimination phase continues through immune selection pressure. Tumor progression leads to the release of tumor-derived soluble factors that are involved in several mechanisms of immune evasion in the escape phase. BM,

bone marrow; iDC, immature dendritic cell; Mϕ, macrophage; NK, natural killer; SLN, sentinel lymph node; TAM, tumor-associated macrophage; TAs, tumor antigens; TDSFs, tumor-derived soluble factors; T_E, effector T cell; TiDC, tumor-associated iDC; Tregs, regulatory T cells.
(Adapted and modified with permission from Kim et al. 2007 (27))

1.2.2 Immunotherapy

In 2013, the scientific journal Science pointed out immunotherapy to be the "break-through" for cancer treatment that year (42). As compared to conventional treatment strategies (surgery, radiotherapy and chemotherapy), immunotherapy uses an entirely different approach to treat cancer, namely by targeting the immune system. This was necessary because tumors are able to create an immunosuppressive TME as stated in the previous section. If a balance is considered between immune suppression and immune activation in each TME, immunotherapy aims to tilt the balance in favour of immune activation. And therefore most members of the oncology community consider this new direction as quite definite. Immunotherapeutic strategies reach out through a very broad field of treatment options ranging over four major categories: 1) adoptive strategies e.g. the use of chimeric antigen receptor (CAR) T cells, 2) immunomodulatory strategies such as immune checkpoint inhibition, 3) passive immunotherapy including mAb for targeted therapy and 4) active immunotherapy with cancer vaccines (43-45). In this chapter all of these categories within GBM treatment will be briefly touched upon, while in the next two chapters the subject of cancer vaccines will be discussed in more detail.

Adoptive T cell transfer aims at generating anti-tumor immunity through directly injecting antigen-specific T cells. These T cells can be TILs or CTLs isolated from e.g. TDLNs, which are injected back into the patient after *ex vivo* expansion. For the next generation of adoptive transfer therapy, genetically modified T cells are used which combine the recognition specificity of antibodies with T cell signal transduction (e.g. CD3-ζ chain) (43). These CAR T cells have the advantage not to be restricted to a human leucocyte antigen (HLA) type as they bind tumor antigens in a major histocompatibility complex (MHC)-independent fashion. Based on positive preclinical results using EGFR variant 3 (EGFRvIII)-directed CAR T cells, these CAR T cells are now tested in a phase I clinical trial on 12 patients to treat human $EGFRvIII^+$ GBM (NCT02209376) (46). EGFRvIII is a tumor-specific antigen (TSA) expressed in nearly 30% of GBMs (47). The last patient in this study was included in December 2018, but preliminary results have already been described in literature. A single dose of CAR T cells was safe with no dose limiting toxicity. Although limited persistence of 30 days post-transfusion was observed, potentially tumor infiltration could be detected. Curiously two patients with the highest level of immune-mediated changes as reflected by $CD8^+$ T cell and/or CAR T cell infiltration, remained alive (48, 49). Final results of the study can be expected this year.

As for other advanced tumor types (lung cancer, melanoma, renal cancer, head and neck cancer), immune checkpoint inhibitors are intensively studied to treat glioma patients. These immunomodulatory anti-cancer agents represent an important discovery in cancer treatment. They block inhibitory molecules and their receptors on effector immune cells and especially effector T cells, resulting in T cell activation (50). For the discovery of these immune inhibitory molecules and the development of cancer therapy by inhibition of these molecules, James P. Allison and Tasuku Honjo were rewarded with the Nobel Prize in Medicine 2018. Cytotoxic T lymphocyte antigen 4 (CTLA-4) is an inhibitory molecule at the surface of T cells that competes with and inhibits the required costimulatory signal of T cell activation. CTLA-4 thus works at an early moment when T cells come in contact with an antigen presenting cell (APC). Treg cells for example constitutively express the molecule partly explaining their immune inhibitory effect. Ipilimumab is a mAb against CTLA-4, but to date, the only proven effectiveness and safety for 'brain tumors' is described in the setting of melanoma patients harboring small brain metastases (51). A second immune checkpoint molecule is programmed cell death protein 1 (PD-1), a molecule of which signaling dampens the antigen specific T cell activity at a later time point at the site of inflammation. PD-1 binds to its ligand PD-L1, leading to suppression of T cell activity by inhibiting its signaling. Moreover, PD-1 can promote Treg cell proliferation and attenuates NK and B cell responses. In contrast to the ligand of CTLA-4, PD-L1 can also be overexpressed by malignant cells; in this way counteracting the anti-tumor T cell immunity. This is also the case for gliomas, in which PD-L1 expression by primary cells has been shown and found to correlate with tumor grade (52). Besides, a high percentage of TILs in glioma are also expressing PD-1, making the PD-1/PD-L1 pathway even more relevant (53). Nivolumab has been shown an effective mAb inhibiting the PD-1 receptor in melanoma; clinical studies for treatment in glioma have been started in 2014 (NCT02017717). This phase III clinical trial evaluates the use of immune checkpoint inhibitors in the treatment of GBM. Randomization of patients with recurrent GBM is performed over three treatment arms: nivolumab alone, nivolumab plus ipilimumab or bevacizumab alone. The nivolumab alone arm of this study was closed prematurely due to reduced OS in recurrent GBM (54). However, when responding, long median duration of response (PFS) was observed (55). Now new trials are searching for an additive role of Nivolumab in newly diagnosed patients with GBM on top of radiotherapy (NCT02617589) or radio- and chemotherapy (NCT02667587), in respectively unmethylated and methylated MGMT glioblastoma.

The use of other mAbs, such as bevacizumab, in the treatment of GBM has already been discussed in a prior chapter. In contrast to this passive immunotherapy, the active form aims to increase the specific immune response against tumor cells (43). Anti-tumor vaccines represent a broad range of approaches including peptide vaccination, tumor cell vaccination and DC vaccination. Moreover they comprise the most studied immunotherapeutic strategy in GBM, and yet, only modest, often inconsistent immune responses and anti-tumor benefits

have been reported (44, 45). More attention to cancer vaccines will be provided in the next two chapters discussing DC immunotherapy and tumor lysate treatment.

Importantly, because of the multiple possibilities of immune suppression and the plasticity of tumor cells, combinations of different strategies may prove to be synergistic. For example, a preclinical study performed by Antonios *et al.* suggests that DC vaccination followed by PD-1 blockade significantly improved survival of glioma-bearing mice in comparison to each therapy alone (56). This has recently lead to the initiation of a phase II clinical trial which started in January 2019, using autologous DCs loaded with tumor lysate antigens alone or in combination with nivolumab (NCT03014804). Moreover, other immunotherapy combinations are already in clinical trials: another study combining nivolumab and DC vaccination (NCT02529072), combination of anti-EGFRvIII vaccine (rindopepimut) and an IL-2 receptor mAb (NCT00626015), rindopepimut together with bevacizumab (NCT01498328) and earlier mentioned combination of immune checkpoint inhibitors ipilimumab and nivolumab (NCT02017717). In Table 1 of the "Addendum – immunotherapeutic treatment in glioma clinical trials" a list of the discussed clinical trials can be found.

1.3 Dendritic cells and their application in immunotherapy

In this chapter, the first part will handle about DCs in general followed by an extensive discussion concerning the most important feature of DCs required to induce a CTL response, namely cross-presentation. Finally, results of a few relevant preclinical and clinical studies will be mentioned.

1.3.1 General characteristics of dendritic cells

Ever since their discovery by Nobel Prize laureate Ralph Steinman, DCs have been subject of intensive research (57). This is most likely attributed to the important connection these cells form between innate and adaptive immunity. DCs are the most complete professional APCs and are found throughout the body sampling their environment. They are responsible for initiating antigen-specific T cell responses by expressing peptide fragments of degraded (foreign) antigens on their surface in the context of MHC molecules. DCs are hematopoietic cells that originate from bone marrow (BM) out of which precursor DCs (pre-DCs) migrate via circulation to different lymphoid and non-lymphoid tissues (58). Pre-DCs give rise to several DC subsets like plasmacytoid DCs (pDCs) and conventional DCs (cDCs; also known as myeloid DCs), which consist of two subsets being cDC1 and cDC2, based on the expression of surface markers CD141 and CD1c respectively (59). Once pre-DCs have settled in peripheral tissue they are considered naive, immature DCs (iDCs). These iDCs are known to actively scan their environment in search for foreign material. Uptake and processing of antigenic material in the presence of the required stimuli, a process which will be discussed in detail in the next section, will give rise to mature DCs (mDCs). In summary, iDCs, prone to take up and process antigens, differentiate into mDCs presenting the antigens and expressing

costimulatory molecules necessary to induce an immune response (60). Importantly, it should be stressed that we use the abbreviation iDC for immature DCs, while many other authors might use this same abbreviation for inflammatory DCs.

Before they can perform their functions, DCs must be attracted to tissue regions in need. In line therewith, chemokines regulate the attraction and migration of DCs (61). Initially, iDCs respond to inflammatory chemokines such as monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α). These chemokines can be produced by leukocytes, endothelial cells and epithelial cells in response to inflammatory molecules. As the iDCs approach the inflammatory region, the higher concentration of these cytokines in combination with pathogen products or antigenic payload will mature the DCs. In doing so, the mDCs switch chemokine receptor expression from inflammatory to lymphoid homing with e.g. CCR7 being the best known. When reaching their place of function, DCs need to take up, process and present antigens to induce an adaptive immune response.

1.3.2 Antigen uptake, processing and (cross-)presentation by dendritic cells

1.3.2.1 Antigen uptake needs to go hand in hand with detection of danger/damage

DCs take up exogenous antigen via numerous endocytic pathways: phagocytosis, (macro)pinocytosis and receptor-mediated endocytosis (62-66). Phagocytosis handles the largest exogenous products that are often insoluble particulate antigens like necrotic/apoptotic cells and opsonised pathogens, but also complete cells, several µm in diameter. The process requires receptor signalling, resulting in pseudopod generation by actin assembly (63). Macropinocytosis, a synonym for cell drinking, is a constitutive process in iDCs as well as macrophages. This way nutrients, soluble molecules and foreign antigens can be taken up non-specifically. This endocytosis condition is also actin dependent and leads to endocytic vesicles, the macropinocytome, without coating (64). This is in contrast to the third internalisation process, receptor-mediated endocytosis, where clathrin-coated or caveolin-coated vesicles are created after internalisation (62). Compared to the other two uptake mechanisms, the receptor-mediated endocytosis is a process in which smaller antigens are taken up (67).

To make sure DCs are able to induce a proper immune response, DCs must be activated. Therefore, so called danger signals have to stimulate the DCs before or when antigen uptake occurs. In case no danger signal accompanies antigen uptake, DCs risk to differentiate into tolerogenic DCs as pointed out by Bonifaz *et al.* (68). These DCs will induce T cell tolerance through either deletion or anergy, the latter process functionally inactivates lymphocytes after antigen contact while staying alive in a hyporesponsive state (69).

Creating an immune response thus requires a joint effort of a lot of molecules, signals and receptors produced by different cells. To begin, pathogens (bacteria, viruses, fungi and parasites), through their cell cycle, produce certain evolutionary conserved molecules that are

not expressed by the host. These include nucleic acids, carbohydrates, proteins and lipids, and are called pathogen-associated molecular patterns (PAMPs) (70). On the other hand, DCs are well equipped to detect these PAMPs via an array of pattern recognition receptors (PRRs) (71). Multiple receptor families have been identified such as Toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs), RIG-I-like receptors (RLRs) and DNA receptors. The most abundantly studied PRRs are the TLRs, a group of receptors searching extracellular space and endosomal compartments (72). Most TLR signalling goes through the cytoplasmic TLR-interleukin receptor (TIR) domain which recruits adaptor molecules like MyD88 and TIRF, ultimately leading to activation of NF-κB, MAP kinases or IRF3/4/7 (73, 74).

As one can imagine, danger signals within cancer comprise of different molecules as is the case for pathogens. In cancer, but also for instance in autoimmune diseases, endogenous molecules called damage-associated molecular patterns (DAMPs), can be released as a reaction to cell death, injury or stress (75). DAMPs are also recognized by PRRs to that extent that the same receptors recognize both PAMPs and DAMPs, with again a principal role for TLRs. DAMPs can originate both from extracellular matrix products including peptidoglycans and glycoproteins as intracellular molecules. The latter group of DAMPs consists of a very heterogeneous group ranging from small ATP molecules to complete organelles (76). Later in this introduction, an important role of DAMPs in DC immunotherapy will be elaborated on.

1.3.2.2 Classical processing and presentation pathways

Antigen processing primarily occurs via two major pathways in DCs: an exogenous, endosomal pathway and an endogenous, proteasomal pathway. The first leading to antigen presentation by MHC class II molecules, while in the latter pathway antigens will be presented in MHC class I context (**Figure 2**). Other differences observed between both pathways comprise cell types capable of performing the process and different effector molecules that are targeted. While MHC-I molecules are expressed by all nucleated cells, MHC-II presentation is restricted to professional APCs such as DCs, macrophages and B cells. Besides, MHC-I presentation aims to generate $CD8^+$ T cell responses, whereas $CD4^+$ T cells will engage antigen-presenting MHC class II molecules to induce an immune response. In the next paragraphs the two 'classic' presentation pathways will be described rather briefly. For more extended information on these two processes, some very nice reviews can be consulted (60, 77-79).

Major histocompatibility complex class II presentation: Exogenous antigens are internalized by different endocytic pathways, leading these antigens to early endosomes, which acidifies by fusing with lysosomes eventually creating the late endosomal MHC class II compartment (MIIC). MHC class II molecules are assembled in the endoplasmic reticulum (ER) as a heterodimer of α- and β-peptide chains. This process is facilitated by the invariant chain (Ii), a

protein that associates with MHC-II molecules protecting the binding cleft from premature ligand capture of e.g. self-proteins. Moreover, this Ii protein directs the MHC-II-Ii complexes to the MIIC. There the acidic environment partially degrades the Ii leaving a small fragment called CLIP (class II-associated invariant chain peptide) within the binding cleft. With the help from a highly homologous molecule to MHC-II, CLIP can be removed from the MHC class II molecule allowing specific peptide fragments of the antigen to take its place. These peptide fragment of fifteen to twenty-four amino acids long are obtained from the exogenous antigen due to protease activity especially by cathepsins in the MIIC endosomes. Finally the MHC-II-peptide complexes can be transported to the plasma membrane presenting their antigenic peptides to T cells.

Major histocompatibility complex class I presentation: As for the MHC-II molecules, MHC class I molecules are also synthesized in the ER consisting of a heterodimer of class Iα heavy chain and β2-microglobulin subunits. Initially these dimers need to be stabilized by chaperone proteins like calreticulin, heat shock proteins (HSPs) and tapasin that eventually will also help in peptide binding. Peptides presented in MHC-I context originate from endogenous cytoplasmic and nuclear proteins that are degraded in proteasomes. Via the transporter associated with antigen processing (TAP), peptides can enter the ER. Here the peptides are further trimmed by ER aminopeptidases until peptides of eight to nine amino acids remain. These octamer or nonamer peptides of the appropriate sequence bind the MHC-I molecule where after the fully assembled peptide-MHC-I complex is delivered to the cell surface.

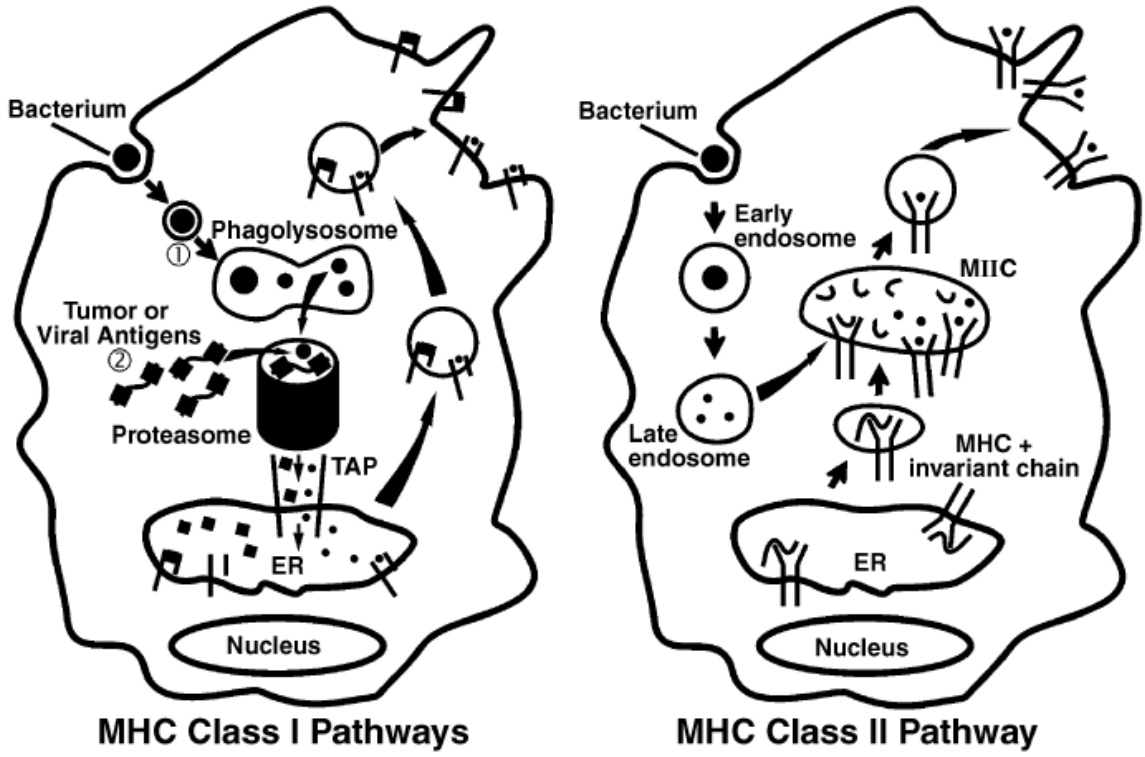


Figure 2. Classical antigen processing and cross-presentation by dendritic cells. On the left, the classical MHC class I pathway (2) and the cross-presentation pathway (1) are depicted. In the simplistic representation of cross-presentation, antigen (here presented in the form of bacterium), is taken up by phagocytosis and/or receptor-mediated endocytosis, undergoes limited proteolysis, and by active transport enters the cytosol. There, the antigens are further degraded via the proteosomal pathway, enter the endoplasmic reticulum (ER) utilizing TAP, and are bound to newly synthesized MHC class I molecules. MHC class I/peptide is subsequently carried by vesicular transport to the cell surface. Endogenous proteins, through the classical pathway, are similarly degraded, enter the ER, are bound to MHC class I molecules, and finally are transported to the cell surface. The MHC class II processing pathway is shown on the right. Antigen is taken up by early endosomes evolving to late endosomes rendering proteolysis. Generated peptides enter the MHC class II-rich vesicular compartment (MIIC) where they are bound in the MHC class II peptide-binding groove and are then transported to the cell surface. MHC class II molecules are synthesized in the ER where invariant chain (Ii) protects the groove from premature binding of self-peptides. Ii is further degraded into a smaller peptide, CLIP, to ready itself for its replacement by the antigenic peptide in MIIC. CLIP, class II-associated invariant chain peptide; TAP, transporter associated with antigen processing.
(Adapted and modified with permission from Lipscomb et al. 2002 (61))

1.3.2.3 Cross-presenting MHC-I pathway

Additionally, a third presentation pathway exists, namely cross-presentation. As already stated above, malignant cell growth, but also viral infection, requires a CTL response ($CD8^+$ T cells). However, the classical presentation pathways described before do not explain the possibility of exogenous antigens being processed and presented in MHC class I context. This hiatus was solved by the discovery of cross-presentation (80-82). In other words, all nucleated cells do express MHC class I molecules, but naive $CD8^+$ T cells cannot use these to generate a CTL response. Therefore, the naive cells first need to become effector cells via activation by DCs. These DCs somehow need to acquire the exogenous antigens and present them in MHC-I context. As the presence of a specialized machinery promoting delivery of antigens into cross-presentation has been shown, DCs are generally considered the main cross-presenting APC *in vivo* (83, 84). As an example, different research groups have shown limited antigen degradation correlates with efficient cross-presentation (85, 86). This limited degradation in DCs is made possible by their ability to actively control the alkalinisation of their phagosomes, low lysosomal proteolysis due to high pH and the expression of protease inhibitors (85-87).

Figure 3 shows that cross-presentation of an exogenous antigenic payload can occur via three intracellular pathways: a vacuolar pathway, a phagosome-ER-cytosol pathway and a CD74 pathway (88). The vacuolar cross-presentation pathway depends on endosomal protein/peptide degradation. It employs lysosomal proteolysis by in particular cathepsin S and other proteases followed by loading onto cell surface recycled MHC class I molecules (89). On the other hand, cytosolic cross-presentation (phagosome-ER-cytosol pathway) requires the

endosomal escape of antigens to the cytosol where they will be degraded by the proteasome (90). This endosomal escape is most likely enabled by the recruitment of ER components to endocytic compartments. For example, there is evidence indicating that parts of the retrotranslocation machinery used for misfolded proteins in the ER, the endoplasmic reticulum-associated protein degradation (ERAD) complex, is present in endosomes/phagosomes (e.g. protein transporter Sec61) (91, 92). After proteasomal degradation, the peptides can go into the classical MHC-I mediated presentation pathway using TAP to enter the ER where they are loaded onto newly formed MHC-I molecules. However, suggestion for endocytic peptide loading also exist. Next to the ERAD complex, endosomes and phagosomes have, for instance, been shown to recruit TAP and the MHC-I loading complex and thus new MHC-I molecules (93). Moreover, the crucial trimming of proteasome-generated peptides for MHC-I presentation has also been shown to be performed by endosomal aminopeptidases (80). The third pathway is the most recent discovered pathway and involves CD74 association with MHC-I molecules in the ER (88, 94). CD74 has already been mentioned above as the invariant chain in classical MHC class II presentation. Through the CD74 endosome-targeting sequence, CD74-MHC-I complexes essentially follow the same way as MHC-II molecules do for antigen presentation. The CD74 molecule dissociates from the MHC class I molecule in the endolysosome, allowing loading with peptides generated by proteolytic cleavage of antigen in the endosome/phagosome (94). Due to the great importance of cross-presentation in e.g. viral infections and immunotherapy, the process is still an important subject of research.

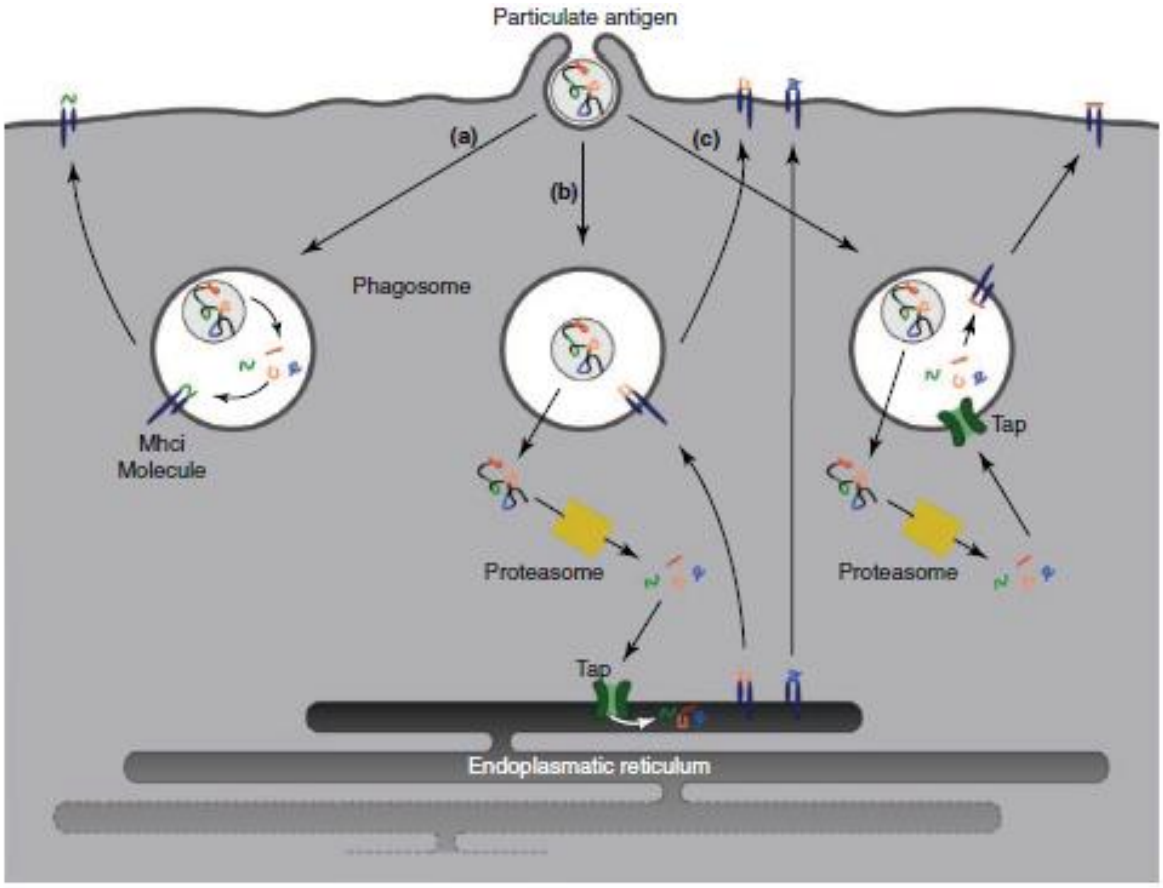


Figure 3. Cross-presentation pathways in dendritic cells. Three cross-presentation pathways are depicted: (a) the vacuolar pathway, (b) the phagosome-ER-cytosol pathway and (c) the CD74 pathway. In the vacuolar pathway (a), antigens are degraded by proteases in the endosomal environment followed by loading onto cell surface recycled MHC-I molecules. Cytosolic cross-presentation (b) starts with endosomal escape of antigens after which these molecules are degraded by the proteasome. Following transport of the peptides to the endoplasmatic reticulum (ER) via TAP, the loading of antigen fragments onto MHC class I molecules will occur on the spot. Finally, MHC-antigen complexes are shuttled directly to the cell membrane or via the endosome. The CD74 pathway (c) requires CD74-MHC-I complexes to enter the endolysosome. Antigens can be processed by proteases in the endosome or in the cytosol followed by re-entering the endosome utilizing TAP. Following CD74 dissociation from the MHC class I molecules, peptides can be loaded onto these molecules and the complex can be transported to the cell surface. TAP, transporter associated with antigen processing.
(Adapted and modified with permission from De Temmerman et al. 2011 (95))

1.3.2.4 The immunological synapse, the dendritic cell-T cell interface

Once DCs have processed the antigens, presented them in MHC context and differentiated to mDCs, they are destined to induce an adaptive immune response. Therefore, naïve T cells need to be differentiated to effector T cells, a process which requires the sequence of at least three signals (61). Signal 1 consists of the TCR-MHC-peptide complex which forms by TCR of $CD4^+$ or $CD8^+$ T cells reacting to MHC class II or class I molecules, respectively. This

contact needs to sustain for a certain time as only prolonged TCR-MHC contact can lead to T cell differentiation (96). Costimulation shapes signal 2 and is necessary for T cell proliferation and differentiation. The most widely studied is the B7 family with CD80 and CD86 expressed on DCs that will bind CD28 expressed by T cells. Importantly, these costimulatory molecules can also be immune inhibitory (e.g. CTLA-4/CD28 and PD-L-1/PD-1). Finally, signal 3 comprises cytokine secretion to polarize the T cells (97, 98). This way $CD4^+$ T cells can be polarized to the different T helper (T_h) cell populations. Together, the clustering of complexes at the DC-T cell interface, following from these signals, is called the immunological synapse (99). Of note, the past five years extra signals have been proposed by different research groups. As an example, imprinting lymphocyte homing instructions to activated T cells, can be considered a fourth signal. This process aims to address the T cells to the tissue where the DCs originate from and thus where the infection is present, as already shown for gut and lungs (100, 101).

The 'three signal model' as discussed before elegantly describes the differentiation and polarization of $CD4^+$ T cells. To be able to create a CTL response, originating from $CD8^+$ T cells, licensing of DCs is required. A CTL immune response requires the specific help of T_h cells and because $CD8^+$ CTLs lack the expression of MHC-II molecules to create and antigen-specific interaction with T_h cells, DCs are necessary to act as the messenger (102). As described by Kurts *et al.* DC licensing is the process of T_h cells converting DCs to a transient state in which they can program CTLs for sustained cytotoxic effector functions and memory differentiation (102). DCs have been shown to store this information received from the T_h cells, making them able to activate and differentiate CTLs at the site of inflammation. For the sake of completeness, next to this classical DC licensing, a second alternative licensing using NKT cells and chemokines, exists (103).

1.3.3 Dendritic cell vaccination

Having extensively described the most important features of DCs and how they manage to induce a strong T cell response, this and the next chapter will handle their therapeutic implementation in cancer immunotherapy. The aim of DC immunotherapy is to induce tumor-specific effector T cells able to specifically reduce tumor mass and immunological memory to control tumor relapse. To begin, DCs need to be provided with tumor antigens, which can be either TSA or TAA. The former being expressed only by tumor cells, while the latter are merely overexpressed as compared to some normal tissues leading to weaker immune induction due to host immune tolerance (104). From a practical point of view, bringing tumor antigens to DCs can be achieved either by patient-derived *ex vivo* culturing of DCs with tumor antigens and a maturation cocktail (adjuvant), or by *in vivo* 'targeting' of tumor antigens to DCs (105). The focus in this section will be on the first vaccination strategy, while the second one will be discussed in the next chapter.

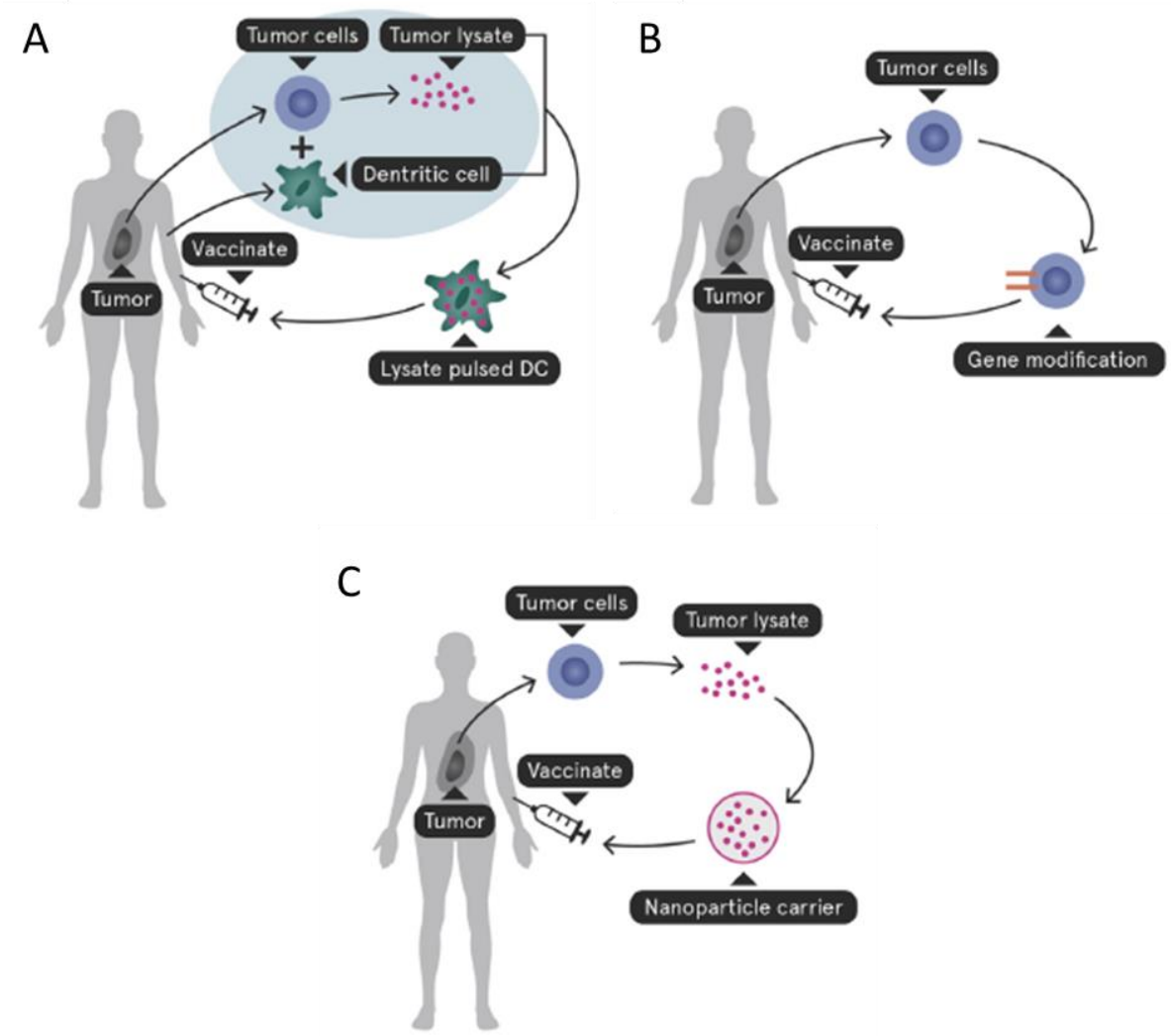


Figure 4. Personalized active immunotherapy approaches using whole tumor lysate. Autologous tumor lysate can be loaded to dendritic cells (DCs) *ex vivo* (A). Afterwards, tumor lysate-pulsed DCs can be injected back into the patient. (B) Whole tumor cells and/or lysate can also be used for cancer vaccination. Therefore, the immunogenicity of the tumor cells can be enhanced by genetically modifying the isolated tumor cells to express co-stimulatory molecules or secrete immune-stimulatory cytokines. (C) Finally, nanoparticles (NPs) can be used as vehicles to deliver tumor antigens to DCs *in vivo*. Tumor lysate fragments can be coupled to the surface of or encapsulated in these particles.
(Adapted and modified with permission from Ophir et al. 2015 (106))

In order to generate tumor antigen presenting DCs, DC vaccination uses patient-derived autologous DCs that are *ex vivo* cultured and loaded with tumor antigens (**Figure 4A**). These cells can be injected back into the patient eliciting potent $CD4^+$ and $CD8^+$ T cell responses and potentially a humoral response (105). Based on early phase clinical trials, DC vaccination in cancer treatment is generally considered both safe and feasible (107-109). In 2010, the FDA approved the first DC vaccination strategy for human use. Sipuleucel-T (Provenge) is an autologous DC-enriched vaccine product for metastatic prostate cancer (110). Importantly, it should be mentioned that during the main clinical trial no anti-tumor effect was observed, nor

was the proposed mechanism of action, via T cells, confirmed (111). Moreover, the observed difference in survival between treated and placebo-controlled patients might be explained by a detrimental effect in the placebo-arm of the trial. Despite of the vast amount of clinical trials performed and still ongoing, up till now no standard DC vaccination strategy has been accepted. A possible explanation is the heterogeneous set-up of the different trials, with differences in e.g. antigen source, origin of the DCs, cultivation of these DCs and injection place. Although this makes interpretation of efficacy very challenging, one meta-analysis and several review papers focussing on glioma therapy have stated (limited) increase in OS and prolonged PFS based on the results of over 20 phase I and II clinical trials (112-115). This conclusion has to be taken with caution, as the outcome might be biased by inherent limitations. For example, all studies implemented in the meta-analyses by Cao *et al.* are phase I and II studies, designed to show feasibility and safety and to perform dose finding. The best way to show efficacy of DC vaccination, would however be a phase III clinical trial, of which only two have been performed or are currently ongoing for newly diagnosed GBM patients. The company Northwest Biotherapeutics manufactures a patient-specific DC product pulsed with autologous tumor lysate, DCVax-L (116). Results of two phase I/II clinical studies including 20 newly diagnosed glioblastoma patients and 19 with recurrent disease, showed encouraging long term (LT) survival, with 33% of patients surviving over 48 months and 27% over 72 months (117). The phase III clinical trial (NCT00045968) was started in 2006, but only last year the first long awaited results of this study have been published (118). Overall, the results were very positive describing over 30% of patients surviving more than 30 months with a median OS of 46.5 months and 24% of patients surviving over 3 years with a median OS of 88.2 months. However, the structure of this phase III clinical trial provokes the major problem for testing e.g. DC immunotherapy in addition to standard therapy. Patients were randomised over two treatment arms: one receiving standard therapy followed by DC immunotherapy, the second standard therapy plus placebo. Following recurrence, all patients were allowed to receive DC vaccination without unblinding. This renders the researchers without a real control group as in the end the placebo treated patients have received DCVax-L. Moreover, the primary end point is limited to PFS, with OS as the second end point. The fact that the same company started a new phase II clinical trial using this DCVax-L treatment, only this time in combination with nivolumab (NCT03014804), might already suggest a necessity for combinations of immunotherapeutic strategies to treat GBM patients. The second phase III clinical trial uses a different approach to create a vaccine. Immunocellular Therapeutics is testing the efficacy of ICT-107 in newly diagnosed glioblastoma patients (NCT02546102). ICT-107 consists of a mixture of six GBM antigens being: tyrosine-related protein 2 (TRP-2), glycoprotein 100 (gp100), human epidermal growth factor receptor 1 (HER-2), absent in melanoma 2 (AIM-2), melanoma-associated antigen 1 (MAGE-1), and interleukin 13 receptor a2 (IL-13Ra2) (119). The early results of a phase II trial using ICT-107 were less promising as the DCVax-L trial but still PFS was increased with 2 months and a trend towards increased OS was observed (120). The estimated date for completion of the

study is December 2021. Hopefully this large randomized controlled phase III study can give a more conclusive idea about the potential benefits of DC vaccination in glioma.

Recalling the heterogeneity between clinical trials testing DC vaccination, two important differences need to be stressed. Firstly, the origin of the DC can strongly differ. On the one hand, there is the *ex vivo* culturing of DCs starting from blood monocytes ($CD14^+$) and hematopoietic stem cells ($CD34^+$), while on the other hand, naturally occurring DCs can also be isolated from patients' blood and put in culture merely for loading and maturation (121, 122). Finally, in one of their latest publications, the group of Jo Van Ginderachter anticipate the use of tumor-associated DCs in (targeted) immunotherapy (123). They showed different DC subsets in the tumor environment having different activities with monocyte-derived DCs being the most active at antigen uptake in comparison to cDCs. However, the cDCs showed highest activity for tumor regression with cDC1s inducing strong anti-tumor CTLs and cDC2s reducing MDSCs plus reprogramming of pro-tumoral tumor-associated macrophages. The second difference showing heterogeneity in DC vaccination was already touched upon by introducing the two phase III trials before. Several methods of antigen loading of DCs are described in literature such as whole cell lysate, selected TAA/TSA, RNA transfection, DC-tumor fusion cells and tumor exosomes (124-127). Whole cell lysate and selected TAA or TSA constitute the two main groups each with their advantages and disadvantages (**Table 1**). The former strategy can elicit an immune response against multiple tumor antigens with a lower risk for immune-escape due to lowering presentation of certain antigens by tumor cells. Using selected tumor antigens first needs the discovery of these antigens but the biggest concern is the risk for immuneselection with tumor cells that stop expressing the targeted antigen(s). The advantages on the other hand are: easier immune monitoring, possibility for off-the-shelve product development and reduced chance for autoimmunity. Although, the latter has not really been observed after application of whole tumor cell lysate based DC vaccination either, as stated by the many phase I and phase II clinical studies showing feasibility and safety. Comparison of clinical studies using both DC vaccination strategies demonstrated an enhanced clinical efficacy for vaccination with whole cell lysate loaded DCs over DCs loaded with molecularly defined antigens (128, 129).

Focussing on whole tumor lysate, multiple methods of generating these lysates are available. Among these methods, the most common used is freeze/thawing of the cancerous cells (130-132). Here, the cells are repeatedly exposed to changes in temperature going from e.g. liquid nitrogen to a warm water bath. Importantly, a large variety in protocols exists: the number of cycles, the temperatures to which the cells are exposed and the incubation time can strongly differ among various research laboratories. Another frequently used method for tumor lysate generation is mechanical disruption of the cells. This can be obtained by mincing of cells, using e.g. decreasing sizes of needles, and/or acoustic sonication (133). To increase the reproducibility of the mechanical disruption of the cells, research labs are tend to the use of automated machines like the GentleMACS tissue dissociator (Miltenyi Biotec, Germany) with

pre-programmed settings for cell lysate generation (133). Finally, chemical cell lysis, using a homogenizing or lysis buffer with the addition of enzyme inhibitors (e.g. protease inhibitors), makes up a third method for obtaining cell lysates. However, this method is only used if the tumor lysate is used for *in vitro* studying like e.g. proteomics (134).

Next to the antigenic source, another important feature, which is more applicable for whole tumor cell lysates, is the immunogenicity of this antigenic payload presented to the DCs. Over the past decade researchers have realized that this antigenic characteristic cannot be ignored when considering *ex vivo* or *in vivo* cancer vaccine applications (135). Most research groups, if not all, using whole tumor lysate cancer vaccines, irradiate the tumor cells after generation of the lysate. Originally, this extra step in lysate generation was implemented as a safety precaution, to make sure all tumor cells are killed before presentation to DCs, this way removing the risk to inject viable tumor cells back into the patient. Ever since the importance of immunogenicity of cancer cells used to generate tumor lysate has been shown, the real advantage of lysate irradiation might have been uncovered. A critical involvement of oxidative modifications and the related oxidative associated molecular patterns (OAMPs) has been pointed out by our research group as well as other groups (136, 137). X-ray irradiation of cells induces oxidative stress which leads to the generation of OAMPs like protein carbonylation. A strong correlation between efficacy of DC vaccines using irradiated tumor lysate and the degree of protein carbonylation in this lysate was observed. In an orthotopic glioma mouse model this lead to a survival benefit for animals treated with DC immunotherapy where irradiated lysate was used to load the DCs as compared to non-irradiated lysate (137). Next to lysate irradiation, another way to improve immunogenicity is to generate tumor lysate through immunogenic cell death (ICD). This form of cell death induces an ER-specific oxidative stress leading to the release of DAMPs and is therefore associated with enhanced immunogenicity (138).

Regrettably, DC vaccination in literature has a variable response rate at least partly caused by the clinical context at which the treatment strategy is tested. Only in recent years DC vaccination has been tested in the condition of minimal residual disease which might benefit the therapy. Other possible explanations for the variable response rate of DC vaccination can be suboptimal loading and subsequent maturation of *ex vivo* grown DCs. Once injected, DCs often have problems reaching the draining lymph nodes as shown by De Vries *et al.*, who showed that as few as 5% of the injected DCs reach the lymph node (139). Besides, pre-clinical data show an indirect activity of injected DCs through lymph node resident DCs (140). Finally, DC immunotherapy using *ex vivo* generated DCs is a time consuming and labour intensive procedure which requires a GMP-facility (good manufacturing product) and thus results in an expensive treatment modality that moreover has an increased risk of errors due to the relative large amount of manipulations. Alternatives that have been and are currently investigated are whole glioma cell vaccines and protein/peptide-based antigen delivery using DC antibodies and thus resulting in *in vivo* targeting of DCs.

Table 1: Advantageous and disadvantageous of antigenic sources and vaccination methods in active immunotherapy using dendritic cells

	Pro's	**Con's**
Antigen source		
Peptide	Short peptides (8-10 AA) directly loaded onto MHC molecules Possibility to monitor immune response Synthesized and purified at relative low cost Modification of synthetic peptides	Poor delivery efficiency Limited number of known antigens HLA specific treatment Potential low affinity for MHC Monovalent immune response
Protein	Multiple epitopes in full length proteins Prolonged antigen presentation	Poor delivery efficiency Limited number of known antigens Possibly directed to MHC type II
Tumor lysate	Comprises of various antigens (even unknown ones) Potential for personalized medicine	Certain amount of tumor cells necessary Challenges in manufacturing Monitoring immune response difficult Availability of self-antigens Loss of antigenicity during production
Vaccination method		
Whole cell vaccine	Limiting risk of tumor immune escape Broad immune response (including neo-antigens) No need for antigen identification Available to all HLA types	Increased risk for auto-immunity (self-antigens) Variable composition of the vaccine High quantity of antigenic material need Lower specificity and efficacy
Protein/peptide vaccine	Feasible off-the-shelf product No need for autologous tumor cells (Relative) tumor specificity Possibility of multi-target vaccination, but higher cost Allows targeting of specific DC subsets	Immune response may be transient Possible low magnitude of immune response Often requires adjuvant co-administration Limited set of known antigens for GBM HLA specific treatment (population heterogeneity) Risk tumor escape (antigen-loss variants) Personalized identification of candidate epitopes
DC vaccine	Shown to induce cellular and humoral immune response	Cost and labour intensive generation of DCs Limited migration DCs to lymph nodes

1.3.4 Active immunotherapy through *in vivo* antigen delivery to dendritic cells

Cancer vaccines aim to induce a cellular immune response that targets tumor cells. Next to DC immunotherapy, two other possible methods of treatment do so, through the employment of DCs: whole tumor cell (lysate) vaccines and peptide vaccines. In contrast to DC immunotherapy, which requires *ex vivo* grown DC cultures, these treatment modalities work through antigen delivery to DCs *in vivo* (**Figure 4B**). Both strategies use different antigen sources, consequently generating different immune responses (**Table 1**). Whole tumor cells and cell lysates can induce a broad immune response and are preferred in glioma treatment due to the heterogeneity of the tumor. Therefore, the main focus of this chapter will be on

tumor cell and lysate vaccinations, followed by peptide treatment which will be touched upon more briefly.

1.3.4.1 Whole tumor cell (lysate) treatment

In the treatment of HGG, only a few research papers describe the application of crude tumor lysate without the addition of an adjuvant next to the lysate vaccination. Often this condition of naked lysate injection is used as a negative control group in these publications. One group described the preclinical potential of the coadministration of tumor lysate together with CpG ODN (141). Subcutaneous treatment was followed by higher T cell numbers and activated DCs in the draining lymph nodes as compared to animals treated with adjuvant or lysate alone. In comparison to the control groups, an increased survival of intracranial glioma growth was observed after tumor lysate CpG vaccination, resulting in more than 50% of the animals being tumor free. Later, the same research group even further improved the vaccination protocol by combining their lysate immunisation with antibodies directed against the OX40 receptor on T cells for further immunostimulation and noticed a synergistic effect (142). Importantly, their investigation showed an immune response independent of $CD8^+$ T cells, which is contradictory as the main focus of cancer immunotherapy lies in that immune response (143). Instead of the classical $CD8^+$ T cell response, it was shown that $CD4^+$ T cells, B cells and NK cells had a major contribution in the tumor treatment. Another research group investigated the possibility to use allogenic tumor cell lysate to treat malignant gliomas (144). Using 2 genetically different rat strains, they showed effective prophylactic treatment of allogenic lysate as well as significant reduction and even eradication of established tumors if combined with syngeneic lysate vaccination. Treatment with only syngeneic tumor lysate failed to induce an anti-tumor immune response. Clinical implementation of this therapeutic treatment strategy is provided by a currently running phase II clinical trial (NCT01903330) in which allogenic/autologous GBM vaccination, using patient inactivated tumor cells mixed with tumor cell lysates derived from three GBM donors, in combination with GM-CSF and cyclophosphamide is tested as an adjuvant treatment to bevacizumab injection. Early results suggest a median OS benefit of the combination treatment (12 months survival) in comparison to bevacizumab plus placebo (7.5 months survival) (145). In contrast to the previous described lysate treatment strategies, where tumor lysate as such is used as a negative control, Jouanneau *et al.* have implemented whole tumor lysate vaccination in their treatment protocol. Although their main focus was in DC immunotherapy of glioma preclinically, they discovered a superior role for lysate to boost the immune system after initial priming with DC vaccination as compared to boosting with lysate loaded DCs (146). Still, whole tumor lysate as a stand-alone treatment was ineffective.

Whereas in preclinical animal models (mostly murine models) initially tumor lysate vaccinations have been used, in human testing whole glioma cells were used both in autologous and allogenic setting. A summary of all published clinical studies describing this type of treatment in HGG can be found in **Table 2**. Nearly all studies have in common that the

glioma cells were irradiated before injected back into the patients, with the aim to make sure no living cells were used in the vaccines. The only study that immunised the patients using irradiated autologous tumor cells without an adjuvant was unsuccessful in improving the outcome for the patients (147). Though, this is a very old study where active immunotherapy is used as a stand-alone therapy and compared to standard therapy at that time being surgery followed by radiation. On the other hand, three pilot studies did show potential for whole cell vaccinations with the induction of immune responses and improvement of survival (148-150). In the study of Schneider *et al.* whole tumor cell vaccination resulted in a comparable survival benefit as chemotherapy (148). The tumor cells were treated with the oncolytic Newcastle disease virus before being injected into the patients. Only one phase I clinical trial using the whole glioma cell approach has been published (151). Out of 5 patients with recurrent malignant glioma treated with irradiated autologous tumor cells in combination with the infusion of GM-CSF at the site of injection, 3 patients showed prolonged survival without additional treatment as compared to historical control patients with recurrent GBM treated with surgery and local chemotherapy.

Table 2: Summary of human clinical trials using whole glioma cell (lysate) treatment in high grade gliomas

Type of vaccination (+ optimisation)	Patients information	Study design	Main conclusion	Refs
s.c. injection of IRR autologous tumor cells	27 patients actively treated, 35 patients without vaccine High grade astrocytoma	Randomized prospective clinical trial	No survival benefit in comparison to surgery + radiation: none of the vaccinated patient survived more than 30 months, while in comparison 20% of the control group survived past this point.	(147)
s.c. injection of IRR allogenic cell line (+adjuvant BCG and levamisole immunostimulation)	20 patients, 58 historical control patients Malignant gliomas	Non-randomised pilot study	No survival benefit experimental group in general. For one cell line, a significant survival benefit ($p < 0.02$) was detected as compared to the historical controls, but not in patients treated with the other cell line (p-value 0.06).	(152)
s.c. injection of IRR autologous tumor cells modified to secrete IL-2	1 patient with GBM	Case report	Anti-tumor immune response detected, in part mediated by cytotoxic $CD8^+$ T cells. Tumor necrosis detected with MRI.	(153)
i.d. vaccination with NDV-modified autologous tumor cells	11 patients actively treated, 11 patients without vaccine GBM	Pilot study	Detection of T cell activity based on DTH viewed at injection site (not with NDV alone). Comparable survival rate in patients on active immunotherapy as compared to chemotherapy.	(148)
i.d. injection of IRR autologous tumor cells in combination with IL-4 transfected fibroblasts	1 patient with recurrent GBM	Case report	Local immune response induced at site of injection (CD4 and CD8 cells).	(154)
i.d. injection of IRR NDV-modified autologous tumor cells	23 patients actively treated 87 patients without vaccine GBM	Non-randomised pilot study	Peripheral DTH reactivity, anti-tumor memory T cells and $CD8^+$ TILs in recurrent tumors. Improved median PFS and median OS in comparison to control group, respectively 40 vs. 26 weeks and 100 vs. 49 weeks	(149)
s.c. injection of IRR autologous tumor cells modified to express GM-CSF and B7-2 antigen	3 patients with GBM (3 patients with melanoma)	Pilot study	Inflammatory response shown by DTH, but no anti-tumor specific immunity was demonstrated. Only 1 patient, with minimal residual disease when treated, showed prolonged PFS after immunisation.	(155)
i.d. injection of autologous formalin-fixed tumor tissue (+adjuvant tuberculin microparticles)	12 patients with primary, recurrent GBM	Pilot study	Stable disease in 5 patients with 1 patient showing complete tumor regression. Median OS after vaccination was 10.7 months, with 5.0 months for non-responders and 20.3 months for responders.	(150)
s.c. injection of IRR autologous tumor cells (+ pump-infusion with GM-CSF at injection site)	5 patients Recurrent malignant gliomas	Non-randomised phase I study	DTH after vaccination in 2 out of 5 patients. Prolonged survival in 3 patients without additional treatment (42, 62 and 88 weeks after second surgery).	(151)

BCG = Bacillus Calmette-Guerin, DTH = delayed type hyperreactivity, GBM = glioblastoma, i.d. = intradermal, IRR = irradiated, NDV = Newcastle disease virus, OS = overall survival, PFS = progression free survival, s.c. = subcutaneous, TIL = tumor-infiltrating lymphocytes

1.3.4.2 Peptide treatment

The investigation of peptides for active immunotherapy has gained more interest by the HGG treating community which has led to more progress as compared to whole cell (lysate) vaccinations. Most of the time synthetic peptides are used with the ability to induce an immune response and thus serve as an immunogenic antigen. These antigens can be divided in TSAs, which are often the result of mutations and are never expressed in healthy tissues (e.g. EGFRvIII, IDH1), and TAAs. The latter group consists of overexpressed proteins (EphA2 and TRP-2), viral antigens, cancer/testis antigens normally restricted to germ line cells (MAGE) and differentiation antigens (gp100).

One clinical study, currently performed at the Dana Faber Cancer Institute in Boston, is of particular interest. While other clinical studies use peptides of known antigens, in this phase I clinical trial (NCT02287428) a personalized tumor treatment is achieved by defining neoepitopes of the patient's tumor. Up to 20 neoepitopes, originating from mutant peptides expressed by the patient's tumor cells, constitute the neoantigen vaccine NeoVax. Safety and feasibility of this investigational intervention are currently tested in 46 newly diagnosed GBM patients on top of standard radiotherapy with and without TMZ chemotherapy. First results of the study are expected at the end of 2019.

1.4 Nanoparticles to further optimize antigen delivery to dendritic cells

Following the introduction about DCs and immunotherapy, this chapter will handle nanotechnology and its potential application in cancer (immune) therapy. After briefly introducing nanoparticles (NPs), the advantages for oncotherapeutic applications and more specific the possibilities for immunotherapy will be described.

The International Organisation for Standardization (ISO) has worked out a number of technical specifications describing nanotechnologies vocabulary which are joined in the ISO/TS 80004. According to this ISO/TS 80004 vocabulary nanotechnology can be defined as "the application of scientific knowledge to manipulate and control matter in the nanoscale to make use of size- and structure-dependent properties and phenomena, as distinct from those associated with individual atoms or molecules or with bulk materials" (ISO/TS 80004-5:2015). Moreover the nanoscale ranges from 1 nm to 100 nm and thus NPs are defined as particles with a size within this nanoscale range. In the next paragraphs the main parts of this definition will be further explained.

Many research groups use a different, less strict definition for NPs. In line with the definition of microparticles being particles in the size range of 1 µm to 1000 µm, these researchers consider all particles sized between 1 nm and 1000 nm (= 1 µm) to be NPs (156, 157). Within this dissertation we also consider NPs to be particles in the 1-1000 nm dimension.

1.4.1 Nanoparticles in cancer: diagnosis and treatment

The last years/past decade, the idea of non-targeted and targeted therapy got some new attention with the opportunity to use NPs. Due to their small size, NPs possess unique physicochemical properties differing from the bulk materials of the same composition and rendering them to be excellent candidates for diagnostic and treatment modalities (158, 159). In this part the key properties for anticancer NPs will be touched upon. Most of these properties will be given extra attention later.

To begin, NPs can be tailor-made with tuneable optical, electronic, magnetic and biological properties. The large variation in these features brings along a vast amount of applications leading to usage in many different fields like electronics, chemistry, manufacturing, biology and health.

NPs can be engineered to have different sizes, shapes, chemical compositions and surface chemical characteristics. On the one hand size and shape are important factors that regulate the intracellular uptake, while on the other hand chemical composition of the NPs and especially their surface can determine which cells are most likely to take up the NPs (160, 161). These characteristics will be further described in chapter 1.4.2.2 Potential advantages of NPs in immunotherapy.

One of the most important characteristics of NPs is their nanoscopic size. In comparison to bulk material of the same composition, NPs have a very high ratio of surface area to volume. As an example imagine a 1 cm cube is cut into 10^{21} cubes that are 1 nm in size each. This will result in the same overall mass and volume, but the surface area will increase 10 million times (162). This large ratio of surface area to volume makes NPs more reactive which can be useful for some treatment modalities.

To improve biocompatibility, the surface of NPs is often coated with polymers and bio-inert materials can be used. On the one hand, once administered into the human body, the latter NPs have a minimal interaction/reactivity with its surrounding tissue. On the other hand, surface polymers can have additional purposes like biorecognition molecules used for targeting certain cell types.

NPs are able to benefit from the enhanced permeability and retention (EPR) effect that arises in tumor tissues. Combining the published work of Matsumura *et al.* and Gerlowski *et al.* from 1986 created the foundations of the hypothesis of EPR effect (163, 164). Both publications describe the presence of a tumoritropic accumulation of macromolecules due to hypervasculature, resulting in a higher permeability of the microvasulature of tumors. Moreover, the lymphatic drainage system seems to be impaired in tumor tissue (165). As a consequence systemic application of therapeutic anti-cancer agents with or without the help of NPs is able to generate an anti-tumor effect through passive targeting. Therefore the EPR effect is called the royal gate in the drug delivery field. The most well-known example for

passive targeting through the EPR effect is Doxil® (doxorubicin encapsulated within polyethylene glycol-coated NPs), which was approved for clinical use in 1995 to treat multiple types of cancer (166).

1.4.1.1 Nanoparticles in cancer diagnosis

Multiple optical imaging systems based on the use of nanotechnology have been developed to enhance cancer diagnosis. A first example are dendrimers and liposomes containing radionucleotides. Within these particles, the radionucleotide is being concentrated leading to an increased signal as compared to the separate radionucleotide (167). Another group of nanotechnology-based cancer diagnosis devices are fluorescent NP systems like quantum dots. These are semi-conductor nanocrystals containing a metallic core surrounded by an outer shell which can be conjugated with different tumor-targeting ligands. One research group in China published their results of quantum dots labelled with aptamer 32. Due to the aptamer, the quantum dots are able to specifically target glioma cells with the EGFRvIII. Preclinical testing using a murine U87-EGFRvIII model showed strong fluorescent signal clearly visualising the tumor borders (168). Although the result of this work would only be of benefit for a part of the glioma patients, this experimental work holds great promise for future guided surgery in glioma. Next to radionucleotide and fluorescence detection another technique that can benefit from nanotechnology is MRI. Small iron oxide particles with a magnetic particle core can be coated with biocompatible polymers resulting in super paramagnetic iron oxide NPs (SPIONs) (169). Being able to create a larger magnetic field, these NPs are able to increase the contrast of MRI. Although the behaviour of electrons in nanomaterials is more constrained, the two most important features of electrons remain their ability to move between specific energy levels (energy transition) and their spin. These characteristics strongly depend on size with all electrons spinning in the same direction for SPIONs, whereas the electrons spin in opposite direction in macroparticles (170). The magnetic field generated by SPIONs is stronger because of the additive effect of all electrons spinning in the same direction.

The above mentioned examples are only a few of a very large group of diagnostic improvements achieved by implementing nanotechnology, but this is not the main focus of this dissertation.

1.4.1.2 Nanoparticles in glioma treatment

As already stated in the introduction concerning glioblastoma, countless treatment strategies have been addressed to treat GBM. Next to the conventional strategies of surgery, radiation and chemotherapy, a few immunotherapeutic treatment modalities were discussed. In this part, different promising NP-based glioma treatment strategies will be briefly touched upon. Most of these strategies are still in preclinical phase.

The most obvious treatment strategy using NPs is NP-based drug delivery. NPs can improve drug delivery for various reasons with amongst other things: prolongation of the half-life of drugs in systemic circulation by reducing their immunogenicity, improve the solubility of poorly water-soluble drugs, sustained drug release (and thus lower the frequency of administrations), deliver drugs in a targeted manner minimizing systemic side effects and combination therapy of two or more drugs simultaneously (158, 171). Also TMZ, the most widely used chemotherapeutic drug in GBM, has been subject to improvement with NPs, e.g. chitosan loaded NPs and PLGA NPs (172, 173). Importantly, in the treatment of brain tumors one extra obstacle needs to be taken into account, namely passage over the BBB. Receptor-mediated transcytosis can be utilised as a selective pathway for crossing this barrier. Therefore, multiple targeting ligands which mimic receptor binding fragments of endogenous ligands have been developed. Endothelial cells in the brain display a set of transport systems and receptors such as transferrin receptors, acetylcholine receptors and glucose transporters, that can serve as an entrance ticket for NPs (171, 174, 175).

Next to improved drug delivery, NPs themselves can also serve for treatment. Metal NPs like iron oxide and gold NPs, can be used for thermal treatment also known as hyperthermia (169, 176). These particles need to consist of a metal, magnetic core with a biocompatible outer layer. The magnetic core can generate heat by moving the electrons of the core between different energy levels and this heat can be utilised to destroy tumor cells. In Europe one treatment strategy using magnetic iron oxide NPs has been approved for GBM, prostate and pancreatic cancer: NanoTherm® (MagForce Nanotechnoligies, UK). The application of the ferroliquid needs to be intratumoral, where the NPs of only 15 nm in size will stay in the tumor tissue due to their aminosilane coating (177). By applying an alternating magnetic field to the tumor region, the NPs can efficiently convert energy into heat, destroying tumor tissue. The results of 60 patients with first recurrence of GBM treated with NanoTherm® resulted in an average survival of 13.2 months in comparison to 6 months with conventional treatments. Though, it would be fair to say that the published results are generated by a combination of magnetic hyperthermia and radiotherapy as the whole group was treated by a combination of both (177).

The more recent and emerging strategies using nanotechnology are the engineering of the (tumor) cell genome or TME and the combination of nanotechnology and immunotherapy. Both treatment modalities hold great promise for the future, but are still in their infancy stage. The first comes forth from an increasing knowledge of the immunosuppressive TME and new technologies like silencing RNA and CRISPR-Cas 9, which facilitate cell genome engineering (178). Moreover, NPs act as ideal candidates to transport nucleic acids into the brain and further into the tumor cells. As an example of TME-based therapy, the results from preclinical work to which our group contributed are briefly illustrated next. Using chitosan NPs, siRNA targeting galectin 1 was transported to the brain of a glioma mouse model through intranasal instillation (179, 180). The change in TME due to galectin 1 knock down, with normalisation

of tumor vasculature and downregulation of MDSCs and Treg cells, was accompanied by increased survival for glioma-bearing mice. Next to changing the TME, the immune system can also be activated to elicit anti-tumor immunity. By nanotechnology entering the field of immunotherapy a new therapy concept is created, nanoimmunotherapy. Two possibilities exist with either passive or active nanoimmunotherapy, the former using nanocarriers for antibodies that target cancer cells, the latter using nanocarriers for antigens to elicit an active immune response against the cancer cells (181). In the next part the focus will be on nanoimmunotherapy.

1.4.2 Nanoparticle based approaches to elicit antitumor immunity

One way for NP-based immunotherapy to promote the anti-cancer effects of the treatment is to antagonize the immunosuppressive environment. This can be achieved by introducing immunomodulatory molecules to the tumor tissue, creating an opportunity for the immune system to respond to the threat of cancer. An alternative treatment exists in the possibility to create and stimulate the induction of an active immune response directed against tumor cells (**Figure 4C**). Nanotechnology can be employed to develop therapeutic vaccines, so called nanovaccines, that aim to create a T_{h1} and CTL response *in vivo* (182-184). Of great importance for generating a therapeutic T cell response and thus for nanovaccine technology, are APCs that can direct the immune response. As is the case for standard active immunotherapy, active nanoimmunotherapy relies on the participation of mainly DCs to induce an anti-cancer immune response. Indeed, NPs can be used to improve antigen delivery to and thus increase antigen presentation by DCs. The success of anti-cancer nanovaccines is based on optimal modulation of APCs to promote the desired responses. Again, a distinction can be made between whole tumor cell lysate formulations and antigen loaded NPs, each with their advantages and disadvantages (185).

1.4.2.1 Nanoparticle uptake by antigen presenting cells

In comparison to soluble antigens, particulate antigens show different uptake mechanisms and kinetics. Considering particulate antigens within the nanoscopic size to comprise of antigens loaded within or conjugated to the surface of NPs, it has been shown that these particulate antigens are more prone into creating an appropriate immune response as compared to soluble antigens (66). Presumably, this observation can be considered a consequence of a facilitated intracellular uptake by APCs due to their particulate shape along with sizes within the viral size range generating pathogen-mimicking particles (67).

Different cell internalisation mechanisms can be distinguished which are strongly influenced by the NPs' physicochemical properties like size, shape and charge (67, 186). On the one hand, phagocytosis is an internalisation process more or less restricted to professional phagocytic cells e.g. macrophages and DCs. In a first step of the process foreign particles are tagged by opsonin proteins which are recognized by the macrophage and finally lead to particle ingestion. On the other hand, various endocytic pathways exist in nearly all cells of

the body. These non-phagocytic pathways consist of four main mechanisms (186). Clathrin-mediated endocytosis is the predominant endocytic mechanism. It can be receptor dependent, requiring ligand-receptor complexes, and receptor independent, the latter characterized by a slower internalisation rate. As an example, **Figure 5** illustrates the main steps in this internalisation process of NPs by DCs. Caveolae-mediated endocytosis is a more regulated process that involves complex signalling. Importantly, the cytosolic vesicles residing from this uptake mechanism are non-lysosomal and do not contain any enzymatic cocktail (187). Therefore, caveolae-mediated endocytosis is often the preferred pathway for particle uptake. Opposing to the previous endocytic pathways, micropinocytosis does not require chaperone molecules and thus seems to be less cargo dependent. However, charge of the particles will positively influence uptake through this process. Cationic NPs will generally associate better with the cells' negatively charged plasma membrane (188). Finally, a fourth endocytic uptake mechanism is clathrin- and caveolae-independent, but lipid raft dependent. This pathway is important for hydrophobic NPs as they will reside near plasma membranes and concentrate at the lipid rafts created in these membranes (189).

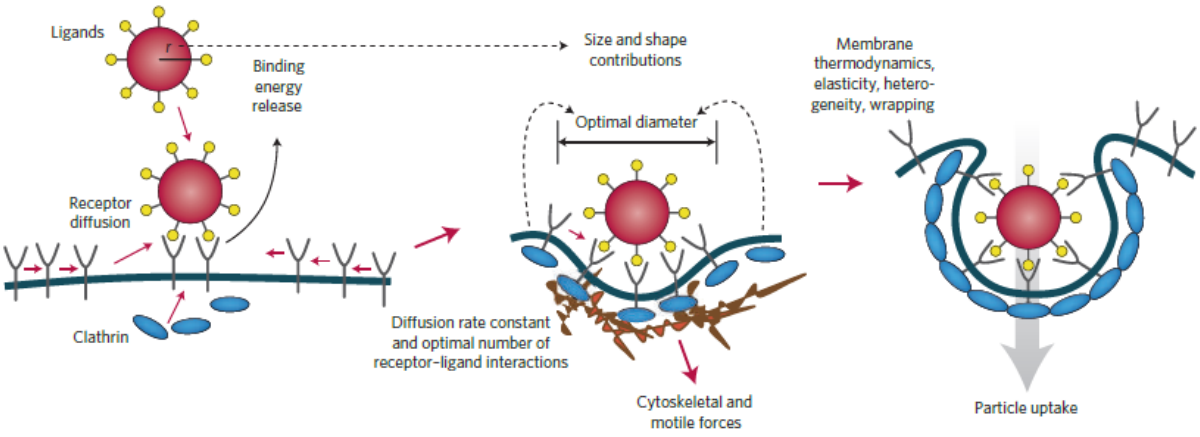


Figure 5. Nanoparticle internalisation via clathrin-dependent endocytosis by dendritic cell. In a first step, the nanoparticle (NP) needs to make contact with the membrane surface. Therefore, specific (e.g. ligand-receptor) and/or non-specific (e.g. hydrophobicity of particles) binding interactions must overcome resistive forces. Once the NP interacts with the membrane, clathrin molecules (blue ellipses) are drawn to the contact site. Due to the combined force of the developed clathrin lattice and cytoskeletal interactions, the particle can be engulfed by the dendritic cell.
(Adapted and modified with permission from Nel et al. 2009 (190))

Next to the physicochemical characteristics, the route of administration is of great importance for NP uptake by APCs. Depending on the injection site, different cell types will be more likely to take up the nanovaccines and for that reason determine the immune response. Intraperitoneal injection of NPs has a greater chance for ending up in macrophages as these cells are the predominant phagocyting immune cells in the peritoneal cavity (191, 192). The skin contains a lot of DCs with dermal DCs in the dermal layer and Langerhans cells in the epidermal layer (105). Therefore, nanovaccines aiming at the active induction of an anti-

cancer immune response are typically injected intradermally or subcutaneously. Once skin-DCs have internalized the NPs, they will migrate to the local lymph nodes. As large populations of DCs reside in lymphoid organs, intranodal injection or intralymphatic injection, although difficult to perform, can also be interesting routes of administration. These vaccination strategies leave out the necessity of nanovaccine transport to the lymph nodes after administration (193). Importantly, comparable intracellular trafficking as is the case for soluble antigens follows nanovaccine internalisation (194).

1.4.2.2 Potential advantages of nanoparticles in immunotherapy

What makes NPs so attractive for implementation in cancer immunotherapy? To begin, NPs comprise of a very heterogeneous group of materials with versatile physicochemical properties. These properties, like size, shape and charge, can enhance antigen uptake by DCs, but also determine which cell will most likely internalize the nanovaccine. Importantly, various studies have shown inherent adjuvant functions of NPs in nanovaccines. Multicomponent loading leaves the option open of multivalent presentation of antigens and co-delivery of antigens and adjuvant. Moreover, NPs are able to protect their cargo from degradation and ensure a long term release of antigens by creating a depot for antigens or by prolonging circulation time in blood. Finally, both passive and active targeting of DCs lie within the possibilities of NPs, even targeting of specific subsets of these DCs to further guide the immune response is possible.

1.4.2.2.1 Physicochemical properties that determine uptake by DCs

The most important and most examined physicochemical characteristic of NPs influencing biodistribution, cellular interactions and cell internalisation is size (195-197). Following intradermal and subcutaneous injection, nanovaccines' biodistribution depend on their size with small NPs (<100 nm) being transported via the lymphatic system to the lymph nodes, where they most likely end up in lymph node resident DCs (198, 199). Larger nanovaccines (>500 nm) are taken up by DCs of the skin as they are trapped in the skin (200, 201). Once skin-DCs have internalized the NPs, they will migrate to the lymph nodes. Constructs with a size in between (100-500 nm) show both migration pathways: cell-based and free transport to the lymph nodes (199). Intranodal injection eliminates the necessity of nanovaccine transport to the lymph nodes. Larger constructs administered in this way have also demonstrated prolonged retention (193). Besides, size can determine which cells will preferentially internalize the NPs. Although it is not a 100% restriction, often an upper limit is considered around 500 nm to distinguish between uptake through phagocytosis, especially performed by macrophages, and endocytosis, by the specialized DCs (62, 201). Comparing *in vitro* uptake of polystyrene NPs with a diameter range of 40 nm to 15 µm, Foged *et al.* reported an optimal particle size for uptake by DCs to be 500 nm and smaller (197). Sizes below 500 nm are thus preferred to passively target DCs, with smaller particles being easier internalised by DCs (202-204). **Table 3** lists the (endocytic) mechanisms of DCs, as described before, according

to the particle size. Micropinocytosis is less size-restricted and acts more like a complementary endocytic mechanism to the others (67, 186).

Table 3. Summary of size-depending, active uptake mechanisms for nanoparticles.

Size range	Internalisation process
< 50 nm	Clathrin- and caveolae-independent endocytosis
50 - 200 nm	Clathrin-mediated endocytosis
200 - 500 nm	Caveolae-mediated endocytosis
> 500 nm	Phagocytosis

One group clearly demonstrated a specific size range of antigen-loaded NPs that can elicit an immune response. Fifis *et al.* obtained a size-dependent immunogenicity as a result of ovalbumin (OVA) conjugated polystyrene NPs. The optimal size for immune cell stimulation was observed to be around 40-50 nm (205, 206). A combined $CD8^+$ T cell response with IFN-γ production and antibody response were detected as a reaction to antigen-loaded NPs in both mice and sheep (205, 207). After intradermal injection, these NPs were found in mature $DEC205^+CD40^+CD80^+$ DCs in the draining lymph nodes. Using transgenic tumor models, more specifically subcutaneous injection of OVA-expressing EG7 tumor cells or EL4 tumor cells expressing E7 human papilloma virus protein, protection from tumor growth (prophylactic) and treatment of established tumors (curative) was shown following treatment with antigen-loaded NPs. Moreover, type 1 T cell responses (IFN-γ) were greater for 40-50 nm OVA-loaded NPs, whereas larger particles (93-123 nm) induced stronger Th2 immune responses (IL-4) (208). Other groups have shown comparable results with smaller NPs being better able to induce T_{h1} responses as compared to larger ones (203).

Next to size, the geometrical shape of nanovaccines also helps defining the immune response by strongly influencing the internalisation by cells. In general, spherical particles are considered the standard shape for nanovaccines, but numerous pathogens like viruses and bacteria display an ellipsoid shape. Therefore various research groups have studied the uptake of different shapes of NPs. Mathaes *et al.* observed a less potent DC activation due to rod-like NPs as compared to spherical ones (209). This observation might be a result of non-spherical NPs being able to resist internalisation by APCs. This is in line with the results of Champion *et al.* who found that, compared to spherical polystyrene particles, elongated particles are able to resist phagocytosis by macrophages (210). Importantly, Kumar *et al.* showed different types of immune response following internalisation of antigen-loaded NPs with geometrically different shapes by DCs (203). While spherical NPs induced a T_{h1} response, characterized by IFN-γ, rod-shaped NPs induced a humoral T_{h2} immune response. These observations lead to the conclusion that shape can affect the nanovaccines' ability to induce an immune response in various ways.

A third physicochemical property of importance for NP internalisation is charge. Contradictory observations have been made considering the *in vitro* settings and the *in vivo* environment. Positively charging the surface of particles increases the uptake by DCs *in vitro* even if their size is above 500 nm (197). The same electrostatic effect that improves uptake of cationic particles *in vitro* limits their uptake *in vivo*, where the environment is full of negatively charged components. These negative charges might immobilize the cationic NPs and prevent transport through tissues. Also, cationic NPs provoke more toxicity in phagocytic cells, mainly through the formation of ROS (211). Two possible solutions for the problem are to shield the positive charge for instance with the hydrophilic molecule PEG or to work with anionic NPs (211, 212).

1.4.2.2.2 Nanoparticles as immune adjuvants

Generally, a vaccine adjuvant is considered to be something that improves the immune response when added to the antigen. Most adjuvant effects of NPs arise from the tuneable physicochemical properties of these particles. Sustained release, prolonged antigen presence, direct stimulation of DCs, improvement of antigen internalisation by DCs and promotion of cross-presentation are examples of possible adjuvant effects achieved by NPs. It is already described above that size, shape and surface charge are able to enhance DC uptake (197, 209). Moreover, NP size can cause a depot effect to occur when antigen-loaded NPs are injected intradermally or subcutaneously (200, 201). Interestingly, the nanocarrier itself can also play an adjuvant role by direct stimulation/maturation of the DCs as well as antigen processing and presentation. Fifis *et al.* compared their OVA-loaded polystyrene NPs with a range of conventional adjuvants like alum, monophosphoryl lipid A (MPLA) and complete/incomplete Freund's adjuvant. The induced immune responses, both CTL and humoral, elicited by the nanovaccine were comparable to Freund's adjuvant immunisation, but more strikingly, significantly higher than that of alum and MPLA immunisation (205). Also, biodegradable poly(γ-glutamic acid) (γ-PGA) NPs loaded with OVA have been shown to induce upregulation of costimulatory molecules (CD40, CD80, CD86) and the lymph node homing chemokine receptor CCR7 upon internalisation by DCs (213). Furthermore, improved cross-presentation efficiency in DCs was observed using these γ-PGA NPs (214). Enhancing OVA-mediated cross-presentation of DCs, and as a consequence realizing a stronger anti-tumor response, is demonstrated by many other NPs such as poly(D,L-lactic-co-glycolic acid) (PLGA) NPs, poly(propylene sulphide) NPs and polyethyleneimine coated aluminum hydroxide NPs (188, 215-217). However, often antigen-only systems are not able to provide all the necessary signals to induce the desired immune response. To remedy the limited efficacy due to low immunogenicity, NPs can be used to co-deliver antigens and adjuvants to DCs (194).

1.4.2.2.3 Engineering DC activation with particles carrying molecular adjuvants

The most studied approach to positively influence the immune response obtained by nanovaccines is to co-deliver antigens with an adjuvant. Already introduced in the DC and

immunotherapy introduction of this dissertation, strong stimulation of DCs can be aimed for through activation of PRRs like the TLRs (218). Hence, TLR agonists are frequently studied in nanoimmunotherapy as an adjuvant for antigen-loaded NPs. Co-delivery of antigens and immune stimulants is described in an article by Hamdy *et al.* in which PLGA NPs were loaded with OVA as an antigen and MPLA, a TLR4 agonist, as an adjuvant. These nanovaccines induced both a potent $CD4^+$ and $CD8^+$ T cell response (219). In another study, Xu *et al.* used lipid-calcium-phosphate NPs that encapsulated a melanoma peptide in combination with the TLR9 agonist CpG (220). Comparing free peptide/CpG vaccination with NP encapsulated vaccination in the treatment of a murine melanoma model and a lung metastasis model only showed significant inhibition of tumor growth in the nanovaccine treated animals.

1.4.2.2.4 Targeting of DCs

On the one hand, passive targeting of NPs to APCs is possible through manipulating the physicochemical properties of the NPs as well as changing the route of administration. On the other hand, receptor-ligand interactions can be employed to target NPs towards specific cell types and even subsets of these cells. Targeted delivery of antigens to DCs is a potential approach to increase the amount of antigen delivered into these cells and NPs constitute the perfect platform for this purpose (221). DCs express both Fc receptors, required for antibody-mediated antigen delivery, and lectin receptors which can bind endogenous proteins as well as sugar residues on pathogens. Both receptor types can be targeted by antibodies directed against these receptors and/or sugar molecules. In a first example, antigen-loaded and IgG-coated liposomes have shown to protect mice from tumor growth with Fc receptor-targeted liposomes performing six times better than non-targeted ones (222). Amongst the most popular DC targeting moieties are the CLRs (223) e.g. mannose receptor (224), DC-SIGN (225) and CLEC9A (226). Silva *et al.* used mannose-functionalized NPs loaded with antigen and adjuvant to induce strong anti-tumor immunity in melanoma mouse models (224). Other possible receptors for DC targeting are DEC-205 and CD11c/CD18 (227).

Importantly, as described by Ueno *et al.*, subsets of DCs can also be targeted, which has significant consequences if one considers that some DC subsets are more specialized in cross-presentation then others and are different in their ability to induce a Th_1 or Th_2 $CD4^+$ response (228). It has been shown that in mice the $CD8\alpha^+$ DCs are the most specialized cross-presenting DCs and thus targeting of this subset might be favourable for anti-tumor responses. Idoyaga *et al.* demonstrated specific internalisation of antigens directed to $CD8\alpha^+$ DCs using antibodies against CLEC9A or DEC-205, leading to a strong CTL response (229). Another research group produced PLGA NPs with antibodies against different receptors for targeted antigen delivery to pDC and found high IFN-γ production (230).

1.4.2.3 The use of nanoparticles for delivering tumor antigens to dendritic cells

Up till this point, only studies describing single antigen-loaded NPs have been discussed. Still another antigen source, being whole tumor cell lysate, is extensively examined for application in nanoimmunotherapy. Each method of antigen delivery has its advantages and disadvantages that correspond to those discussed in the introduction concerning 'classical' DC immunotherapy (**Table 1**). In this part, a resume of lysate-loaded NPs in the treatment of cancer is given. All discussed studies have implemented different advantageous characteristics that follow from the use of nanoparticulate systems like co-delivery with adjuvants, active targeting molecules and stealth effects.

Table 4 provides us an incomplete list of recent immunotherapeutic research articles that utilized tumor lysate-loaded nano- and microparticles to induce an anti-cancer immune response in preclinical cancer models. Most research is performed on PLGA particles as this is an FDA approved, biodegradable polymer already widely used in several drug products for human use (194). One study, performed by Hanlon *et al.*, compared the effectiveness of soluble tumor lysate and NP-lysate conjugates *in vitro* (231). Human PBMCs were used to generate DCs and these cells were loaded with either soluble lysate or NP-associated lysate. Using PLGA NPs to deliver the tumor lysate induced production of pro-inflammatory cytokines as well as expression of costimulatory molecules able to induce a $CD8^+$ T cell response. Another research group showed anti-tumor responses in a transgenic prostate mouse model, that had previously resisted 'classical' DC immunotherapy (232). PLGA microspheres that encapsulate tumor lysate and CpG ODN (CpG oligodeoxynucleotide) as an adjuvant were injected in combination with Poly(I:C) (polyinosine-polycytidylic acid) loaded microparticles leading to a reduced tumor growth in tumor-bearing mice. Finally, one research group aimed to treat glioblastoma using lysate-loaded PLGA microspheres (233, 234). In their research papers, they also investigated the possibility to use only the plasma membrane fraction of tumor lysate. Lysate-loaded particles were able to induce splenocyte proliferation and T_{h1} cytokine secretion following co-culture of these splenocytes with irradiated tumor cells. Unfortunately, no survival benefit accompanied these findings.

Table 4: Preclinical, particle-based tumor lysate nanovaccines (summary)

Type of nanocarrier	**Type of tumor**	**Optimisation (targeting/adjuvant)**	***In vitro*/*in vivo* testing**	**Immunological features Main conclusion**	**Refs**
PLGA microparticles (2.29 µm)	Breast cancer (lung metastasis)	Not applicable	*In vivo* (s.c.)	Particles efficiently internalised by DCs; 42% reduction in lung metastasis using prime-boost vaccination protocol	(235)
PLGA microspheres (12 µm)	Glioma	Not applicable	*In vitro*/*in vivo* (s.c.)	Increased secretion of IL-2 and IFN-γ in microsphere treated mice; no survival benefit using PLGA microspheres, only for irradiated glioma cells	(233, 234)
PLGA NPs (200-500 nm)	HNSCC	Not applicable	*In vitro* (human patient cells)	Increased secretion of IFN-γ and decreased IL-10 production by $CD8^+$ T cells stimulated by DCs produced from PBMC *ex vivo* loaded with NP-lysate conjugates (4/5 patients) Same cytokine profile observed when TIL were stimulated with comparable DCs	(236, 237)
PLGA NPs (200-500 nm)	Ovarian cancer	Not applicable	*In vitro* (human patient cells)	Strong Th1 cytokine profile produced by $CD8^+$ T cells stimulated with DCs (produced from PBMC) *ex vivo* loaded with NP-lysate conjugates	(231)
PLGA NPs (100-300 nm)	Melanoma	Not applicable	*In vitro*/*in vivo* (i.d.)	Increased secretion of IL-2 and IFN-γ; diminished tumor growth by DCs *ex vivo* loaded with PLGA NPs	(238)
PLGA microparticles (2.4 µm)	Melanoma	CpG ODN (+ Treg depletion)	*In vivo* (i.p.)	Uptake by $CD11c^+$ DCs; expansion and proliferation tumor-specific cells with increased IFN-γ secretion; particle treatment induced Treg expansion; protection from tumor growth by combining lysate-loaded particles and Treg depletion	(239)
PLGA microparticles (8-9 µm)	Prostate cancer	CpG ODN (+ Poly(I:C) microspheres)	*In vitro*/*in vivo* (s.c.)	Substantial CTL responses as well as reduced tumor growth in a transgenic mouse model following PLGA particles containing Poly(I:C) and lysate + CpG ODN	(232)
PLG matrix disks (250-425 µm)	Melanoma	GM-CSF and CpG ODN	*In vivo* (s.c.)	Subcutaneous pocket of PLG matrix containing GM-CSF and CpG ODN attracts and activates DCs leading to generation of CTLs and inhibition of Treg cells; monotherapy leading to complete regression of tumors	(240)
Chitosan NPs (120 nm)	Melanoma	Mannose targeting	*In vitro*/*in vivo* (s.c.)	*In vivo* uptake by DCs; increased serum IFN-γ and IL-4 levels; retarded new tumor growth and decreased growth of established tumors	

Chitosan microparticles (1-2 µm)	Ovarian cancer	Enteric polymers + M-cell targeting ligand	*In vivo* (parenteral)	Elevated $CD8^+$ T cell, $CD4^+$ T cell and B-cell populations in lymphoid organs; mixed Th1 and Th2 immune response; significant delay in tumor growth	(241)
Fusogenic liposomes (400 nm)	Melanoma	Not applicable	*In vivo* (i.d.)	Inhibitory effect on tumor growth; *ex vivo* loaded DCs more effectively as compared to lysate-loaded liposomes	(242)
Solid lipid NPs (180 nm)	Endometrial and ovarian cancer	Mannose targeting	*In vivo* (i.p.) *In vitro* (human patient cells)	Pre-clinical assessment: higher uptake + maturation DCs; human patient PBMC produced DCs take up NPs and create an optimal immune response with Th1 cytokines, T cell proliferation and CTL response	(243)
Carbon nanotubes (500-800 nm)	Liver cancer	Not applicable	*In vivo* (s.c.)	Specific anti-tumor response; tumor-bearing mice are cured from tumor growth	(244)

HNSCC = head and neck squamous cell carcinoma, i.d. = intradermal, i.p. = intraperitoneal, PLG = poly(lactide-co-glycolide), PLGA = poly(lactic-co-glycolic acid), s.c. = subcutaneous

1.5 Murine glioblastoma models to study immunotherapy

Preclinical research models are of great importance for studying glioma and more specifically interactions with therapeutics and the immune system. In order to replicate glioma behaviour *in vivo*, meaningful characteristics like histopathology, molecular profiles, tumor biology and invasiveness of glioma must be preserved. On the other hand, important features of experimental glioma models are reproducible and predictable growth characteristics, *in vivo* recapitulation of disease characteristics, easy transplantation in the animals and immortalisation of the cell line (245). An ideal mouse model needs to combine relevant glioma characteristics with these paramount features. Ever since the development of the first experimental brain tumor model in 1939 by Seligman and Shear (246), a vast amount of murine glioma models has been developed which can be divided over three groups: syngeneic immunocompetent models, genetically engineered animals and human cell line or patient-derived immunocompromised xenografts (247). Each group has its advantages and disadvantages and therefore it is important to choose the tumor model(s) wisely. Syngeneic, engrafted models lack spontaneous tumor growth, are more or less limited to growth in the brain parenchyma and rarely recapitulate tumor-of-origin phenotype. Nevertheless, due to good reproducibility, shorter latency periods for tumor formation and predictable growth characteristics, the tumor remains well circumscribed. On the other hand, spontaneous oncogenesis in genetically engineered mice, through deletion of tumor suppressor genes or induction of oncogenes, goes at the expense of latency to tumor development, problems with reproducibility and high costs. Finally, because of the need for immunocompromised mice in the xenograft models, these models are of limited use for immunology research.

For the type of research performed during this PhD research, namely immunotherapy research, the presence of an intact immune system was crucial. Therefore, the immunocompetent, syngeneic GL261 and CT2A glioma models were used. This way the TME, including vasculature and stromal cells as well as immune cells, could contribute to the generated anti-tumor response. Moreover, instead of working with subcutaneous tumor models, we chose to work with two orthotopic models which allowed important tissue specific interactions to take place. Originally, the GL261 mouse model was chemically induced with methylcholanthrene in the C57BL/6 background and is one of the best characterized syngeneic models (248). Although histopathologically the tumors most resemble ependymoblastomas, they closely mimic GBM phenotype (249). From an immunological point of view, GL261 cells show low levels of MHC-I, no MHC-II and low expression of co-stimulatory molecules CD80 and CD86 (248). Moreover, the expression of unique tumor antigens, like Ephrin A2 (EphA2), are present and can be used to generate a GL261-specific immune response (250). Importantly, GL261 cells are known to be moderately immunogenic leading to the absence of spontaneous tumor regression. The second tumor model, the CT2A glioma mouse model, was also developed by chemical induction with methylcholanthrene in the C57BL/6 background (251). CT2A tumors show multiple high

grade astrocytoma features including high cellular density and an infiltrative nature. Given this high mitotic index and cell density, microvascular proliferation and haemorrhage pseudopalisading necrosis, the CT2A model recapitulates several features of GBM as well (245). In comparison to other established glioma cell lines, the CT2A cell line is significantly more invasive and proliferative *in vivo*. The model is mainly used for studying glioma stem cells in an immunocompetent environment and is known to generate neurospheres when cultured in serum-free media (252).

2 Research objectives

Immunotherapeutic strategies for GBM are extensively studied as an adjuvant treatment modality. DC vaccination aims for T cell activation to attack remaining tumor cells after standard therapy. Therefore, autologous DCs can be *ex vivo* loaded with tumor antigens and injected back into the patient. One important factor is the antigenic source with whole tumor lysate being preferred for GBM due to the heterogeneous character of gliomas and the potential loss of target-antigen expression by the cancer cells. Previous work of our research group pointed out the importance of immunogenicity of the lysate used for DC immunotherapy: implementation of X-ray irradiation of tumor lysate strongly increased lysate immunogenicity, but also the risk of manipulation and the duration of the DC maturation process. Therefore, we were wondering whether targeting *in vivo* DCs could replace the *ex vivo* ones.

For more than a decade, DC immunotherapy has been studied by many research groups, including ours, but unfortunately this has not led to any major breakthrough in the treatment of GBM. Despite the observations of objective immune responses like IFN-γ producing CTLs, no clear clinical improvement has been shown except for a fraction of long term survivors. Apart from the immune suppressive environment the tumor resides in, this limited improvement can perhaps be partly attributed to the use of *ex vivo* loaded DCs. The limited yield and viability of DCs *in vitro*, an incomplete uptake of lysate fragments and the potential absence of accompanying maturation stimuli and a decreased homing to lymphoid tissue once injected, all contribute to a potentially decreased potency of cultured DC to induce an anti-tumor immune response. With the rise of NPs and especially the implementation in cancer (immuno)therapy, the scientific field gained a renewed interest in active immunotherapy. NPs are able to improve DC immunotherapy for example by combining antigen and adjuvant internalisation, by rendering the opportunity to target DCs *in vivo* and even target specific DC subsets.

The general aim of this research project was to replace the use of *ex vivo* DCs by ‘targeting’ *in vivo* DCs with autologous tumor lysate in the setting of preclinical GBM. The requirement for autologous cell cultures in DC immunotherapy causes this therapy to be labour intensive and expensive, leading to an unmet need to decrease the cost for DC immunotherapy production and to create a broader applicable product in future. Moreover, every step less in human manipulation is a step further away from possible mistakes and contamination. Investigating the potential of autologous tumor lysate treatment in GBM is further supported by the fact that in some clinical trials testing DC immunotherapy, prime-boost treatment schedules are used where boosting is performed with lysate alone. Finally, we were wondering if the tumor lysate vaccination could benefit from the implementation of NPs in this treatment. Therefore, tumor lysate was conjugated to the surface of NPs which can

passively target DCs after subcutaneous injection. In this setting we moreover hypothesized that the concentration/amount of lysate necessary to induce an immune response can be further decreased when compared to *ex vivo* loaded DCs.

To study our hypothesis, we evaluated vaccination with naked autologous lysate and lysate-NP conjugates (nanovaccines) in the murine GL261 glioma model, the most validated immunocompetent model available to pre-clinically investigate potential immunotherapeutic strategies in GBM. Moreover, we introduced a new orthotopic mouse model, the CT2A model, in our research group to evaluate the treatment modalities in a more aggressive tumor model.

With the current research project, we wished to achieve the following aims:

I. To investigate the potential of autologous immunogenic lysate vaccination in pre-tumoral glioma vaccination strategy.
II. To identify the immune mechanisms involved in this treatment strategy.
III. To combine lysate vaccination with clinically relevant therapies such as TMZ chemotherapy and checkpoint blockade to generate therapeutic glioma treatment options.
IV. To pursue optimisation of lysate vaccination through the development of a nanovaccine treatment strategy.
V. To evaluate the nanovaccine along the lines of lysate treatment.

3 Methodology and materials

3.1 Glioma cell lines and cell culture

GL261 glioma cells were kindly provided by Dr. Ilker Eyüpoglu from the University of Erlangen (Germany). CT2A cells were a generous gift from Prof. Holger Gerhardt from the Vesalius Research Center (VIB, Leuven, Belgium) with MTA obtained from Prof. Thomas N. Seyfried (Boston College, USA). Both C57BL/6 syngeneic tumor cell cultures were maintained in a humidified atmosphere at 37°C under 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin (pen/strep) and 2 mM L-glutamine (L-Glu) (all from Lonza, Verviers, Belgium).

3.1.1 Tumor lysate generation and FITC labelling

Starting with a concentration of 40 x 10^6 tumor cells per ml 1% PBS/FCS (phosphate buffer saline (PBS) containing 1% FCS), lysate was created by performing 6 freeze-thaw (F/T) cycles consisting of 3 minutes liquid nitrogen and 3 minutes 56°C warm water bath. Afterwards the lysate was irradiated with 60 Gy total dose to increase immunogenicity (137). Quantification of protein concentration was performed by Bradford protein assay (Bio-Rad, Temse, Belgium). Finally, tumor cell avitality was checked with trypan blue dye exclusion.

In some experiments, lysate was labelled with fluorescein isothiocyanate (FITC) for the detection by microscopy and flow cytometry (253). In short, 20 µl FITC (Sigma-Aldrich, Diegem, Belgium) dissolved in 5 mg/ml dimethyl sulfoxide (DMSO, WAK-chemie Medical, Steinbach, Germany) was added per milligram of protein in labelling buffer containing 0.05 M boric acid (Sigma-Aldrich) and 0.2 M sodium chloride (Fischer Scientific, Merelbeke, Belgium) at pH 9.2 using a 10.000 MWCO (molecular weight cut-off) Amicon Ultra 15 centrifugal filter (Merck, Overijse, Belgium). After 2 hours incubation at room temperature, unbound FITC was removed by gel filtration using an Econo-Pac® 10DG Column (Bio-Rad). Finally, the protein concentration was measured with a SmartSpec Plus Spectrophotometer (Bio-Rad, California, USA) at 280 nm and 492 nm. The second filter at 492 nm was used to correct the protein concentration measured at 280 nm for the strong FITC signal that might interfere:

$$Protein\ concentration = A280nm - (A492nm\ x\ 0.35\%)$$

3.2 Generation of NP-lysate conjugates

Surface carboxylated polystyrene NPs and fluorescent polystyrene NPs (yellow-green Fluoresbrite carboxylate microspheres), both with a size of 50 nm, were bought from Polysciences Inc. (Hirschberg, Germany). To conjugate lysate to the NPs, the latter were sonicated (Bransonic ultrasonic bath 1510, Branson, St. Louis, USA) for at least 15 minutes

and brought into a pre-activation mixture consisting of 50 mM N-hydroxysulfosuccinimide (sulfo-NHS, Thermo Scientific, Merelbeke, Belgium) and 4 mg/mL 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC, Sigma-Aldrich) in a 50 mM 2-(N-morpholino)ethanesulfonic acid sodium salt (MES)-buffer (MP Biomedicals, France) (254). Pre-activated NPs were gel-filtrated (GF) using Zeba Spin Desalting Columns (40K MWCO, Thermo Scientific) to remove the excess of activation molecules (255). Afterwards, lysate was added and incubated for 60 minutes on a rotating wheel. The final NP-lysate conjugates were stored at 4°C before use. Lysate manipulation for initial optimisation of NP-lysate conjugates was performed by filtering lysate using a 0.2 µm syringe filter (Thermo Scientific).

Size of NPs and conjugates was analysed with dynamic light scattering (DLS) using a VASCO particle size analyser DL135 (Cordouan Technologies, France). Two mathematical algorithms were used for size distribution information. (1) Assuming the presence of only one main size with Gaussian distribution, the Cumulant algorithm was only used for size measurements of unconjugated standard NPs. Homogeneous size is accepted when the PDI < 0.1 (polydispersity index). (2) The Mastercurve statistical algorithm starts from the idea a multi-modal, disperse sample is measured and thus represents a more correct size information for NP-lysate conjugates or lysate samples. The presented data for size depict the summary of the intensity distribution.

Using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) conjugation of lysate fragments and NPs was studied. PAGE was used to separate proteins and, for these experiments, free lysate fragments from conjugates. Lanes of NuPAGE 10% Bis-Tris gel (Life Technologies, USA) were loaded with 10 µg lysate or NP-lysate conjugates. Afterwards, a current of 160 V was applied for 45 minutes and Coomassie blue staining (Bio-Rad) was used to visualize the presence of lysate fragments in the gel.

3.3 DC generation and culturing

Bone marrow derived DCs were obtained by isolation of femur and tibia from 8 to 10 weeks old female C57BL/6J mice (Envigo, Horst, The Netherlands) based on literature (256-258). Bone marrow was flushed using PBS followed by erythrocyte lysis using an ammonium buffer consisting of 155 mM ammonium chloride (NH_4Cl; MP Biomedicals, Germany), 10 mM potassium bicarbonate (KHCO3; Merck) and 0.5 M EDTA (ethylenediaminetertraacetic acid; Sigma-Aldrich) in sterile water. Finally, progenitor cells were counted using an automatic cell counter (Micros 60, Horiba ABX, France) and cultured at 10^6 cells per ml DC-medium consisting of RPMI-1640 medium (Lonza) supplemented with 10% FCS, pen/strep, L-Glu, 50 µM β-mercaptoethanol (Sigma-Aldrich) and 20 ng/ml GM-CSF (Peprotech, USA). At days 3 and 5 medium was refreshed and at days 6 and 7 the cells were prepared for flow cytometry or microscopy. On day 7 immature DCs (iDCs) could be collected to develop mature DCs using 100 µg glioma lysate per 10^6 cells in the above medium supplemented with

1 µg/ml *Escherichia coli* LPS (Sigma-Aldrich). To check for maturation, maturation markers of DCs were stained (**Table 5**). Flow cytometry was performed using a FACSort flow cytometer or a LSR Fortessa Analyzer (BD Biosciences, Erembodegem, Belgium) and data analysis was performed using respectively BD Cellquest software (BD Biosciences) or FlowJo software (Tree Star, Ashland, OR, US).

3.3.1 Analysis of lysate uptake by dendritic cells and presentation in MHC class I molecules

DCs at day six of culture (iDCs) were seeded on glass coverslips in 6-well plates for 24 hours and incubated with FITC-labelled lysate for 90 minutes. Afterwards, cells were washed extensively, followed by fixation and nucleus staining with DAPI (Sigma-Aldrich). To study uptake, surface FITC quenching with trypan blue was conducted using 10 min incubation in 0.1% (w/v) trypan blue solution (259). In order to show antigen presentation by MHC class I molecules, cells were washed after the 90 min incubation and matured during 24 hours. Cells were stained with PE-anti-MHC-I for 30 minutes before fixation and nucleus staining (253). Finally, the coverslips were placed on microscope slides and an Olympus BX41 Fluorescence Microscope (USA) and LSM 510 meta confocal microscope (Zeiss, Germany) were used to examine the samples. Multiphoton detection of DAPI staining was performed to avoid photobleaching of the fluorescent signal.

3.3.2 MTT assay

DCs at day six of culture (iDCs) were plated in 96-well plates at a density of 80.000 cells per well. After 24 hours incubation at 37°C, polystyrene NPs or NP-lysate conjugates were loaded onto the cells. Each condition was tested in six fold and visually checked with a light microscope before starting MTT staining. Incubation of DCs with NPs or NP-lysate conjugates for 1, 2, 4, 24 or 48 hours was followed by replacement of culture medium with 100 µl of a 0.5 mg/ml MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich). After 2 hours incubation at 37°C, the MTT solution was removed and replaced by 100 µl of pure DMSO. After 15 minutes of shaking, the optical density at 570 nm and 620 nm, the latter as a reference wavelength, was measured using an ELISA reader (Thermo Labsystems, Franklin, US).

3.4 Orthotopic glioma mouse models

Female C57BL/6J mice were treated prior to (pre-tumor treatment) or shortly after (therapeutic treatment) tumor inoculation. Unless stated otherwise, all experiments were performed with 100 µg lysate of a 1 mg/ml concentration. For all *in vivo* experiments freshly prepared lysate or NP-lysate conjugates, called nanovaccine in these *in vivo* experiments, were used and all injections were administered in a total volume of 100 µl except for the adjuvant co-injection experiment where 150 µl was injected. Subcutaneous injection was followed by daily to once-another day inspection of the injection site for one week, checking

for signs of irritation/inflammation. For tumor inoculation, mice were intracranially injected with 5 x 10^5 glioma cells in a volume of 10 µl (260). Briefly, mice were anaesthetized and fixed in a stereotactic frame. Tumor cells were injected in the right hemisphere, 2 mm posterior and lateral from the bregma at 3 mm depth. Afterwards, follow-up of the mice was performed two to five times a week, depending on the severity of illness, by weight and scoring based on a neurological deficit scale adapted by Maes *et al.* (260). Mice surviving longer than three times the median survival of mock treated animals were considered long-term survivors. All animal experiments were approved by the bioethics committee of the KU Leuven and conducted according to international guidelines.

a) Pre-tumor treatment

Mice of 8 to 10 weeks old were intraperitoneally or subcutaneously (in the abdominal wall, near the inguinal lymph nodes) injected 14 and 7 days before tumor inoculation. Treatment modalities consisted of PBS-treated (mock) controls, DC vaccination and lysate treatment or PBS-treated controls, NP controls, lysate controls and nanovaccine treatment. The same amount of 100 µg lysate was used in all treatment conditions, dissolved in a total volume of 100 µl. Nanovaccine consisted of 1 mg/ml lysate conjugated to 5 mg/ml NPs. Mock-treated animals received an equal volume of PBS and NP controls an equal amount of NPs as in the nanovaccine condition. For DC vaccination, DCs were *ex vivo* grown from bone marrow as described above. At day 7 immature DCs were loaded with 100 µg of lysate per 10^6 DCs, matured with LPS and used for treatment 24 hours later. At day 0, mice received intracranial tumor inoculation as described above. In a group of long term surviving mice, tumor inoculation was repeated at the contralateral (left) hemisphere, without any additional treatment. Naïve mice, older than 20 weeks, were used as an age-matched control condition. In another set of experiments, tumor specificity was tested using a Lewis lung carcinoma (LLC) cell line, obtained from Prof. Patrizia Agostinis (KU Leuven, Belgium). Therefore long-term surviving animals and age-matched controls were inoculated subcutaneously with LLC cells or intracranially with glioma cells.

b) Nanovaccine pre-tumor treatment combined with adjuvants

In the explorative experiment using nanovaccine-adjuvant co-injection, different TLR ligands were injected concomitantly with the nanovaccine therapy. Instead of 100 µl volume, the injections consisted of an extra volume of 50 µl containing 50 µg of either R848, Poly(I:C) or CpG ODN (all InvivoGen, California, USA). Only minutes before administration to the animals, nanovaccine and TLR ligands were joined in a treatment cocktail consisting of 100 µg lysate, conjugated to NPs, and 50 µg of free TLR ligand.

c) Pre-tumoral peptide (nanovaccine) treatment

Finally, an explorative survival experiment was performed through pre-treatment of mice with a peptide mixture of synthetic peptides. Glycoprotein 100 (gp100), tyrosinase-related protein

2 (TRP-2) and Ephrin A2 (EphA2) are three known onco-proteins for the GL261 cell line (250, 261). Of these three onco-proteins the synthetic CD8 immunodominant epitopes were ordered from LifeTein (New Jersey, USA): $gp100_{25\text{-}33}$ KVPRNQDWL, $TRP\text{-}2_{180\text{-}188}$ SVYDFFVWL and $EphA2_{682\text{-}689}$ VVSKYKPM. Mice were pre-treated with 100 µg of a peptide mixture containing an equal amount of 33 µg from each synthetic peptide according to the same vaccination protocol as introduced in the pre-tumoral treatment protocol. Control groups of PBS treated animals and DC vaccination using *ex vivo* generated DCs loaded with the same peptide mixture (100 µg). Another experimental treatment was obtained by conjugating the peptide mixture to polystyrene NPs to generate a peptide-nanovaccine. An extra step in the conjugation protocol was to remove the unbound peptides by dialysis in PBS using a 10 kDa MWCO Slide-A-Lyzer® G2 dialysis cassette (Thermo Scientific).

d) Therapeutic treatment

In these survival experiments lysate treatment was combined with temozolomide (TMZ) administration or PD-1 blockade. The combination with TMZ chemotherapy was performed for both lysate and nanovaccine treatment, while PD-1 immune checkpoint blockade was only executed in combination with lysate treatment. Tumor inoculation was performed as described above and followed by combinatorial treatment. (a) TMZ was prepared as described previously (262). In brief, the content of Temodal® capsules (MSD, Hertfordshire, UK) together with an equal amount of L-Histidine was dissolved in a phosphate buffer and administered in a total volume of 200 µl by oral gavage (Schering-Plough, Belgium). Prior to treatment, mice were randomised over the different groups. Mice received 20 mg/kg bodyweight as a low dose or 40 mg/kg bodyweight as an intermediate dose. TMZ was administered at days 5, 7, 9, 12, 14 and 16 and subcutaneous lysate or nanovaccine injections at day 21, 28 and 35. (b) Anti-PD-1 mAbs (RMP1-14) and isotype controls (rat IgG) were obtained from Eperius Biopharmaceuticals (Utrecht, the Netherlands). Antibodies were dissolved in saline and injected intraperitoneally at day 7 and 12 following tumor inoculation (180). PD-1 blockade was combined with early lysate treatment performed at day 2 and 9.

3.5 Brain immune cell isolation and characterisation

Mononuclear cells in the brain of pre-treated, CT2A-inoculated mice were isolated 7, 14, 21 or 28 days post tumor inoculation as previously described (260). Briefly, isolated brains were digested in Collagenase D (Roche, Anderlecht, Belgium) and DNase (Invitrogen), and filtered prior to Percoll gradient centrifugation (Sigma). After recovering the mononuclear cell interphase, assessment of the immune cells was done by flow cytometry. Therefore surface staining for T cell markers and MDSC (myeloid derived suppressor cell) markers was performed following zombie yellow viability staining (**Table 5**). Foxp3-PE staining kit (eBioscience, Vienna, Austria) was used according to the manufacturer's protocol to detect intracellular Foxp3. For the intracellular IFN-γ staining, cells were stimulated for 3 hours *in vitro* with 100 ng/mL phorbol myristate acetate, 1 mg/mL ionomycin and 0.7 mg/mL

monensin (all from Sigma-Aldrich). After viability and surface staining, cells were permeabilized with a buffer containing 0.5% saponin and 0.5% bovine serum albumin (both from Sigma-Aldrich) prior to IFN-γ staining.

Systemic immune response was checked by isolating spleen, inguinal and axillary lymph nodes. Spleen cells and lymph node cells were processed separately. Cells were passed through a 70 µm cell strainer (Greiner Bio-One, Frickenhausen, Germany) and single cells were stained as described for the brain immune cells.

3.6 Lymph node biodistribution of lysate, NPs and nanovaccine

Fluorescent polystyrene YG-NPs (yellow-green Fluoresbright carboxylated polystyrene NPs, Polysciences) as well as FITC-labelled lysate were used to study biodistribution of NPs, lysate and NP-lysate conjugates. Non-fluorescent NP injection was used as a negative control condition. Mice were sacrificed 12, 16 or 24 hours following injection. Inguinal, axillary, cervical, auricular and mesenteric lymph nodes were isolated and processed separately (with the exception of 'distant' cervical and auricular lymph nodes, which were pooled). Lymph node cells were obtained through lymph node smashing and filtering over a cell strainer. Obtained cells were surface stained with antibodies described in **Table 5** and studied by flow cytometry.

3.7 Functional testing – systemic T cell proliferation

At day 14 following tumor inoculation of pre-treated mice, the spleen was isolated and a single cell suspension of splenocytes was generated as described before. Single cells were cultured in T cell medium consisting of RPMI-1640 medium with 10% FCS, pen/strep, L-Glu, 50 µM β-mercaptoethanol and supplemented with non-essential amino acids and pyruvate. CFSE stock solution was prepared according to the manufacturer's instructions and used at a final concentration of 50 µM CFSE solution for 10^6 cells (CellTrace™ CFSE cell proliferation kit for flow cytometry, Invitrogen). Cells were incubated with CFSE during 10 min at 37°C while regular shaking. Afterwards, CFSE labelled splenocytes were stimulated with phytohaemagglutinin (PHA) or mDCs *ex vivo* loaded with tumor lysate at a responder:stimulator ratio of 10:1. After 5 days of co-culture, cells were surface stained for T cell markers (CD45, CD3, CD4 and CD8) and flow cytometry was performed.

3.8 Spleen, bone marrow, skin cell isolation

To make sure lysate or nanovaccine treatment in combination with TMZ chemotherapy is plausible, the potential negative influence of TMZ treatment on systemic T cells and skin DCs was checked. Spleen cells and bone marrow were obtained as described above. For the isolation of skin cells, a 2 cm^2 piece of skin from the abdominal wall, where vaccination of lysate or nanovaccine would take place, was removed and processed as described in literature

(263). This skin piece was incubated in cell medium containing 2.5 mg/ml dispase (Sigma-Aldrich) for 90 minutes to separate dermis from epidermis. Next dermis and epidermis were divided with a forceps, further digested in Collagenase D (Roche) and DNase (Invitrogen) for 2 hours at 37°C and filtered with a 70 µm cell strainer (Greiner Bio-One). Immune cell assessment was performed by surface staining of T cell and myeloid cell populations followed by flow cytometry (**Table 5**). Tests were performed for tumor-free untreated animals, glioma-bearing untreated animals, tumor-free TMZ treated animals and glioma-bearing TMZ treated animals. TMZ treatment with 20 mg/kg bodyweight was performed at days 4, 6, 8 and 11 and all animals were sacrificed at day 14 following contingent tumor inoculation.

3.9 Statistical analysis

Two-way analysis of variance (ANOVA) testing and non-parametric Kruskal-Wallis one-way ANOVA with Bonferroni correction were used for comparing multiple groups. The non-parametric Mann-Whitney U test was used to compare two groups. Due to skewness of the data, they are represented as median (± interquartile range). Survival analyses were performed using the Log-rank (Mantel-Cox) test. All data were analysed using Graphpad Prism software 6 (Graphpad Software, San Diego, USA) and statistical significance was considered if $p < 0.05$.

Table 5. Antibodies for flow cytometry.

Antigen	Fluorochrome	Company
Surface stainings – DC maturation		
CD11c	APC	eBioscience
CD80	PE	eBioscience
CD86	PE	BD
CD40	PE	BD
H2Kb (MHC-I)	PE	BD
IA/IE (MHC-II)	PE	BD
Surface stainings – Brain immune cells (T cell populations)		
CD45	AF700	eBioscience
CD3	FITC / PE	eBioscience
CD4	PerCP Cy5.5 / eF780	eBioscience
CD8	BV421	BD
NKp46	APC	Biolegend
Intracellular stainings – Brain immune cells (T cell populations)		
Foxp3	PE	eBioscience
IFN-γ	PerCP Cy5.5	BD
Surface stainings – Brain immune cells (MDSC populations)		
CD45	AF700	eBioscience
CD11b	BV421	BD
MHC-II	PerCP Cy5.5	Biolegend
Ly6C	AF647	Serotec
Ly6G	FITC	BD
Surface stainings – Biodistribution experiment		
CD45	BV650	Biolegend
CD11b	BV605	Biolegend
CD11c	PE-Cy7	eBioscience
IA/IE	PE	BD
PDCA-1	APC	eBioscience
B220	PerCP-eF710	eBioscience
CD8	BV421	BD
Viability marker	Zombie Yellow	Biolegend
Viability marker	Zombie NIR (near infrared)	Biolegend

4 Results

4.1 Immunotherapy with subcutaneous immunogenic autologous tumor lysate increases murine glioblastoma survival

Unfortunately, the future perspectives for patients with GBM remain dismal with a median overall survival of less than 15 months following intensive treatment. Therefore, adjuvant treatment strategies are being pursued such as DC-based active immunotherapy with either *ex vivo* grown DCs or *in vivo* targeting of these cells. The latter condition might be preferred because of efficiency, variable response rates of *ex vivo* grown DCs plus limited migration to lymphoid tissue, as well as practical and economic reasons (time-consuming, labour-intensive process requiring sterile handling leading to an expensive vaccine).

In the treatment of GBM, only a few research papers have been published describing the use of naked tumor lysate. In all of these publications the treatment exists of autologous or allogenic tumor lysate in combination with an adjuvant such as cytokines (GM-CSF) or TLR-agonists (CpG). Another way to improve the efficacy of tumor lysate vaccination might be to increase the immunogenicity of this lysate. Within our group preclinical optimisation of DC immunotherapy has been studied by generating different formulations of tumor lysate. All these formulations had different immunogenicity, which turned out to be very important for DC activation and maturation. Changing the protocol to generate lysate will change its immunogenicity.

Based on the knowledge our research group obtained concerning the immunogenicity of our lysate product, we investigated the potential effect and immune mechanisms of autologous immunogenic tumor lysate, without the addition of DCs or adjuvants, to treat GBM in a preclinical mouse model.

4.1.1 Uptake of lysate and presentation of lysate fragments by DCs *in vitro*

The first goal of the study was to investigate the internalisation of tumor lysate fragments by murine DCs *in vitro*, followed by the activation of these cells and antigen presentation in MHC context (preferably MHC class I). As described earlier in literature for lysate uptake by human monocyte-derived DCs (253), FITC-labelled lysate was generated and added to *in vitro* DCs. A first indication of internalisation was obtained by visualisation of FITC positive DCs using 2 independent techniques being flow cytometry and fluorescence microscopy. Incubation for 90 minutes of lysate-FITC complexes and DCs, resulted in the presence of fluorescently labelled cells (**Fig. 6**). Even as early as 30 minutes after combining tumor lysate fragments and DCs, lysate was localized at the surface of the cells (data not shown). However, we could not discriminate between actual uptake of lysate and surface adhesion to DC membranes till the latter event was excluded. Incubating FITC loaded DCs in a 0.1% trypan blue solution enabled quenching of the surface FITC signal providing first evidence of actual internalisation of tumor lysate fragments by DCs *in vitro* (**Fig. 7A**). Confirmation of this observation was obtained by confocal microscopy, which allows three dimensional fluorescence imaging (**Fig. 7C**). Moving through the optical slices along the Z-axis showed a positive intracellular fluorescent signal diffusely spread over the cytoplasm of the DCs. Moreover, to examine whether the internalisation by DCs was an active physiologic process, loading of DCs with FITC labelled lysate was performed at 37°C and 4°C, a temperature at which all cell metabolism is shut down (253). The absence of any FITC signal in the 4°C condition suggested the uptake mechanism of lysate fragments to be an active mechanism (**Fig. 7A,B**).

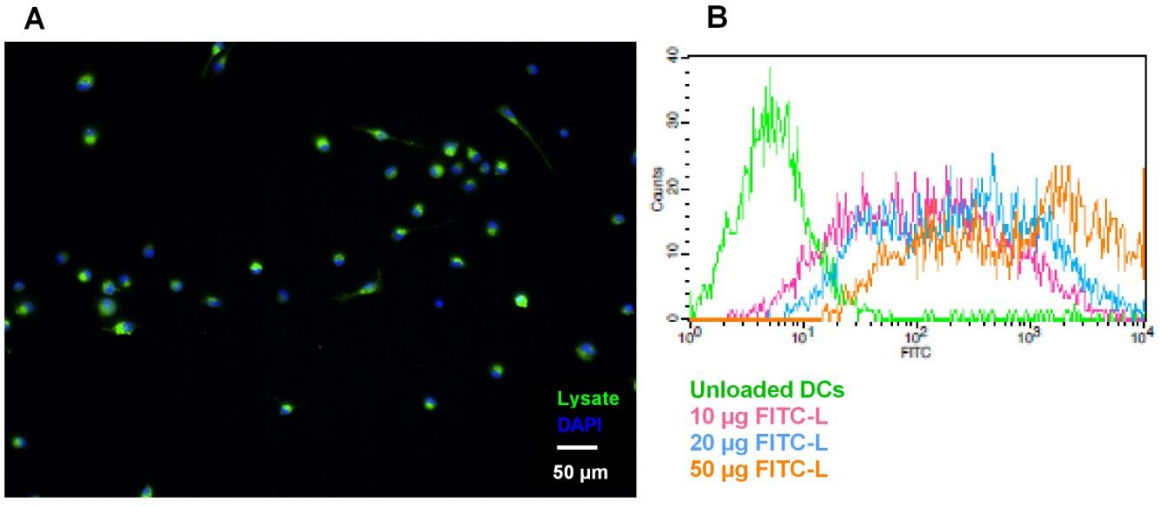


Figure 6: Uptake or surface adhesion of lysate fragments by dendritic cells *in vitro*. *In vitro* differentiated DCs were incubated with FITC labelled lysate for 90 minutes, afterwards washed and stained with DAPI nucleus stain for fluorescence microscopy **(A)** or prepared for flow cytometry **(B)**. In the flow cytometry experiment different concentrations of FITC labelled lysate were loaded to the DCs. Fluorescence image was visualized with a 200x magnification.

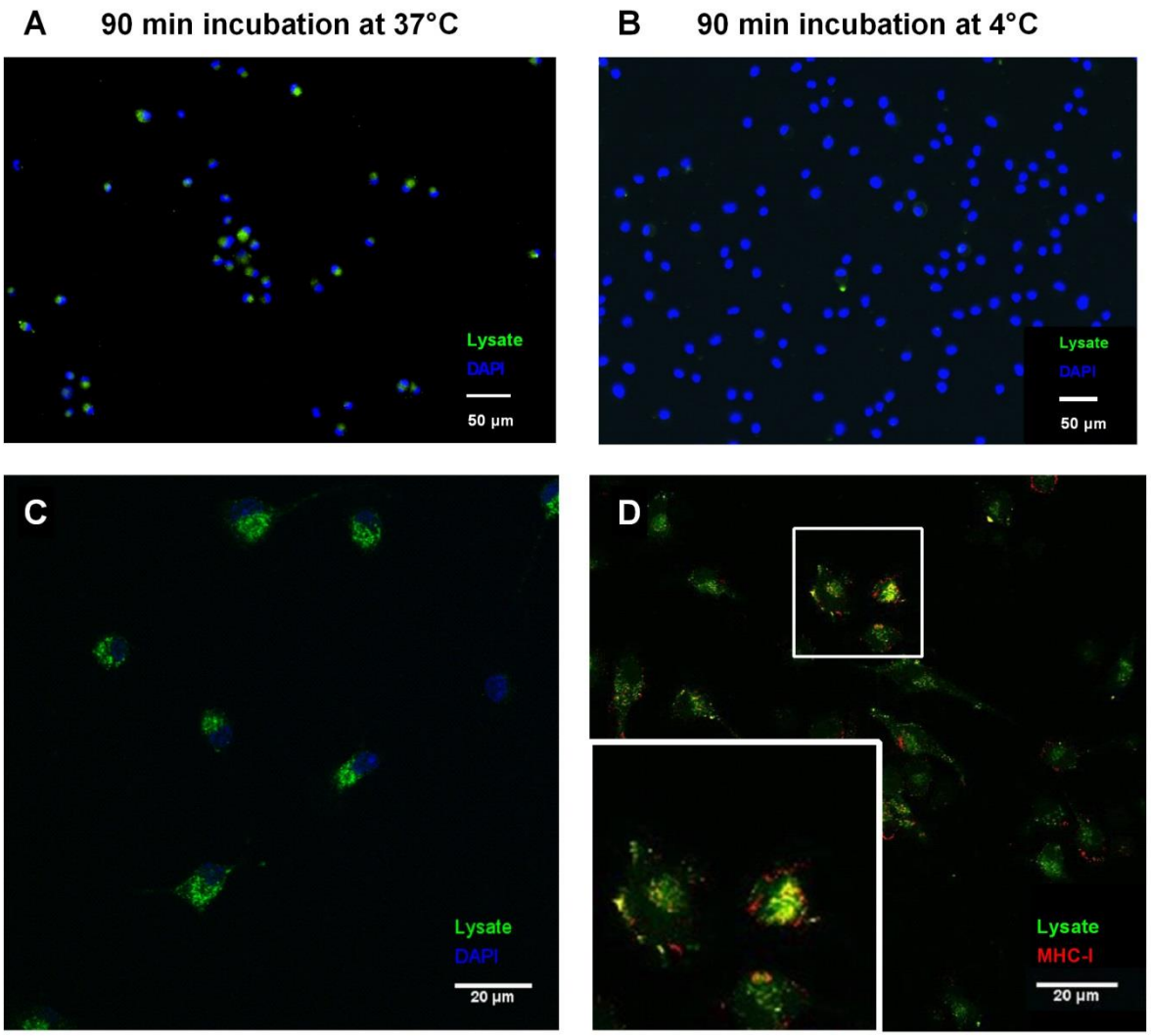


Figure 7: Uptake and cross-presentation of lysate fragments by dendritic cells *in vitro* is an active process. *In vitro* differentiated DCs were incubated with FITC labelled lysate for 90 minutes, afterwards washed and stained in the uptake experiments **(A-C)** or matured with LPS in the cross-presentation experiments **(D)**. For the uptake experiments **(A-C)**, trypan blue quenching of surface FITC signal (green) was used. **(A),(B)** To show uptake is an active process, incubation at 4°C was performed in comparison to 37°C. At 4°C no active, ATP-requiring processes take place. **(C)** Z-stack images, obtained with confocal microscopy, were used to visualize DCs and prove real uptake of lysate. Picture **(C)** represents one slice of the confocal z-stack with DAPI nucleus staining (blue) and FITC signal (green). **(D)** Maturation of DCs was ended after 24 hours by washing cells, fixation and staining. **(D)** Confocal microscopy showed colocalization (yellow signal) of fragments of lysate (FITC, green signal) and MHC class I molecules (PE, red signal). The box at the bottom is a magnification of the marked cells. Images were visualized with a 200x magnification for **(A-B)** and a 400x magnification for **(C-D)** and all pictures are representative of at least three independent experiments.

For the completion of the *in vitro* study we aimed to show activation of DCs and processing of lysate by DCs, both necessary to create an immune response against tumor cells. Multiphoton confocal microscopy was used to demonstrate the colocalization of FITC-lysate fragments with MHC class I molecules (**Fig. 7D**). Besides, indirect proof of DC activation was shown by the upregulation of different maturation markers and the lymphoid tissue homing receptor CCR7 on the surface of lysate loaded DCs when lysate was presented to the DCs (**Fig. 8**). Together these results indicate that (cross-)presentation of lysate fragments in MHC-I context is possible when lysate is loaded to DCs.

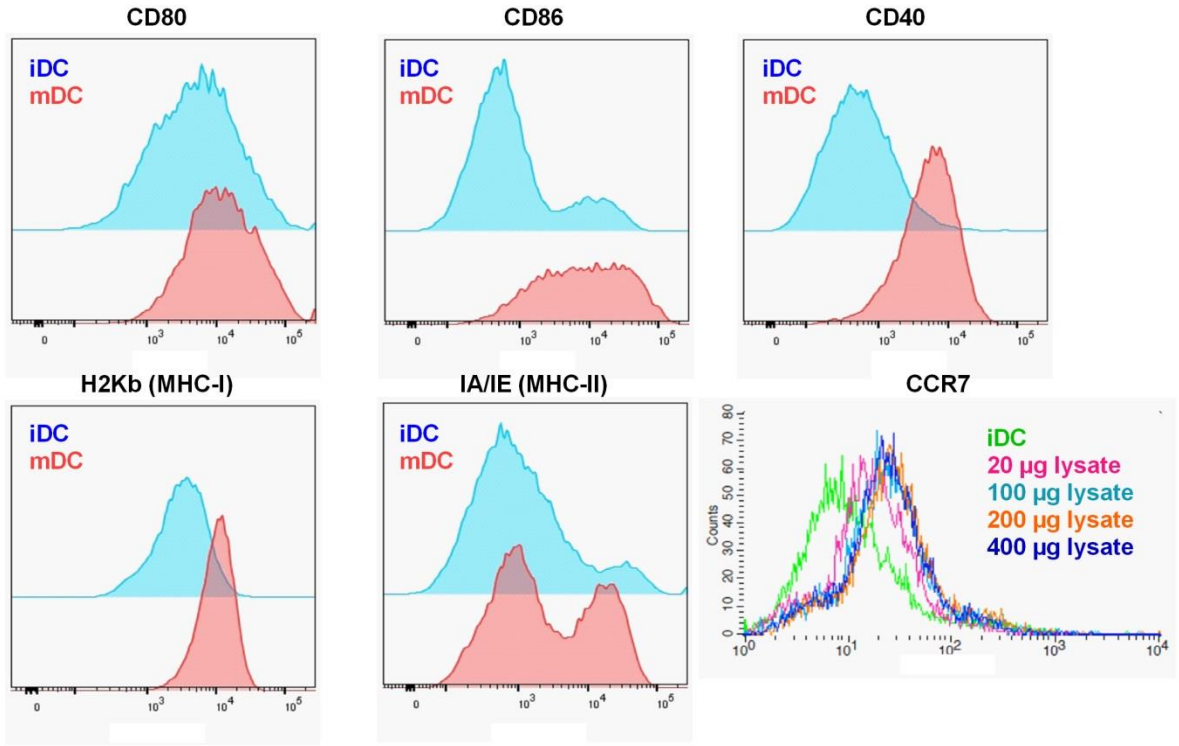


Figure 8: Upregulation of surface maturation markers of lysate-loaded dendritic cells. Flow cytometry was used to study the expression of maturation markers on lysate-loaded DCs. *In vitro* differentiated DCs were incubated with lysate for 90 minutes, washed and after 24 hours incubation, in the presence of LPS, stained. Representative graphs for DC maturation markers CD80, CD86, CD40; for MHC class I and MHC class II molecules and for the lymphoid tissue homing factor CCR7 are shown. Expression on immature DCs is depicted in blue while marker expression by mature DCs in presented in red. For CCR7 expression, different concentrations of lysate were used without major differences in expression. Graphs are representative of at least three independent experiments.

4.1.2 Pre-tumor lysate treatment improves survival in a murine glioma model

Next to the *in vitro* confirmation of lysate internalisation and processing by DCs, we wanted to provide evidence for (pre)clinical activity of autologous lysate. The eminently preclinical model used to test immunotherapeutic treatment in GBM is the GL261 glioma model. Our

research group has already been using this fully immunocompetent, orthotopic murine model for over a decade (245, 248, 264). Here we aimed to compare tumor growth in the presence or absence of lysate vaccination. Due to the aggressive nature of GL261 glioma, a prophylactic immunisation schedule was executed administering tumor lysate 14 and 7 days before tumor inoculation (**Fig. 9A**). To begin, the vaccinations were conducted intraperitoneally, which is in line with the *in vivo* experience generated in our group for DC immunotherapy. Next to an increased median survival, 25% of the lysate-treated mice were defined long term survivors as compared to the mock treated group. However, as we hypothesize DCs being the most important APC to induce an immune response following lysate immunisations and these cells being present in the skin to a relative larger extent than in the peritoneal cavity, the subcutaneous injection route was studied next (265). Similar results as shown by intraperitoneal treatment were observed when mice were injected subcutaneously, resulting in an increase in median survival as well as a certain percentage of long term survivors in comparison to mock treated animals (**Fig. 9B**). Of note, a third treatment condition group, being subcutaneous treatment with *ex vivo* lysate-loaded DCs, showed no difference as compared to lysate treatment.

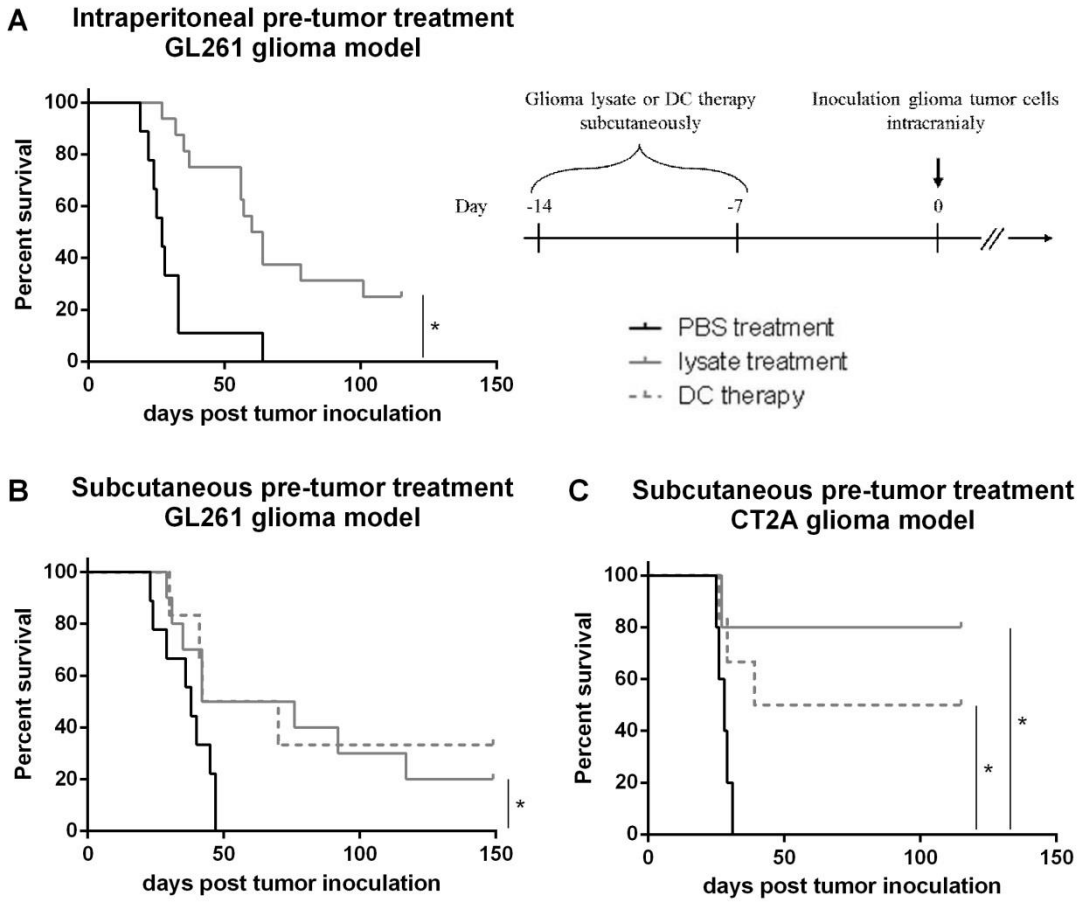


Figure 9: Pre-tumor lysate treatment results in similar survival improvement as compared to dendritic cell therapy in 2 glioma mouse models. Mice were treated with autologous lysate 14 and 7 days before tumor inoculation with 5 x 10^5 glioma cells. In the GL261 glioma model both **(A)** intraperitoneal and **(B)** subcutaneous injection of lysate (n=16 and n=10 respectively) were studied in comparison to PBS treatment (n=9). **(C)** For the CT2A glioma model only subcutaneous lysate treatment was tested. Moreover, in the two bottom graphs (subcutaneous injection), dendritic cell therapy (n=6) was performed and applied as a literature based control condition. For graphs **(A)** and **(B)** data of two independent experiments were pooled. Statistical significance was calculated by Log-rank test, * $p < 0.05$

We then studied if the above *in vivo* results could be confirmed in a second mouse model. The CT2A cell line is predominantly used to study glioma stem cells, but due to several GBM features as there are the high mitotic index, the cell density and the strong infiltrative nature of the tumor cells, we ought this cell line also to be able to generate an intracranial glioma model in fully immunocompetent mice (245, 252). Retaining the pre-tumor treatment described above for the GL261 model, increased median survival in the CT2A model was accompanied with 80% of lysate-treated mice surviving tumor inoculation over 120 days (**Fig. 9C**). Although the DC therapy using *ex vivo* grown cells also improved survival of mice, it seemed less effective as lysate treatment in this experiment. Combining the survival results in both mouse models clearly indicates the potential of autologous lysate therapy.

Wondering if a more universal immunotherapy treatment can become a possibility, some research groups started using lysate of multiple HLA-matching tumor cell lines (266). With that idea in mind and the presence of two different glioma cell lines, this hypothesis was tested to a certain extent. In contrast to literature, where allogenic therapy is described, our observations in the GL261 and CT2A glioma cell lines was limited to syngeneic, non-autologous treatment. Although arising from different cells, both cell lines remain syngeneic for C57BL/6 mice (245). Therefore, pre-treatment of mice with tumor lysate from one cell line was followed by tumor inoculation with tumor cells of the other cell line. This resulted in partial response, where the effect of treatment was shown to be effective in one combination, while the other treatment arm took limited advantage out of the non-autologous therapy (**Fig. 10**). On the one hand, syngeneic CT2A lysate immunisations resulted in increased survival of GL261 glioma cell inoculated animals, with three out of four mice surviving more than 150 days. On the other hand, CT2A inoculated mice showed far less benefit from pre-tumor GL261 lysate treatment with only one mouse surviving tumor inoculation over 100 days and the absence of statistically improved survival. Showing one-way cross-reactivity, these results point out the potential of non-autologous, HLA-matching lysate treatment as well, though with the major drawback being that not all patients would be helped with this kind of treatment.

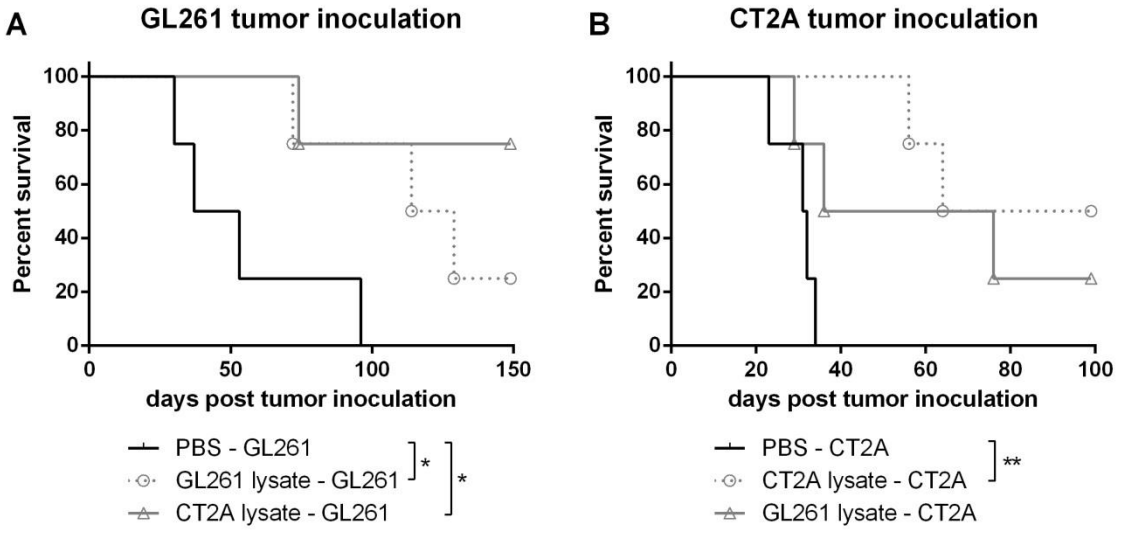


Figure 10: Lysate of CT2A glioma cells is effective in treating mice with an orthotopic tumor inoculation of GL261 glioma cells. Both GL261 and CT2A cell lines are syngeneic cell lines for C57Bl/6J mice. Using one tumor cell line to pre-treat and tumor inoculation with the other cell line, cross-reactivity between both cell lines was investigated. For all conditions four mice per group were used. Statistical significance was calculated by Log-rank test, * $p < 0.05$; ** $p < 0.01$

4.1.3 Early after tumor inoculation, lysate treatment induced a T cell influx combined with diminished immune suppression in the brain

Continuing the work in a more mechanistical way, we continued the research by investigating the immune response induced by lysate treatment. Because of the larger interest in the cellular immune response, brain infiltrating immune cells, with a focus on T cell populations and MDSCs, in glioma-bearing mice were studied. The CT2A model was preferred due to its invasive properties and more resemblance with GBM characteristics (245, 252). Brain immune cells of pre-treated glioma-bearing mice were isolated 7, 14, 21 or 28 days post tumor inoculation. Comparison of the proportions of different T cell populations and MDSCs for mock treated mice and lysate-treated animals was enabled by flow cytometry. The gating strategy used for the population analysis is depicted in Figures 1 to 3 of the "Addendum – flow cytometry information". **Table 5** in the Materials and Methods section summarizes the antibodies used.

As early as day 7 after tumor inoculation an influx of total T cells ($CD3^+$) was observed resulting in an increased proportion of $CD4^+$ T cells and not the $CD8^+$ T cell percentage (**Fig. 11**). While the relative amount of $CD4^+$ T cells expanded, the frequency of $CD4^+Foxp3^+$ Treg cells was declined at this time point. Another immunosuppressive cell population that was relatively decreased at early time points, day 7 and 14, was the monocytic MDSCs defined as $CD11b^+Ly6C^+$ leukocytes. At day 21, but not day 14 nor day 28, a comparable increase in

total T lymphocytes accompanied by a decreased proportion of Treg cells was noticed. Together these results point out that pre-tumor lysate vaccinations shift the cellular immune suppression present in the brain of glioma-bearing mice to one of immune activation (**Fig. 11**).

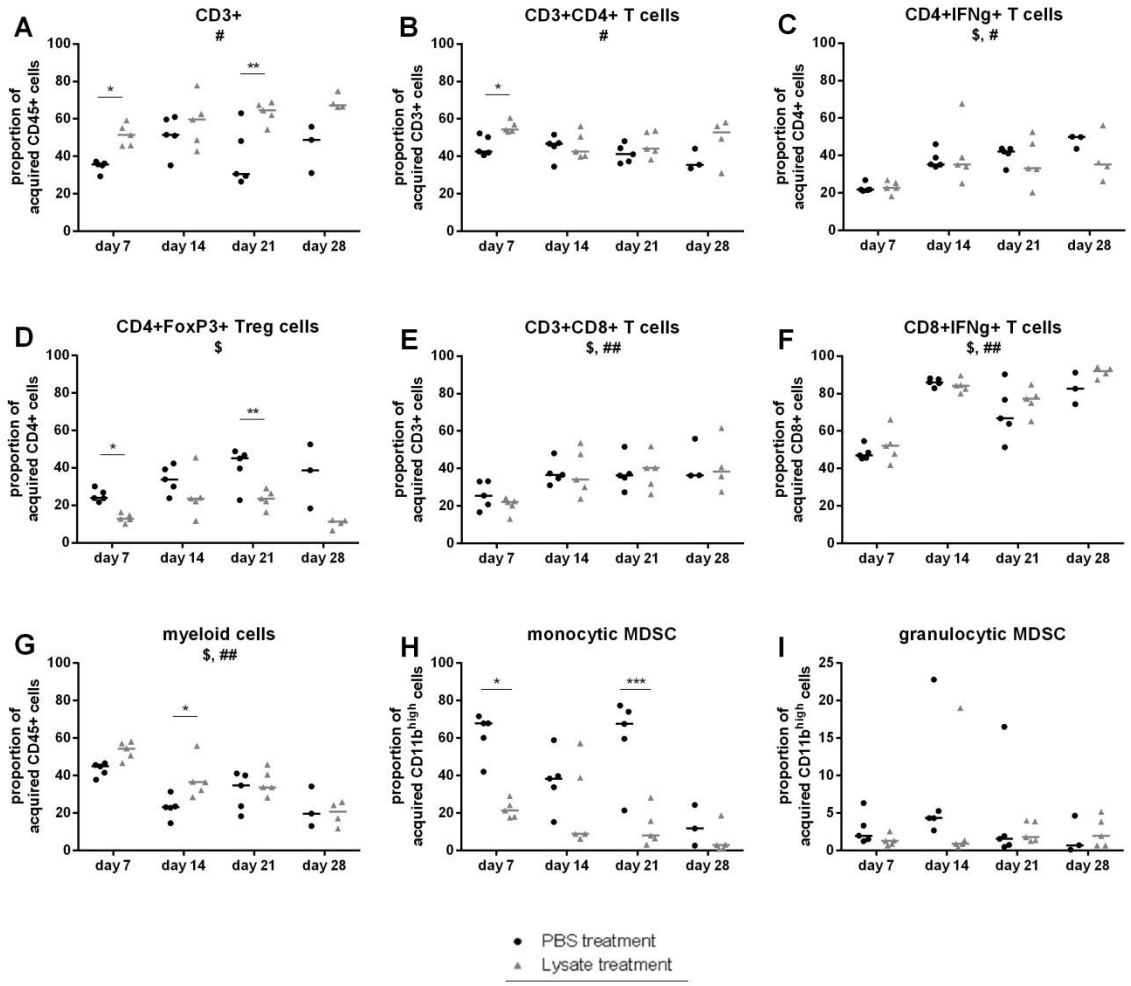


Figure 11: Early after tumor inoculation, lysate treatment induced a T cell influx combined with diminished immune suppression in the brain. Brain immune contexture of pre-tumor treated mice was studied 7, 14, 21 and 28 days after tumor inoculation. Graphs present kinetic analyses of different T cell population proportions and immunosuppressive MDSC type fraction in PBS treated (●) or lysate treated (Δ) mice. Cell populations were defined by different stainings with **(A)** $CD3^+$ lymphocytes as single cells, ZY-, CD45+, CD3+ gated to CD45+; **(B)** $CD4^+$ T cells as single cells, ZY-, CD45+, CD3+, CD4+ gated to CD3+; **(C)** Tregs as single cells, ZY-, CD45+, CD3+, CD4+, FoxP3+ gated to CD4+; **(D)** IFNγ-producing $CD4^+$ T cells as single cells, ZY-, CD45+, CD3+, CD4+, IFNγ+ gated to CD4+; **(E)** $CD8^+$ T cells as single cells, ZY-, CD45+, CD3+,CD8+ gated to CD3+; **(F)** IFNγ-producing $CD8^+$ T cells as single cells, ZY-, CD45+, CD3+,CD8+, IFNγ+ gated to CD8+; (G) myeloid cells as single cells, ZY-, CD45+, CD11b+ gated to CD45+; (H) Monocytic MDSC as single cells, ZY-, CD45+, $CD11b^{high}$, Ly6C+ gated to $CD11b^{high}$; (I) Granulocytic MDSC as single cells, ZY-, CD45+, $CD11b^{high}$, Ly6G+ gated to $CD11b^{high}$. Statistical significance was calculated by two-way ANOVA. Groups of mice consisted of 5 mice except for day 28, when 1 or 2 mice already died due to

glioma growth in the lysate and PBS treated conditions respectively. Significant differences between two treatment conditions, PBS or lysate, are indicated by asterisks: * $p < 0.05$; ** $p < 0.01$. The symbols $ and # below the graph title indicate significant changes over time within the PBS treated and lysate treated populations respectively. $ or # $p < 0.05$; ## $p < 0.01$

Longitudinally, the increased proportion of total T lymphocytes was correlated with the increased relative amount of $CD8^+$ T cells (**Fig. 11**). The observation that this expansion in $CD8^+$ T cells was accompanied by an increase of IFN-γ producing $CD8^+$ T cells over time is of great importance. Albeit this observation applies for both lysate-treated and mock-treated mice, this knowledge contributes to the conclusion that declined immunosuppression and increased immune activation need to go hand in hand to result in a survival benefit. Another observation supporting this conclusion is the reduction in the proportion of $CD11b^+$ myeloid cells over time. Focussing on the changes in $CD4^+$ T cells over time, lysate-treated mice, who showed an early increase in the proportion of $CD4^+$ T cells as compared to PBS treated mice, exhibited a decrease in their frequency starting between day 7 and day 14 (**Fig. 11**). The latter observation points out an important role for $CD4^+$ T cells especially at the earliest time point. Overall it can be concluded that the immune response is driven both by $CD4^+$ and $CD8^+$ T cells, while the proportion of myeloid cells decreases.

Next to the brain immunocontexture, the peripheral immune response was studied for autologous lysate-treated and PBS-treated mice. T cell populations in the spleen and lymph nodes, the latter by pooling the cells from inguinal and axillary lymph nodes, were isolated 21 days after tumor inoculation, stained with the same flow cytometric antibodies as for the brain infiltrating immune cell study and eventually analysed by the same gating strategy (see Addendum). While none of the investigated cell populations showed any difference in the lymph nodes, a decreased proportion of Treg cells was observed amongst the splenocytes of lysate treated animals (**Fig. 12A**). This downregulation of immune-suppression was not accompanied by an increased immune activation as no differences were detected for IFN-γ producing T cells. Therefore it can be concluded that these data suggest the presence of a downregulated systemic immune-suppression by autologous lysate treatment.

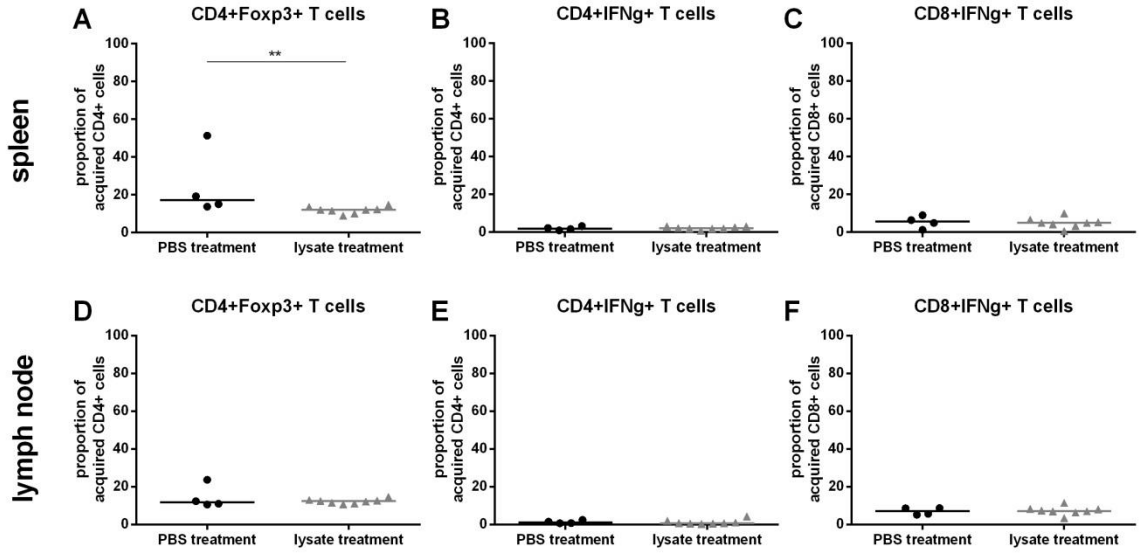
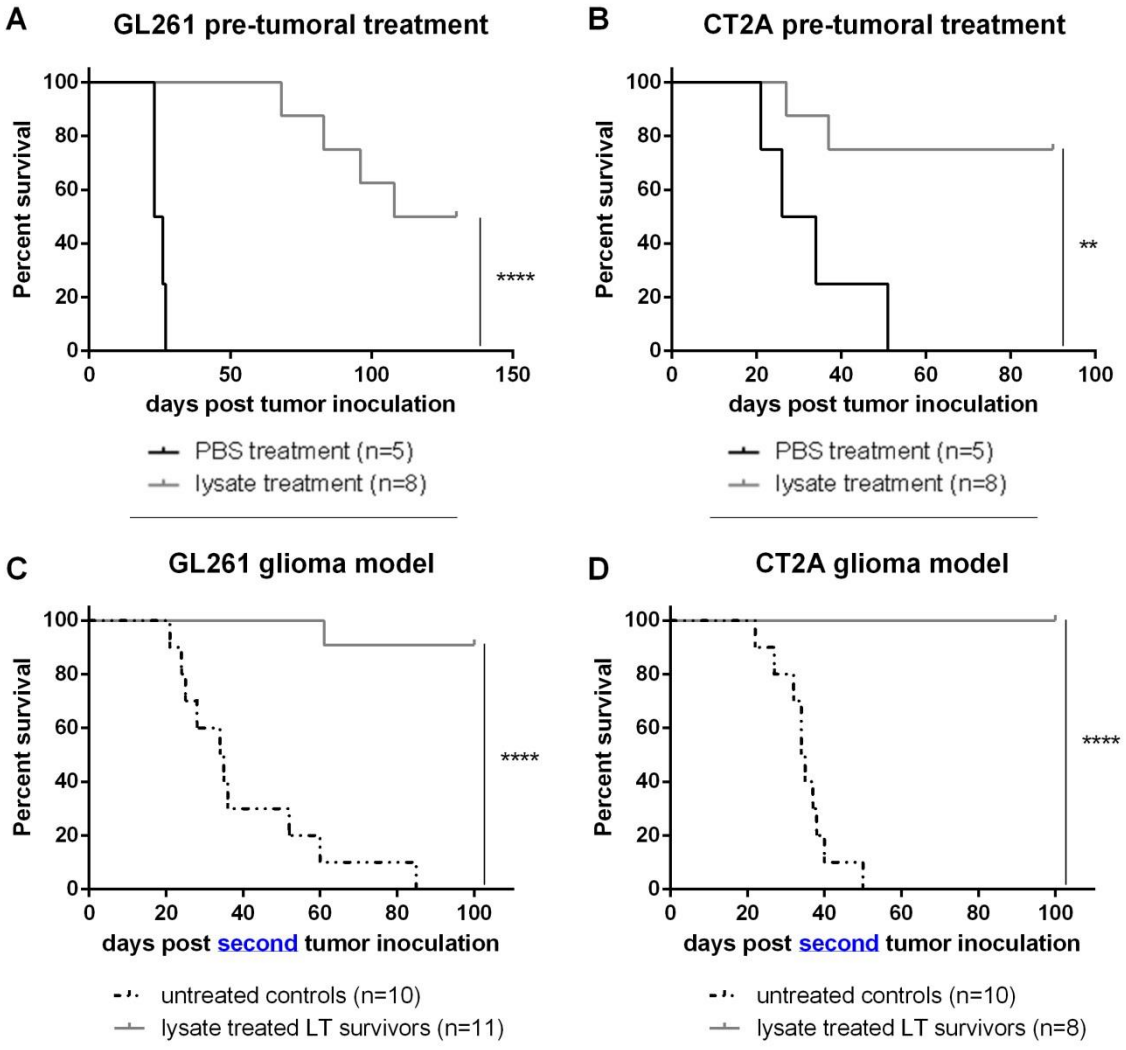


Figure 12: Splenocyte T cell populations suggest downregulation of peripheral immune-suppression by autologous lysate treatment. For pre-tumor treated mice, immune contexture within spleen and draining lymph nodes (inguinal and axillary pooled) was studied 21 days after tumor inoculation. Graphs represent analyses of different T cell population proportions in PBS treated (●) or lysate treated (Δ) mice. Cell populations were defined by different stainings with **(A,D)** Tregs as single cells, CD45+, CD3+, CD4+, FoxP3+ gated to CD4+; **(B,E)** IFNγ-producing $CD4^+$ T cells as single cells, CD45+, CD3+, CD4+, IFNγ+ gated to CD4+; **(C,F)** IFNγ-producing $CD8^+$ T cells as single cells, CD45+, CD3+,CD8+, IFNγ+ gated to CD8+. Statistical significance was calculated by non-parametric Mann-Whitney U test. Groups of mice consisted of 4 mice for PBS treatment and 8 mice for lysate treatment. Significant differences between two treatment conditions, PBS or lysate, are indicated by asterisks: ** $p < 0.01$.

4.1.4 Pre-tumor lysate treatment induces a long-lasting immunological memory

Another characteristic of the adaptive immunity is the presence of memory cells, which can fasten an immune response in case of second exposure or relapsed disease. In search for an indication of an immunologic memory, mice were pre-treated with lysate more than 100 days prior to tumor inoculation. The experiment was conducted in the autologous setting of both GL261 and CT2A glioma models resulting in strong protection from tumor growth (**Fig. 13A,B**). Confirmation of these results was obtained by re-inoculating long term survivors with the same amount of autologous tumor cells at the contralateral side of the brain, without additional treatment. Spreading over the 2 glioma models only 1 out of 19 mice did not survive the second tumor challenge over 100 days again suggesting the presence of a profound immunological memory (**Fig. 13C,D**). Moreover, we were able to show specificity of this immunological (memory) response using subcutaneously injection of Lewis lung carcinoma (LLC) cells. In this study another group of long term surviving mice was inoculated with LLC cells, which resulted in massive tumor growth for all mice injected with

these cells and thus showing specific protection induced by GL261 or CT2A lysate treatment (**Fig. 13, table below**). All of these results demonstrate that pre-tumor lysate treatment gave rise to a tumor-specific memory response, protecting mice for long periods of time and from new tumor inoculation.



E

Tumor incidence in long term survivors and age-matched controls implanted intracranially with glioma cells, or subcutaneously with LLC cells		
	Tumor inoculation age-matched controls	Rechallenge of long term-survivors
GL261	10/10	1/10
LLC	5/5	6/6
CT2A	10/10	0/10
LLC	4/4	3/3

Figure 13: Long-lasting and tumor-specific immunological memory induced by pre-tumor lysate treatment. In graphs **(A)** and **(B)**, mice were treated with lysate > 100 days prior to tumor inoculation with 5 x 10^5 glioma cells of the corresponding cell line. In both glioma models, GL261 and CT2A, lysate treated mice were compared to PBS treated control animals. **(C),(D)** Mice that survived the first

tumor inoculation were rechallenged at the contralateral side of the brain with 5 x 10^5 glioma cells of the same tumor cell line as the first time without new treatment. New untreated controls were used as a control condition. **(E)** Tumor specificity was tested by inoculating other groups of long term survivors with LLC cells subcutaneously. In graphs **(A-D)** data of two independent experiments were pooled. Statistical significance was calculated by Log-rank test, ** $p < 0.01$; **** $p < 0.0001$

4.1.5 Subcutaneous, pre-tumor peptide vaccination results in comparable treatment efficacy as compared to peptide-loaded DC therapy in the GL261 glioma model

The scientific community regarding antigen-specific active immunotherapy has always been divided over two camps: the one group pledges allegiance to the use of whole tumor lysate as an antigen source, while the other group aims for more broadly applicable therapies by using (synthetic) peptides or proteins of known antigens. Wondering if peptide vaccination would also be possible in our murine glioma model, we pre-treated mice with a peptide mixture of synthetic peptides. Glycoprotein 100 (gp100), tyrosinase-related protein 2 (TRP-2) and Ephrin A2 (EphA2) are three known onco-proteins for GL261 cell line (250, 261). Of these three onco-proteins the synthetic CD8 immunodominant epitopes were utilised: $gp100_{25\text{-}33}$ KVPRNQDWL, $TRP\text{-}2_{180\text{-}188}$ SVYDFFVWL and $EphA2_{682\text{-}689}$ VVSKYKPM. A peptide mixture containing an equal amount of each peptide was addressed to test the feasibility of peptide vaccination in the GL261 mouse model. Pre-tumor treatment of mice with *ex vivo* grown DCs loaded with 10 µM peptide mixture per million DCs, resulted in an increased median survival as compared to a mock treated group (**Fig. 14**). Surprisingly, subcutaneous injection of 10 µM peptide mixture without adjuvant was able to induce a similar result. Median survival increased from 24.5 days to 49 days post tumor inoculation together with 20% of mice surviving over 120 days. From this it can be concluded that subcutaneous peptide injection, targeting known onco-proteins, can lead to survival benefit even without the addition of *ex vivo* grown DCs.

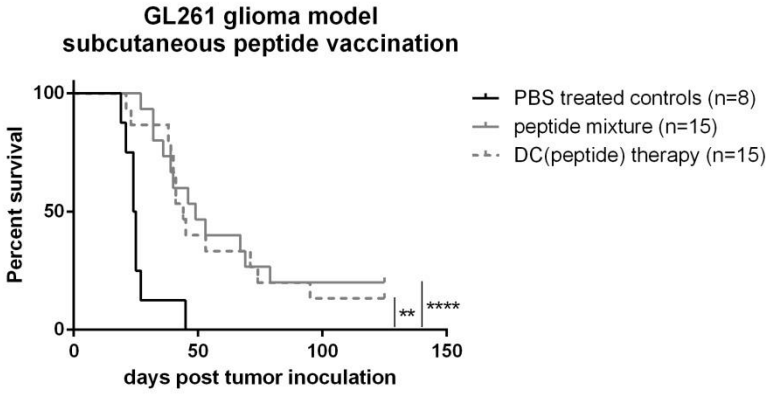


Figure 14: Subcutaneous, pre-tumor peptide vaccination results in comparable treatment efficacy as compared to peptide loaded DC therapy in the GL261 glioma model. Mice were treated 14 and 7 days before tumor inoculation with 5 x 10^5 GL261 cells. Treatment consisted of a mixture of the CD8 immunodominant peptides of 3 different onco-proteins (gp100, TRP-2 and EphA2) injected subcutaneously as such or when loaded to DCs. Statistical significance was calculated by Log-rank test, ** $p < 0.01$; $p < 0.0001$

4.1.6 Combining temozolomide and lysate in a therapeutic treatment model improves median survival of glioma-bearing mice

Because of the relevance for the translation to a human setting, the final set of experiments aimed to develop a therapeutic lysate therapy. Because a preliminary experiment using lysate treatment as a stand-alone therapy at days 3, 7 and 11 post tumor inoculation did not show any survival benefit (**Fig. 15**), we continued by investigating a more clinical relevant situation. Immunotherapy for GBM in clinical setting will often be preceded by a direct anti-tumoral therapy such as chemotherapy. Therefore, we developed a therapeutic treatment strategy combining TMZ chemotherapy and lysate vaccination in the murine GL261 model. To begin, the potential negative myelosuppressive effect of chemotherapy treatment on the immune system was studied. Using low concentrations of TMZ (20 mg/kg bodyweight) resulted in a limited effect on the presence of T cells in spleen and bone marrow and myeloid cell populations in the skin (**Fig. 16**), which is comparable with published data by Litterman *et al.* (267).

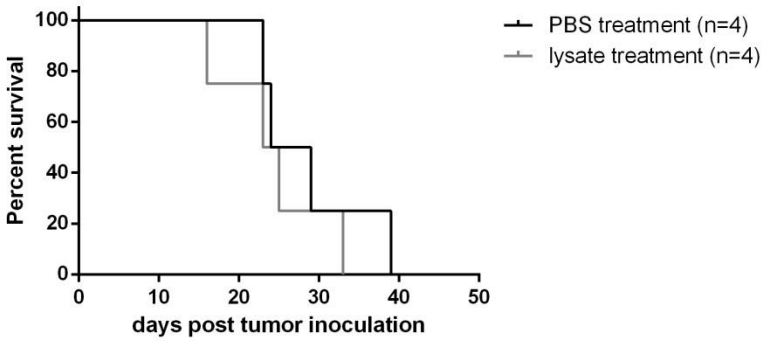


Figure 15: Standalone therapeutic lysate treatment did not result in a survival advantage for glioma-bearing mice. After tumor inoculation with 5 x 10^5 GL261 cells, mice were subcutaneously treated with autologous lysate at days 3, 7 and 11. Injection of lysate was compared to PBS treatment.

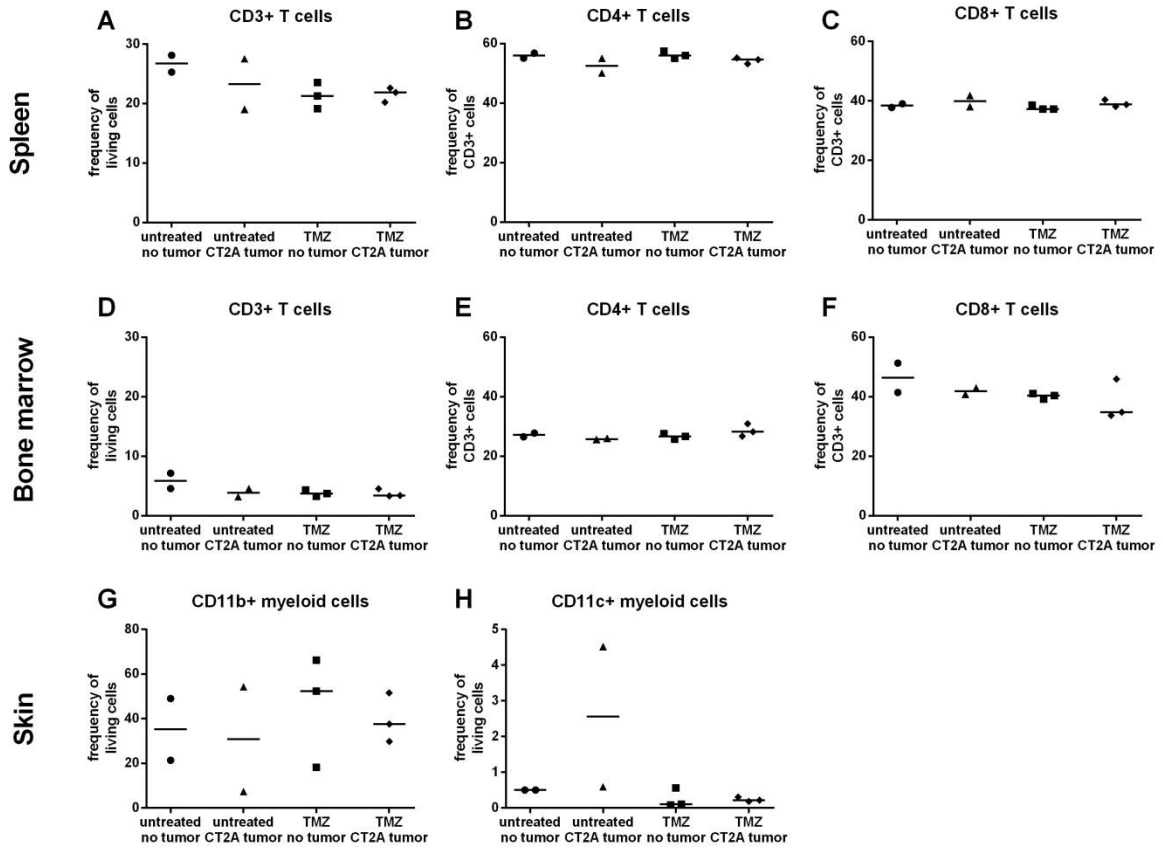


Figure 16: Limited systemic effect of low concentration temozolomide treatment. T cell populations within spleen and bone marrow as well as myeloid cell populations within skin were studied to visualize temozolomide effect on systemic immunity. Half of the mice were inoculated with 5 x 10^5 CT2A cells, the other half was left healthy. Within each group 2 mice were left untreated and 3 mice received 20 mg/kg body weight of temozolomide, orally administered 6 times between day 5 and 16 after tumor inoculation. Cell populations were defined by different stainings with **(A,D)** $CD3^+$ lymphocytes as single cells, ZY-, CD45+, CD3+; **(B,E)** $CD4^+$ T cells as single cells, ZY-, CD45+, CD3+, CD4+; **(C,F)** $CD8^+$ T cells as single cells, ZY-, CD45+, CD3+,CD8+; **(G)** CD11b+ myeloid cells as single cells, ZY-, CD45+, CD11b+; **(H)** CD11c+ myeloid cells as single cells, ZY-, CD45+, CD11c+. Statistical significance was calculated by non-parametric Kruskal Wallis test.

Finally, the combination therapy of TMZ and lysate vaccination in glioma-bearing mice was tested. Following tumor inoculation, mice received six applications of 20 mg/kg bodyweight TMZ between day 5 and 16 and subsequently were injected with autologous lysate at day 21, 28 and 35 (**Fig. 17, scheme**). Although all mice finally died due to tumor growth, combining TMZ and lysate administrations statistically improved median survival. Based on median survival, mice treated with the combination therapy performed 80% better than PBS treated animals and 20% better as compared to TMZ monotherapy (**Fig. 17**). The absence of a 'lysate alone' condition is due to the fact that most PBS treated mice already show disease symptoms at day 21, which would be the first day to treat the mice in the 'lysate alone' group. This idea

is supported by the results of the preliminary experiment described above showing the absence of any survival benefit from early lysate treatment. Collectively, immunotherapy using autologous lysate treatment has potential as an adjuvant therapy next to TMZ administration for glioma.

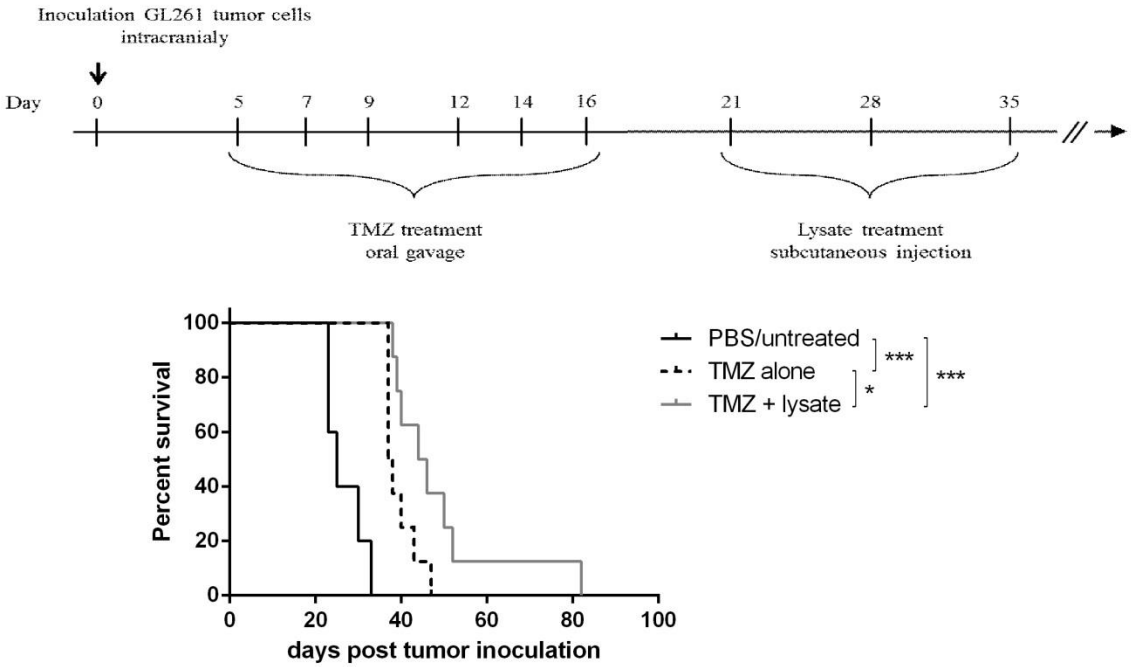


Figure 17: Combining Temozolomide and lysate in a therapeutic treatment improves median survival of glioma-bearing mice. To introduce a therapeutic treatment strategy, lysate injection was combined with chemotherapy (temozolomide). First 20 mg/kg body weight of temozolomide was orally administered 6 times between day 5 and 16, followed by subcutaneous lysate injection at days 21, 28 and 35. The combination of temozolomide and lysate was compared to PBS treated controls and temozolomide monotherapy. One representative experiment out of two; n=5 in the PBS control group and n=8 in both test conditions. Statistical significance was calculated by Log-rank test, * $p < 0.05$; *** $p < 0.001$

On top of the lysate-chemotherapy combination, a complete immunotherapeutic strategy was aimed for by combining autologous lysate treatment with immune checkpoint blockade using anti-PD-1 mAbs. Following tumor inoculation, mice were treated with 100 µg anti-PD-1 mAb at days 7 and 12 together with early autologous lysate vaccination at day 2 and 9 (**Fig. 18 scheme**). Only animals treated with PD-1 blockade in combination with autologous lysate showed a survival benefit as compared to mock treated animals (**Fig. 18**). Unfortunately, anti-PD-1 monotherapy showed to be ineffective within our hands. Therefore, an effective combination of lysate vaccination with anti-PD-1 checkpoint blockade cannot be excluded.

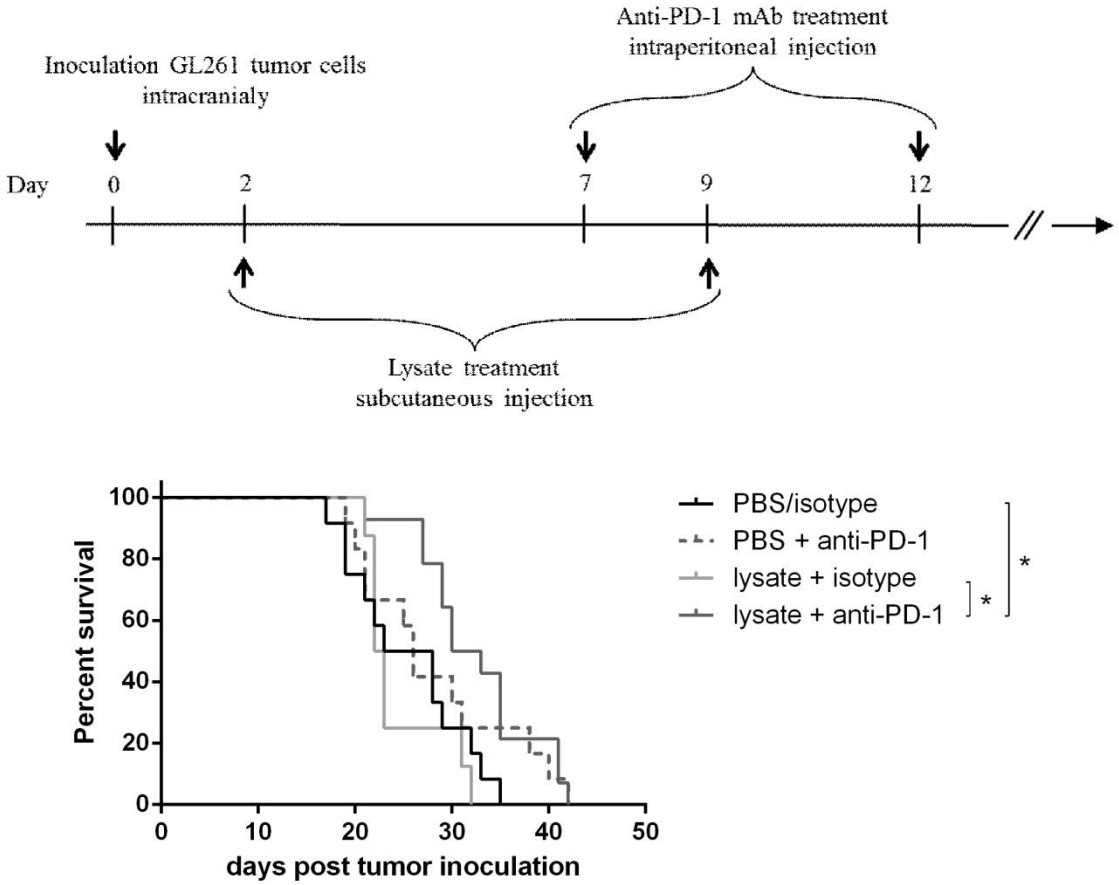


Figure 18: Combining PD-1 blockade and lysate in a therapeutic treatment improves median survival of glioma-bearing mice. Combining lysate treatment and immune checkpoint blockade was executed by early, subcutaneous lysate injection at days 2 and 9 and intraperitoneal administration of 100 µg anti-PD1 mAb at days 7 and 12. The combination of PD-1 blockade and lysate was compared to PBS treated controls, lysate monotherapy and anti-PD1 mAb monotherapy. Data from two independent experiments were pooled; n=12 in the PBS control group, the anti-PD-1 monotherapy group and the combinatorial treatment condition; n=8 in the lysate monotherapy condition. Statistical significance was calculated by Log-rank test, * $p < 0.05$

4.2 Production of whole tumor lysate-loaded nanoparticles as an anti-cancer nanovaccine

In our previous work, we have demonstrated a positive effect of lysate vaccination on the survival of glioma-bearing mice. Influx of T cells in the brain in combination with a reduction in immune suppression were observed and the induced immune response even resulted in a tumor-specific immunological memory. However, targeting *in vivo* DCs is open for improvement and NPs appear to be ideal candidates to achieve this goal. The main advantages for using NPs in active immunotherapy are their variable physicochemical properties, cargo protection from degradation, multicomponent loading with targeting moieties and/or adjuvants and the possibility for an inherent adjuvant function.

In this regard, we aimed for a preclinical proof-of-concept study for using lysate-loaded NPs in the treatment of GBM that can be expanded in future. The work was limited to an easy to obtain polystyrene NP surface-loaded with whole tumor lysate without the addition of targeting molecules nor adjuvants. Passive targeting of these NP-lysate conjugates was aimed for by means of size as DCs have been described to preferably internalise particulate antigens with a size below 500 nm.

With this background in mind, we prepared a study with the following aims:

I. To generate a nanovaccine with stable conjugation of lysate to polystyrene NPs within the size range of 200 nm to 500 nm.
II. To show *in vitro* internalisation of the nanovaccine by DCs followed by antigen processing and presentation.
III. To evaluate nanovaccine therapy in a preclinical glioma mouse model.
IV. To determine the potential mechanism of action for nanovaccine treatment.
V. To investigate the feasibility of a nanovaccine using peptides of known tumor antigens in the murine glioma model.

4.2.1 Gel filtration of pre-activated nanoparticles is required to create nanoparticle-lysate complexes within the preconceived size range of 200 to 500 nm

The first goal of the study was to generate NP-lysate conjugates and optimise this conjugation protocol in a way that the final product can be used for *in vitro* and *in vivo* testing. Utilising carboxylated polystyrene NPs enabled covalent coupling of antigens to the surface of the NPs. As stated in the introduction, DCs have a high endocytic capacity for particles within the size range of 200 to 500 nm, while larger complexes are mainly scavenged by macrophages which have poor antigen presenting function (67, 186). Keeping in mind the 'transport' function of the NPs for lysate to the DCs, the optimal size of the end product should not exceed 500 nm. To this end, the size of different 'generations' of NP-lysate conjugations was measured by DLS. Simultaneously, the prepared complexes were incubated with DCs *in vitro* to check viability of the cells.

The first generation of NP-lysate complexes consisted of carboxylated polystyrene NPs, 50 nm in size, and non-manipulated irradiated tumor lysate. As compared to the size of individual NPs 50 ± 3 nm (mean ± SD), the NP-lysate conjugates significantly increased in size to 868 ± 299 nm (**Fig. 19**). One possible reason for this major size increase was the heterogeneity in size of lysate fragments. Performing a comparable size measurement for crude tumor lysate displayed fragments ranging from only a few nanometers in size up to a couple of micrometers, the latter probably consisting of parts of or even complete apoptotic cells (data not shown). Therefore a second generation of NP-lysate conjugates was developed using certain parts of the tumor lysate. As an example, lysate fragmentation could be achieved by centrifugation of the lysate resulting in the isolation of plasma membrane fragments (268). Unfortunately this did not solve the problem of large lysate size, still measuring lysate pieces up to 1 µm (data not shown). A more successful technique to limit the size of lysate fragments was filtering it using a 200 nm syringe filter normally used to eliminate fine particles and microorganisms from liquid samples. Filtered lysate showed no pieces larger than the filter pores, showing efficient filtration (data not shown). However, the hydrodynamic size, as measured by DLS, for filtered lysate-NP conjugates still increased beyond 500 nm (**Fig. 19**). Next to the heterogeneity of lysate fragment sizes, a second and more important reason for the excessive size increase of NP-lysate conjugates of the first generation is the use of a surplus of EDAC and NHS for NP pre-activation. These activation molecules can also activate carboxyl groups present in the lysate, leading to crosslinking of lysate fragments. This hypothesis led to the third generation of NP-lysate conjugates where the excess of EDAC and NHS was removed by gel filtration (GF). Separation of pre-activated NPs from the excess of activation molecules before adding the lysate resulted in NP(GF)-lysate conjugates with a hydrodynamic size of 340 ± 67 nm, as obtained by 14 independent measurements (**Fig. 19**). Taken together, these results show the possibility to generate NP-lysate complexes within the size range of 200 to 500 nm when the process of gel filtration is added to the protocol.

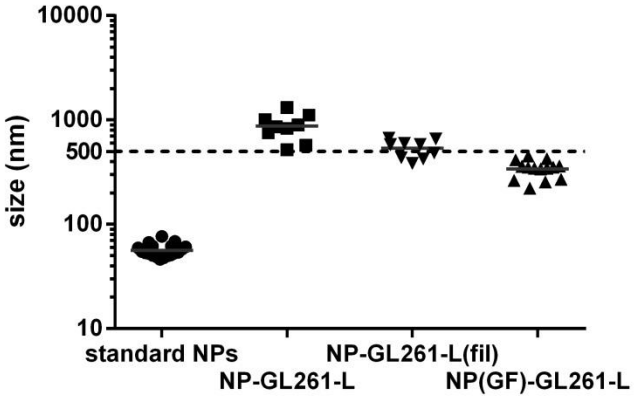


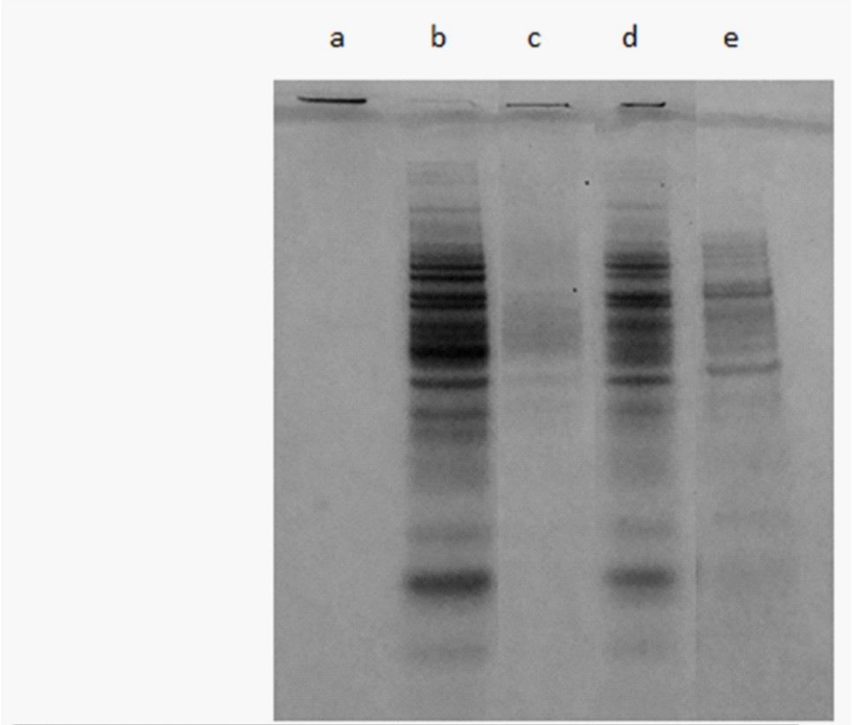
Figure 19: Size characterization of nanoparticles and NP-lysate complexes. Dynamic light scattering was used to measure the size of standard NPs before conjugation with lysate and NP-lysate complexes. Obviously, conjugation of lysate to the NPs increased the size significantly, but beyond the preconceived size range of 200 to 500 nm. Gel filtrating the pre-activated NPs lead to complexes with sizes within this range. Data presented as mean and standard deviation of at least 8 independent measurements.

In search for an optimal NP(GF)-lysate conjugate, different concentrations ranging from 0.1 mg/ml till 2 mg/ml of lysate and 1 mg/ml till 15 mg/ml NPs were combined. Due to the formation of visible aggregates, the highest concentration of NPs was put aside for further experiments (data not shown). Finally, the two combinations most frequently used within the further course of this dissertation are 1 mg/ml lysate + 10 mg/ml NPs and 1 mg/ml lysate + 5 mg/ml NPs. Unless stated otherwise, these concentration combinations were used for *in vitro* and *in vivo* testing respectively.

4.2.2 Fragments of lysate are stably coupled to nanoparticles

Being able to produce NP(GF)-lysate conjugates within the targeted size range, the conjugation of lysate to the polystyrene NPs was checked. Due to the coupling of free amine groups in the lysate and carboxyl groups at the surface of the NPs, especially proteins and peptides are aimed for in the conjugation process. Besides, as a large group of antigens are from protein origin, SDS-PAGE was used to examine the conjugation qualitatively. For this technique proteins are denatured and negatively charged by SDS and afterwards loaded onto a polyacrylamide gel. By the influence of a current, the charged molecules will move through the gel towards the positive electrode. To visualize this migration, protein fragments in the gel were stained with Coomassie blue. We hypothesized that stable conjugation of lysate to NPs would sterically hinder the diffusion process through the gel, leading only to a migration of free lysate fragments. Thus, loss of signal corresponds to conjugation of lysate fragments to the NPs.

As shown in **lane b of Figure 20**, adding just lysate indeed resulted in a smear of protein fragments while NPs alone (**lane a**) did not stain within the gel. Comparing NP-lysate conjugates without and with gel filtration (**Fig. 20, lane c and d respectively**) and produced with the same concentration of 1 mg/ml lysate and 5 mg/ml NPs, showed a clear difference in the amount of free proteins/peptides. As indicated above, cross-linking of lysate created aggregates in which nearly all protein fragments were included, corresponding to the absence of signal for the NP-lysate complexes. NP(GF)-lysate conjugates showed a decreased signal as compared to lysate alone indicating stable conjugation of lysate fragments to NPs (**Fig. 20 lane d and b**). Besides, increasing the amount of NPs further decreased the protein signal after gel electrophoresis (**lane e**). Therefore, it can be concluded that lysate used at 1 mg/ml is indeed stably conjugated to the gel filtrated NPs.



Conc (mg/ml)					
Lysate	/	1	1	1	1
Conc NP	5	/	5	5	10

Figure 20: Stable conjugation of lysate to nanoparticles. Separating free lysate/protein fragments from NP-lysate conjugates was studied using SDS-PAGE. Lanes of the gel were loaded with 10 µg lysate or different NP-lysate conditions. Coomassie blue staining visualized the presence of lysate fragments in the gel. Lane a – polystyrene NPs; Lane b – lysate; Lane c – NP-lysate (1 mg lysate & 5 mg/ml NPs without gel filtration); Lane d – NP(GF)-lysate (1 mg lysate & 5 mg/ml NPs); Lane e – NP(GF)-lysate (1 mg lysate & 10 mg/ml NPs). One representative experiment out of four.

4.2.3 Reduced toxicity of nanoparticle-lysate complexes for dendritic cells when gel filtration is implemented in the conjugation protocol

Because extensive *in vitro* testing with NP-lysate complexes introduces non-biological particles to cells, initial toxicity testing was performed in early stages of the experiments with NPs. Although the polystyrene NPs have been described to be bio-inert, the possibility of toxicity cannot be ruled out (269). The main cells getting into contact with the NPs, especially *in vitro*, are DCs and that is why co-cultures of DCs with NPs were focus of these experiments. Toxicity testing was executed in two ways: on the one hand visualisation of DCs by (fluorescent) microscopy and on the other hand MTT assays.

Comparing different amounts of standard polystyrene NPs which are loaded to DC cultures showed an increasing amount of cell death with increasing quantity of NPs (**Fig. 21A**). When these NP-loaded DC cultures were followed over time, a concentration-dependent toxicity was observed with short term incubation, 1 to 4 hours, leading to no or minor toxicity up till 130 µg of NPs, while for longer incubation times of 24 and 48 hours this was already the case starting from 65 µg of NPs. For higher concentrations of NPs, 260 µg and 520 µg, clear toxicity was present in all incubation time groups. Next, the NP-lysate conjugates, generated with and without the implementation of gel filtration to the protocol, were co-cultured with DCs. Long term co-culturing the NP-lysate complexes with DCs resulted in comparable toxicity between both conditions (**Fig. 21B,C**). Moreover, in comparison to unloaded NPs, cell death started occurring at a higher concentration of 125 µg of NPs. The most striking difference between the NP-lysate complexes was observed for short incubation times. Under these conditions, including gel filtration to the protocol showed to be advantageous, with the absence of NP toxicity for DCs up to the highest concentration of 500 µg NPs. However, this was restricted to the short term incubations, up till 4 hour.

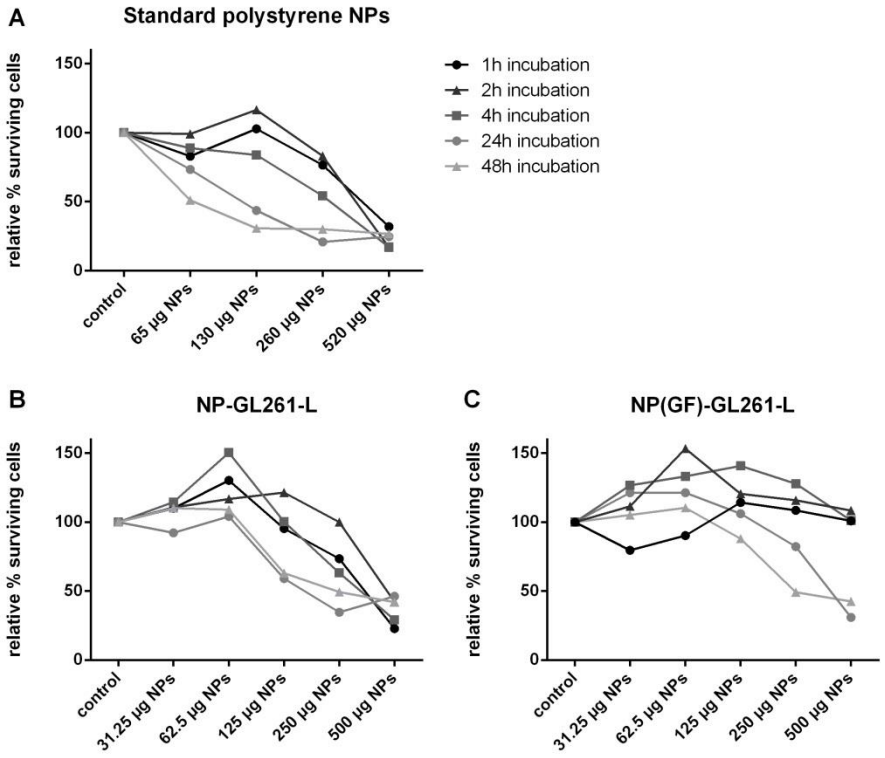


Figure 21: Reduced toxicity of nanoparticle-lysate complexes for dendritic cells when gel filtration is implemented in the conjugation protocol. DCs were incubated with increasing concentrations of NPs **(A)** or NP-lysate conjugates **(B)**,**(C)** for various periods of time. Toxicity in the co-cultures was judged by means of MTT assay.

Figure 22 shows the bright field microscopy images and fluorescence images of cultured DCs loaded with either NP-lysate or NP(GF)-lysate complexes. Judging the phenotype of the DCs in the bright field microscopy images clearly shows healthy DCs in the gel filtration group, while apoptotic and necrotic cells are visualized in the absence of gel filtration during the NP-lysate conjugation protocol (**Fig. 22A,B**). The fluorescent images were enabled by FITC labelled lysate coupled to NPs that was added *in vitro* to the DCs. The condition without gel filtration showed accumulation of NP-lysate complexes near the dying DCs (**Fig. 22C**). Under optimal conjugation circumstances the still healthy looking DCs seem to have taken up the NP-lysate conjugates.

Although both a concentration and time dependent cell toxicity by NPs and NP-lysate conjugates had been shown, a clear improvement by implementing gel filtration in the conjugation protocol of lysate to NPs was observed, especially for short term incubation periods up to 4 hours.

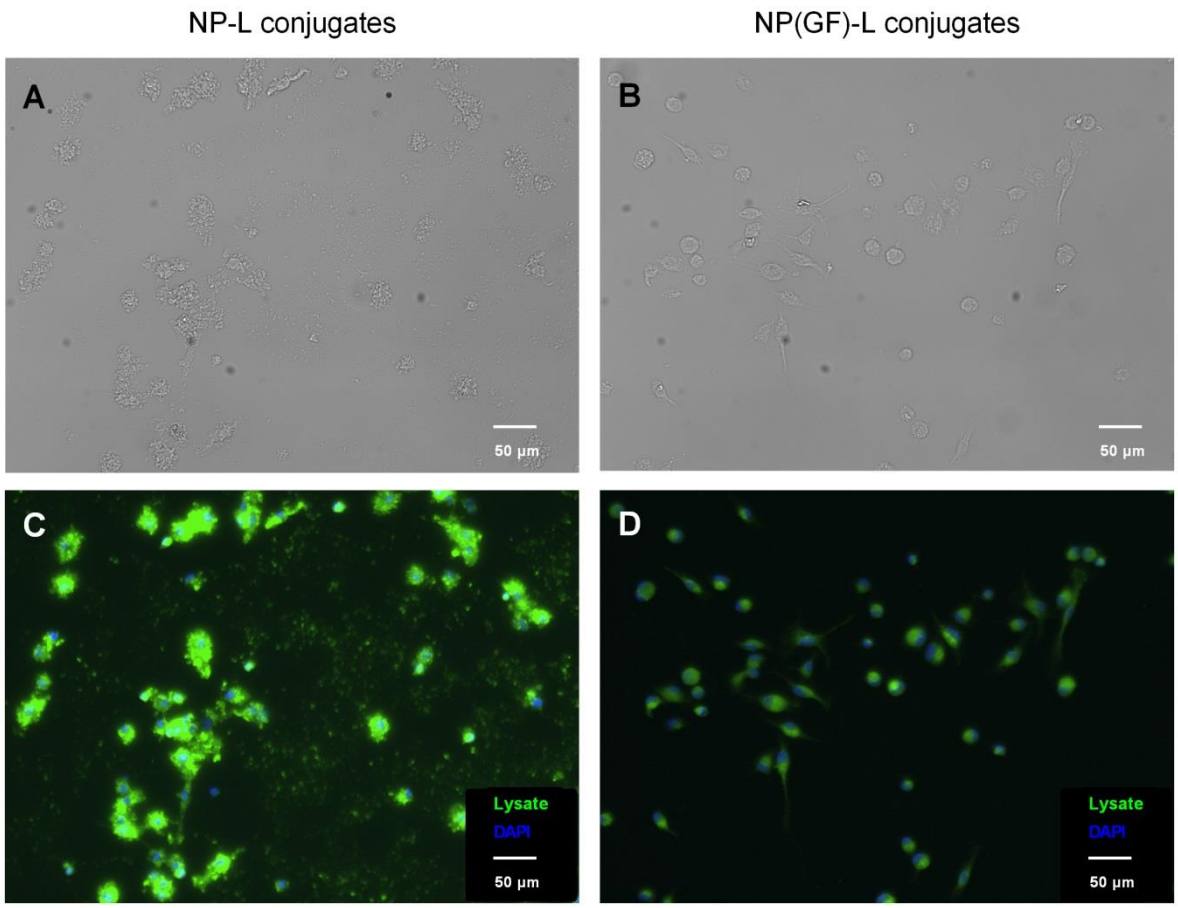


Figure 22: Avoiding crosslinking of nanoparticle-lysate conjugates results in healthier DCs following co-culture with these conjugates. *In vitro* differentiated DCs were incubated for 90 minutes with NP-lysate complexes, for which the lysate was FITC labelled, afterwards washed and stained with DAPI nucleus stain for fluorescence microscopy. Images **(A)** and **(B)** show the bright field visualisation of the fluorescent images **(C)** and **(D)**. Microscopy images were visualized with a 200x magnification.

4.2.4 Uptake of nanoparticle-lysate complexes and presentation of lysate fragments by dendritic cells *in vitro*

Having shown the limited toxicity for NP(GF)-lysate conjugates, especially in short term incubations and for lower NP amounts, the next step was to show uptake of these complexes by the DCs. As already shortly mentioned before, lysate was labelled with FITC, coupled to NPs (NP(GF)-lysate-FITC) and added *in vitro* to iDCs at different concentrations. To discriminate between uptake *versus* surface adhesion, confocal microscopy was performed and z-stacks were used to create a three dimensional examination of the DCs. We could demonstrate that NP(GF)-lysate-FITC complexes were internalized after 90 minutes of incubation (**Fig. 23A**). Similar results were obtained when fluorescent NPs were used for NP-lysate conjugation and loading onto DCs (data not shown). Moreover, this internalisation by

DCs is an active physiologic process, as no fluorescent signal is detected when the NP(GF)-lysate-FITC loading of DCs was performed at 4°C (**Fig. 23B compared to 23C**).

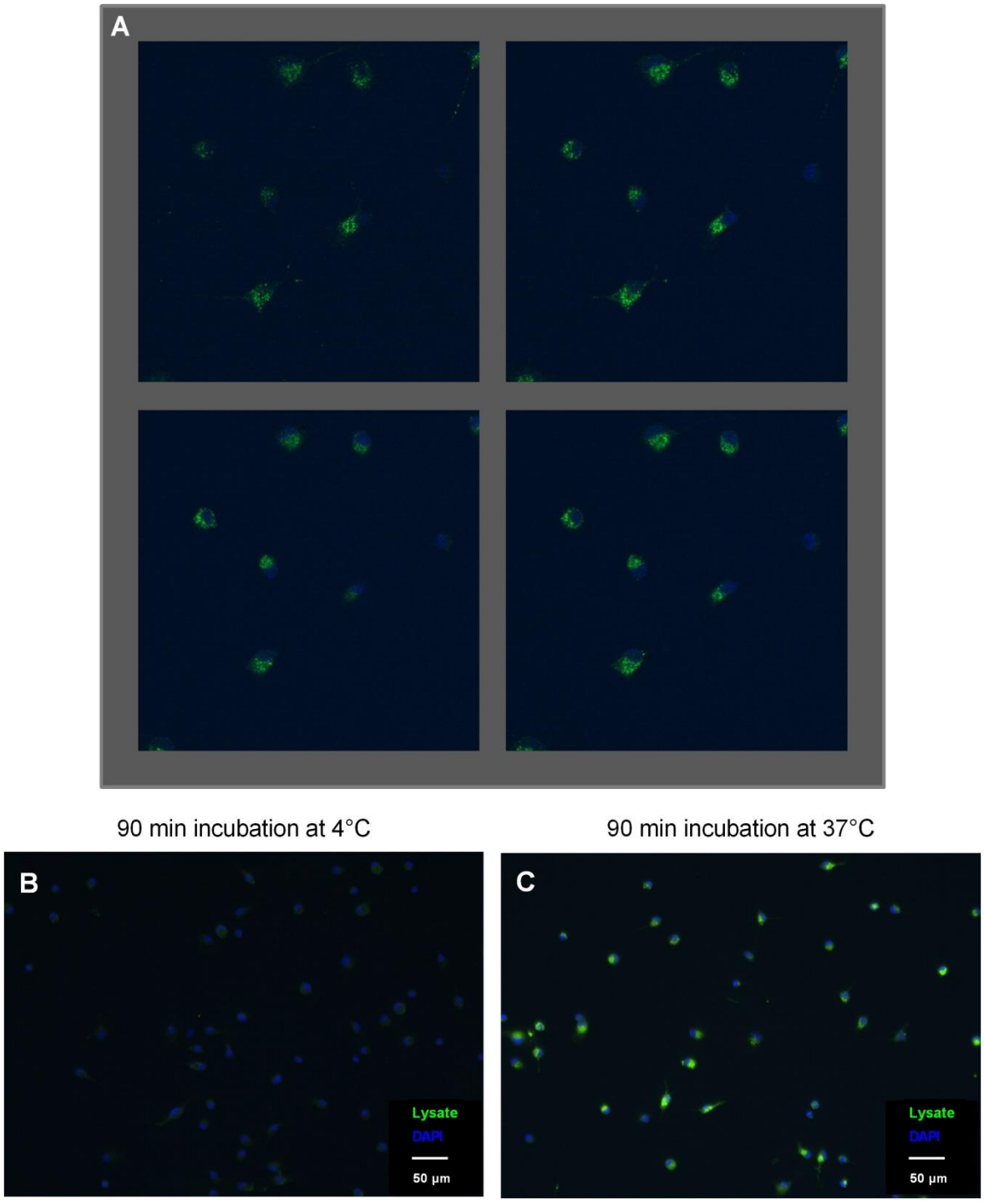


Figure 23: Internalisation of nanoparticle-lysate complexes by dendritic cells *in vitro* is an active process. *In vitro* differentiated DCs were incubated with NP(GF)-lysate-FITC conjugates for 90 minutes, afterwards washed and stained with a DAPI stain. **(A)** Z-stack images, obtained with confocal microscopy, were used to visualize DCs and prove internalisation of the NP-lysate conjugates. The different panels in A represent one confocal z-stack image, bottom to top with DAPI nucleus staining (blue) and FITC signal (green). **(B)**,**(C)** To show uptake is an active process,

incubation at 4°C was performed in comparison to 37°C. At 4°C no active, ATP-requiring processes can take place. Images were visualized with a 400x magnification for **(A)** and a 200x magnification for **(B)** and **(C)**.

As shown by flow cytometry, uptake of NP-lysate conjugates in the presence of LPS, an external maturation factor, was accompanied by increased presence of maturation markers on the surface of DCs. **Figure 24** presents the results of a co-culture experiment where iDCs were loaded with different concentrations of NP-lysate complexes and incubated for 24 hours. Upregulation of CD80, CD86, CD40 as well as MHC class I and MHC class II molecules by the DCs was observed. Besides, a slight increase in the presence of CCR-7, a homing receptor for lymphoid tissue, was detected. Finally, we demonstrated presentation of fragments of FITC-labelled tumor cell lysate in the context of MHC molecules. Using confocal microscopy, colocalization of FITC-lysate with MHC-I molecules was clearly found, indicating that (cross-)presentation of lysate fragments in MHC-I context is possible when lysate is loaded to DCs via polystyrene NPs (**Fig. 25**). These observations demonstrate that the presence of lysate processing and presentation in MHC-I context is plausible when lysate is loaded to DCs with the help of polystyrene NPs.

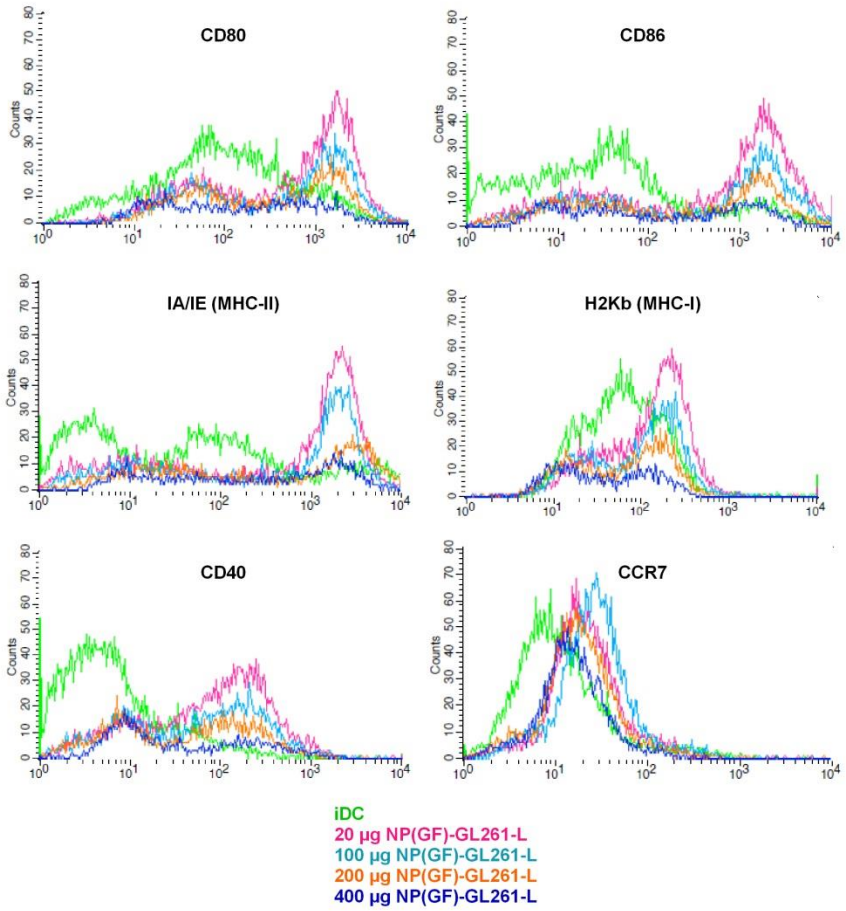


Figure 24: Upregulation of surface maturation markers of dendritic cells following nanoparticle-lysate complex internalisation. Flow cytometry was used to study the expression of maturation markers on lysate-loaded DCs. *In vitro* differentiated DCs were incubated with NP-lysate conjugates for 90 minutes, washed and after 24 hours incubation, in the presence of LPS, stained. Representative graphs for DC maturation markers CD80, CD86, CD40; for MHC class I and MHC class II molecules and for the lymphoid tissue homing factor CCR7 are shown. Expression on immature DCs is depicted in green, while marker expression by mature DCs is presented in pink, blue or orange. Different concentrations of lysate were used without major differences in expression. Graphs are representative of at least three independent experiments.

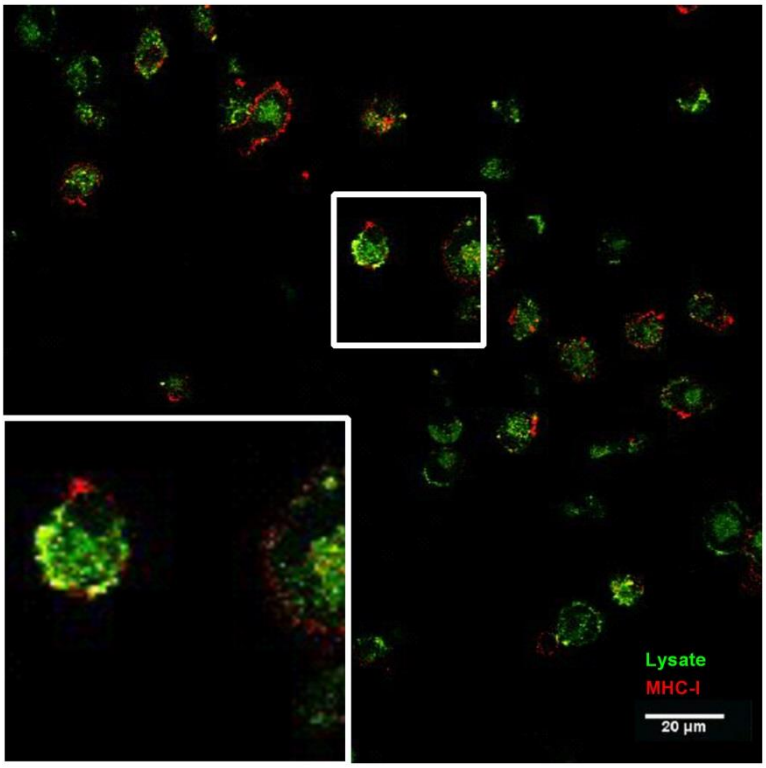


Figure 25: Cross-presentation of fragments of lysate by DCs *in vitro* following nanoparticle-lysate complex internalisation. *In vitro* differentiated DCs were incubated with NP(GF)-lysate-FITC for 90 minutes, afterwards washed and matured with LPS. Maturation of DCs was ended after 24 hours by washing cells, fixation and staining. Confocal microscopy showed colocalization (yellow signal) of fragments of lysate (FITC, green signal) and MHC class I molecules (PE, red signal). The box at the bottom is a magnification of the marked cells. Image was visualized with a 400x magnification and is representative of two independent experiments.

4.2.5 Pre-tumor nanovaccine therapy improves survival in two different mouse glioma models

Preclinical activity of NP-lysate was pursued by the *in vivo* testing of NP-lysate conjugates. In the *in vivo* experiments, this treatment strategy will be referred to as nanovaccine treatment.

The intracranial GL261 and CT2A glioma models in fully immunocompetent mice, as described previously, were used. Again, due to the aggressive *in vivo* growth of both glioma cell lines, immunisation before tumor inoculation was performed. Nanovaccines were administered subcutaneously because of the large amount of DCs in the skin.

In a first set of experiments, nanovaccine administration was compared to DC therapy using *ex vivo* grown DCs. **Figure 26A and B** show the survival curves of nanovaccine-treated and DC-treated glioma-bearing mice in the GL261 or CT2A glioma model. Pre-tumor treatment with nanovaccine in the GL261 mouse model significantly prolonged survival with 30% as compared to PBS controls. However, animals treated with DC immunotherapy did even better leading to a median survival of 52 days post tumor inoculation, while the median survival in the nanovaccine treated group was 37 days and only 26 days for mock treatment. Within the more aggressive CT2A model the nanovaccine was able to provoke a comparable survival benefit as DC therapy, both with respect to PBS injected mice (**Fig. 26B**).

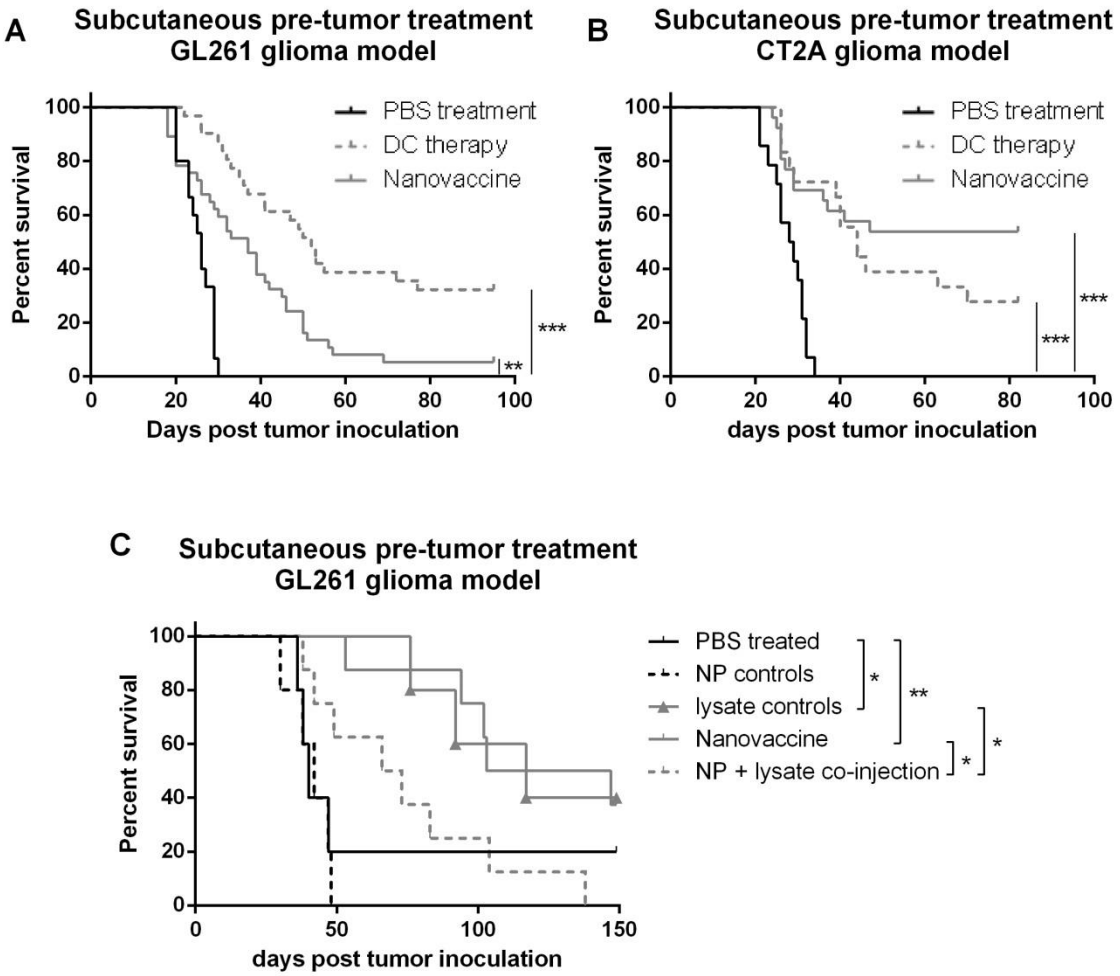


Figure 26: Pre-tumor nanovaccine therapy improves survival in two different glioma mouse models. Mice were subcutaneously injected with nanovaccine 14 and 7 days before tumor inoculation with 5 x 10^5 glioma cells. In the GL261 glioma model **(A)** as well as for the CT2A glioma model **(B)** nanovaccine administration and DC vaccination were studied in comparison to PBS treatment. Moreover, the importance of stable conjugation of autologous lysate to NPs was tested by comparing NP-lysate conjugates (n=8) to co-injection of NPs and lysate in the absence of conjugation (n=8) **(C)**. Autologous lysate injection (n=5) was used as a positive control condition, while the injection of NPs alone (n=5) and PBS injection (n=5) served as a negative control conditions. For graphs **(A)** and **(B)** data of three independent experiments were pooled; n=15 in the PBS control group and n=30 in both experimental conditions. Statistical significance was calculated by Log-rank test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Next the importance of lysate-NP conjugation was tested using on the one hand nanovaccines and on the other hand co-injection of NP and free lysate in the GL261 model. Pre-tumor nanovaccine treatment improved glioma survival significantly in comparison to mock treated animals (**Fig. 26C**). Importantly, survival in the NP control group, injected with unloaded NPs, did not show any difference from the PBS treated group with all animals death within 48 days after tumor inoculation. The condition in which tumor lysate was administered in the absence of NPs and *ex vivo* grown DCs, resulted in a similar survival benefit as for the nanovaccine treated mice (median survival of 117 days and 125 days respectively). Moreover, both treatment modalities generated approximately 40% long term survivors. When lysate and NPs were co-injected without conjugation, the beneficial effect of NP-lysate and lysate alone treatment was reduced significantly (**Fig. 26C**). Although the co-injection did improve survival as compared to PBS treated animals and the NP control group, the survival benefit was limited with an increased median survival from 40 days to 69 days and all mice dying within 138 days post tumor inoculation.

Nanovaccine therapy, consisting of 100 µg lysate for each mouse, significantly prolonged survival in two independent mouse models, showing the efficacy of pre-tumor nanovaccine therapy. Moreover, the necessity to conjugate lysate to the NPs has been shown *in vivo*, indicating the importance of a stable conjugation product.

4.2.6 Pre-tumor treatment with nanovaccines and autologous tumor lysate results in equivalent survival benefit for glioma inoculated mice

Up till this point in our research nanovaccine treatment and autologous lysate treatment still performed equivalent. Building on the idea that DCs prefer to internalize antigens presented in a particulate manner over crude lysate (197), made us wonder if the nanovaccine was able to induce a survival benefit using even lower concentrations than necessary for lysate alone. This would allow its therapeutic use if only limited tumor material would be available, e.g. after tumor biopsy instead of tumor removal. We therefore reduced the tumor amount till 25

µg, a dose that still showed significant DC maturation in the presence of an independent maturation stimulus (**Fig. 24**). Unfortunately, in the GL261 glioma model (as well as in the CT2A glioma model for the lowest dose), nanovaccine treatment with lower concentrations than the 100 µg lysate, which was standardly used, did not significantly improve the survival outcome as compared to PBS treatment (**Fig. 27**).

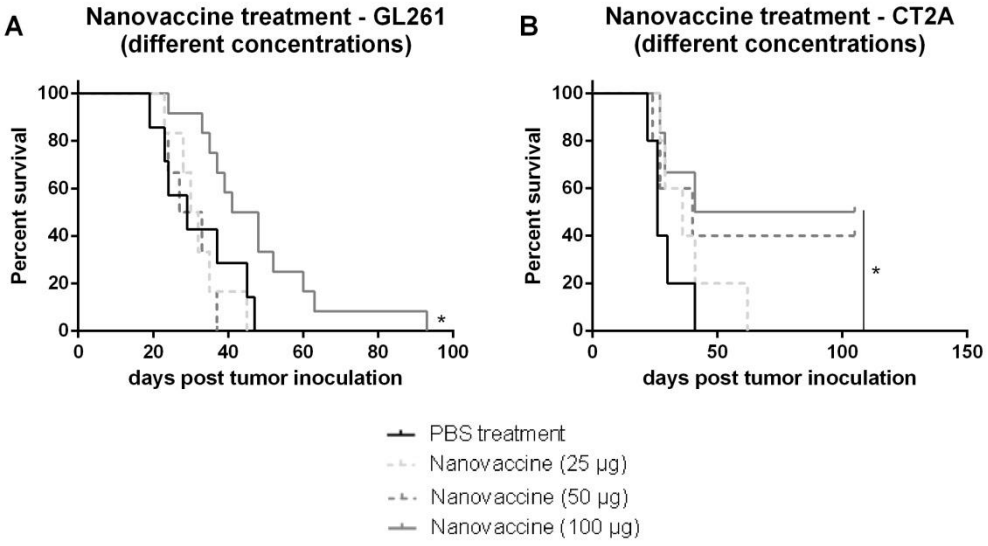


Figure 27: Pre-tumor nanovaccine therapy only able to improve survival at same concentration as autologous lysate. Mice were subcutaneously injected with nanovaccine 14 and 7 days before tumor inoculation with 5 x 10^5 glioma cells. In the GL261 glioma model **(A)** as well as for the CT2A glioma model **(B)** nanovaccine administration was studied at different concentrations ranging from 25 µg to 100 µg protein concentration. Negative control condition of PBS treatment. Statistical significance was calculated by Log-rank test, * $p < 0.05$

4.2.7 Absence of an inherent adjuvant function for polystyrene nanoparticles in the nanovaccines

One possible reason for the limited survival advantage of nanovaccines as compared to DC therapy observed in the GL261 glioma model might be the absence of a maturation stimulus. Despite the observation by Fifis *et al.* showing that comparable polystyrene NPs to the ones we used, can act as an adjuvant for the induction of an immune response (205), we were not able to repeat this observation when using our NP-lysate conjugates. The upper graphs of **Figure 28** show a non-significant, very limited upregulation of CD86 and MHC-I molecules following 24 hours co-culture of DCs with different concentrations of NP-lysate conjugates in the absence of an additional maturation stimulus. Due to the lack of an inherent adjuvant function of the polystyrene NPs within our hands, the *in vivo* nanovaccine administration might further benefit from the addition of a TLR ligand to mature the DCs that internalize the NP-lysate conjugates.

In this section we explored the added value of three different TLR agonists injected in combination with the nanovaccine. The TLR agonists, CpG ODN (TLR 9), R848 (TLR 7 and 8) and Poly(I:C) (TLR 3), were co-injected with the nanovaccine without conjugation to the NPs. The TLR-agonist was injected shortly after the nanovaccine at the same injection place, co-injection was not performed by pre-mixing both contents. Neither of the TLR agonists was able to show an added value for the nanovaccine in terms of survival (**Fig 28C-E**). None of the conditions improved median survival of the treated mice significantly as compared to PBS treated mice or NP control mice. Apart from the confirmation that nanovaccine therapy is effectively increasing the survival period in the GL261 glioma model, this explorative experiment failed to produce positive results. Neither of the TLR agonists was able to improve survival outcome and especially CpG and R848 even seemed to antagonize the nanovaccine treatment advantage at the concentrations used.

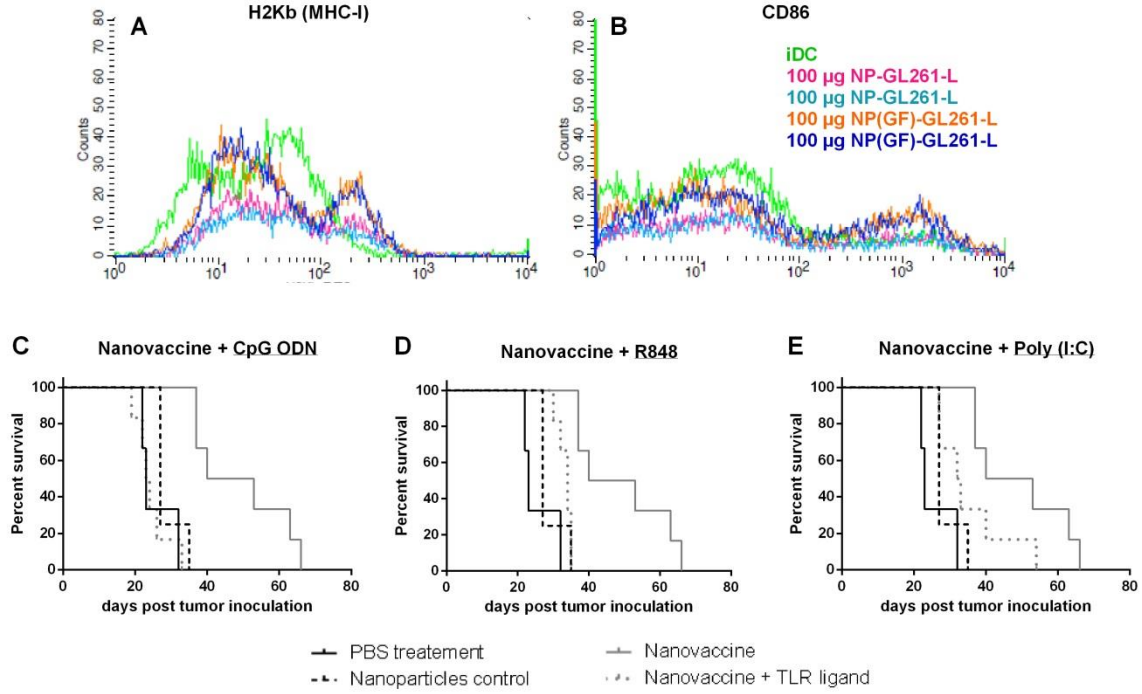


Figure 28: Absence of an inherent adjuvant function for polystyrene nanoparticles in the nanovaccine. Flow cytometry was used to study the expression of maturation markers on lysate-loaded DCs. *In vitro* differentiated DCs were incubated with NP-lysate conjugates for 90 minutes, washed and after 24 hours incubation, in the absence of an external maturation stimulus, stained. Representative graphs for MHC class I **(A)** and DC maturation marker CD86 **(B)** are shown. Expression on immature DCs is depicted in green, while marker expression by DCs loaded with NP-lysate conjugates is presented in pink, blue or orange. Graphs **(C)**,**(D)** and **(E)** depicted below show the survival curves of GL261 glioma inoculated mice that were pre-treated with nanovaccine in the absence or presence of 50 µg of TLR ligand (CpG ODN, R848 or Poly(I:C)). TLR-ligands were co-injected with nanovaccine and not conjugated to the NPs. Control conditions consisted of PBS treatment and NP alone injection. Statistical difference, as calculated by Log-rank test, was only present for nanovaccine treatment ($p < 0.05$). n=5 for control conditions and n=8 for test conditions.

4.2.8 Nanovaccine treatment introduces influx of effector T cells in combination with reduced immunosuppression in the brain

In search for a mechanism of action of nanovaccine treatment, the immune response in the brain of glioma-bearing mice, GL261 and CT2A, was studied. More specifically, proportions of different T cell populations and immunosuppressive MDSCs that have infiltrated the brain were studied in mice pre-treated with PBS or nanovaccine. These brain immune cells were isolated 7, 14, 21 or 28 days after tumor inoculation and examined by means of flow cytometry. The gating strategy used for the population analysis is depicted in Figures 1 to 3 of the "Addendum – flow cytometry information". **Table 5** in the Materials and Methods section summarizes the employed antibodies.

At the earliest time point, day 7, an influx of total T cells ($CD3^+$) accompanied by a relative increase in $CD4^+$ T cells and a relative decrease in $CD8^+$ T cells was noticed in the GL261 model (**Fig. 29**). The same proportional shift to $CD4^+$ T cells was observed in nanovaccine treated CT2A glioma-bearing mice, although not statistically different as compared to mock treatment (**Fig. 30**). In the latter glioma model, a comparable increase in total T lymphocytes at day 21 was accompanied by an increased proportion of IFN-γ producing $CD8^+$ T cells and as important a decreased proportion of $CD4^+Foxp3^+$ Treg cells. Unfortunately, these results were not present in the GL261 model. Besides, as a tumor combatting representative of the innate immune system, no differences were observed for NK cells (data not shown). Moreover, the results of two MDSC subpopulations, monocytic ($CD11b^+Ly6C^+Ly6G^-$) MDSCs and granulocytic ($CD11b^+Ly6C^-Ly6G^+$) MDSCs, are presented in **Figure 29 and 30**. As early as 7 days after tumor inoculation a decreased percentage of monocytic MDSCs was observed for nanovaccine treated animals in both glioma models. Moreover, this observation was maintained at least until day 21 in the CT2A model. Granulocytic MDSCs were proportionally far less present in the brain of glioma-bearing mice, whether or not treated, and did not show any statistically difference. Together these data suggest a combination of reduced immunosuppression, supported by the observations of $Foxp3^+$ Treg cells and MDSCs, and increased immune activation.

Over time, the increased proportion of total T lymphocytes was correlated with an increase in the relative amount of $CD8^+$ T cells (**Fig. 29 and 30**). Importantly, this $CD8^+$ T cell expansion was accompanied by an increase of IFN-γ producing $CD8^+$ T cells in both glioma models for treated as well as untreated animals. Although little change in $CD4^+$ T cells is detected over time in the GL261 glioma model, CT2A glioma-bearing mice treated with nanovaccine demonstrate a declined frequency of this cell population. Despite this reduction of $CD4^+$ T cells, the proportion of IFN-γ producing $CD4^+$ T cells inclined. Finally, immunosuppressive monocytic MDSCs showed a reduction in their relative amount in the GL261 model. No decrease in MDSCs was observed in the CT2A model, potentially due to a general decrease in the percentage of $CD11b^+$ myeloid cells.

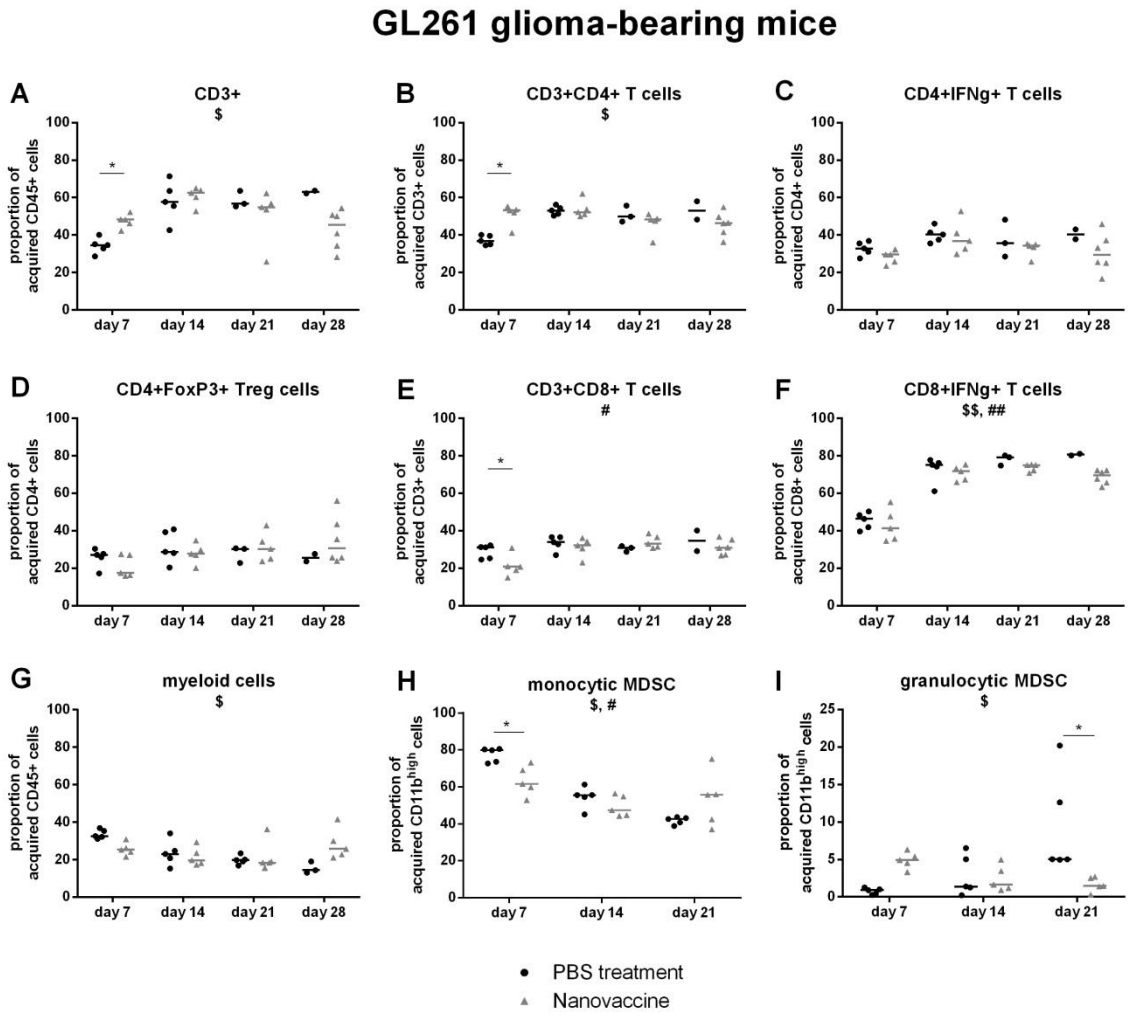


Figure 29: Influx of effector T cells in combination with reduced immunosuppression in the brain of nanovaccine treated GL261 glioma-bearing mice. Brain immune contexture of pre-tumor treated mice was studied 7, 14, 21 and 28 days after tumor inoculation. Graphs present kinetic analyses of different T cell population proportions and fractions of immunosuppressive MDSC types in PBS treated (●) or lysate treated (Δ) mice. Cell populations were defined by different stainings with **(A)** $CD3^+$ lymphocytes as single cells, ZY-, CD45+, CD3+ gated to CD45+; **(B)** $CD4^+$ T cells as single cells, ZY-, CD45+, CD3+, CD4+ gated to CD3+; **(C)** Tregs as single cells, ZY-, CD45+, CD3+, CD4+, FoxP3+ gated to CD4+; **(D)** IFNγ-producing $CD4^+$ T cells as single cells, ZY-, CD45+, CD3+, CD4+, IFNγ+ gated to CD4+; **(E)** $CD8^+$ T cells as single cells, ZY-, CD45+, CD3+,CD8+ gated to CD3+; **(F)** IFNγ-producing $CD8^+$ T cells as single cells, ZY-, CD45+, CD3+,CD8+, IFNγ+ gated to CD8+; **(G)** myeloid cells as single cells, ZY-, CD45+, CD11b+ gated to CD45+; **(H)** Monocytic MDSC as single cells, ZY-, CD45+, $CD11b^{high}$, Ly6C+ gated to $CD11b^{high}$; **(I)** Granulocytic MDSC as single cells, ZY-, CD45+, $CD11b^{high}$, Ly6G+ gated to $CD11b^{high}$. Statistical significance was calculated by two-way ANOVA. Groups of mice consisted of 5 mice except for day 28, where 3 mice already died due to glioma growth in the PBS treated condition. Significant differences between two treatment conditions, PBS and nanovaccine, are indicated by asterisks: * $p < 0.05$. The symbols $ and # below the graph title indicate significant changes over time within the PBS treated and lysate treated populations respectively: $ or # $p < 0.05$; $$ or ## $p < 0.01$

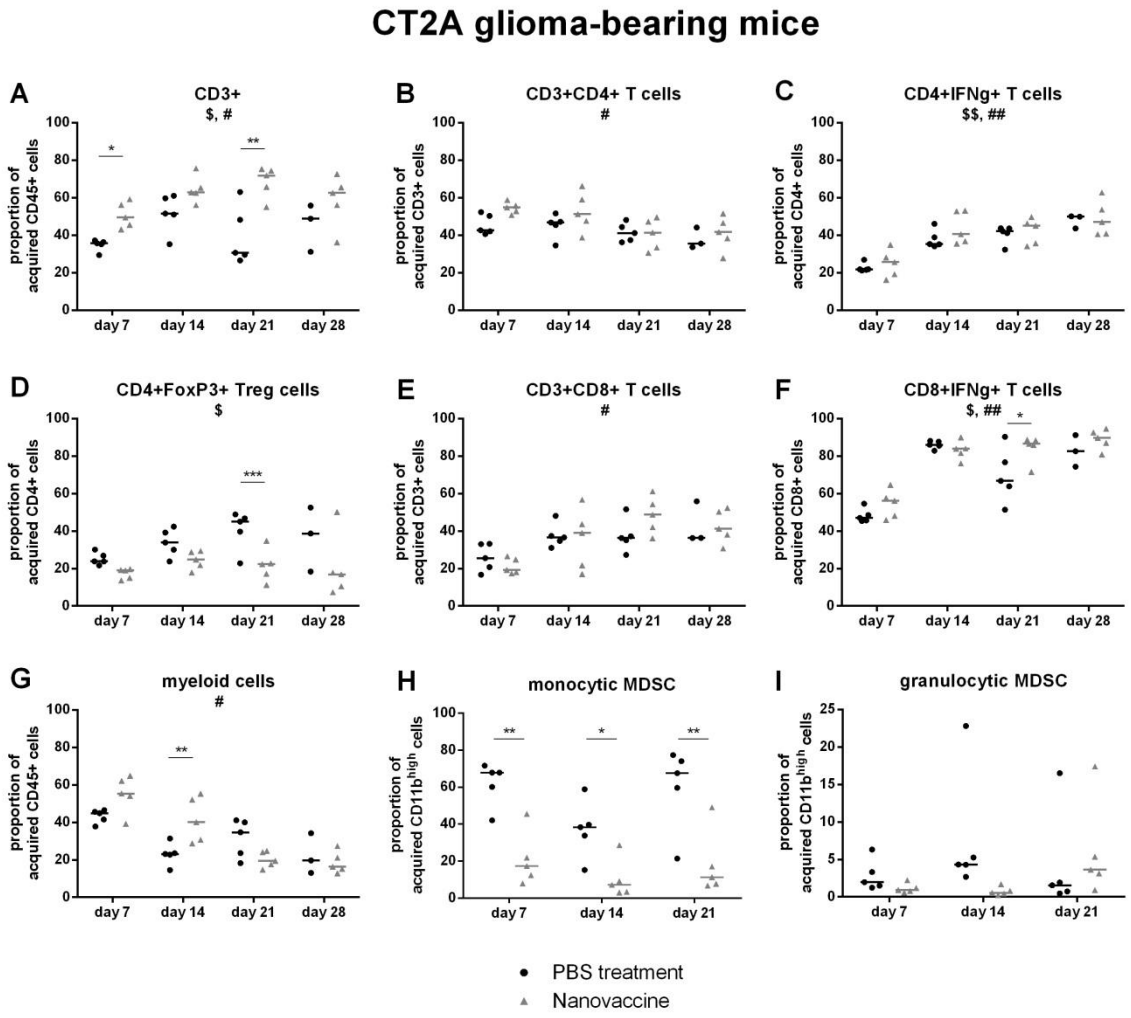


Figure 30: Nanovaccine treatment induces effector T cell influx and reduced immunosuppression in the brain of CT2A glioma-bearing mice. Brain immune contexture of pre-tumor treated mice was studied 7, 14, 21 and 28 days after tumor inoculation. Graphs present kinetic analyses of different T cell population proportions and fractions of immunosuppressive MDSC types in PBS treated (●) or lysate treated (Δ) mice. Cell populations were defined by different stainings with **(A)** $CD3^+$ lymphocytes as single cells, ZY-, CD45+, CD3+ gated to CD45+; **(B)** $CD4^+$ T cells as single cells, ZY-, CD45+, CD3+, CD4+ gated to CD3+; **(C)** Tregs as single cells, ZY-, CD45+, CD3+, CD4+, FoxP3+ gated to CD4+; **(D)** IFNγ-producing $CD4^+$ T cells as single cells, ZY-, CD45+, CD3+, CD4+, IFNγ+ gated to CD4+; **(E)** $CD8^+$ T cells as single cells, ZY-, CD45+, CD3+,CD8+ gated to CD3+; **(F)** IFNγ-producing $CD8^+$ T cells as single cells, ZY-, CD45+, CD3+,CD8+, IFNγ+ gated to CD8+; **(G)** myeloid cells as single cells, ZY-, CD45+, CD11b+ gated to CD45+; **(H)** Monocytic MDSC as single cells, ZY-, CD45+, $CD11b^{high}$, Ly6C+ gated to $CD11b^{high}$; **(I)** Granulocytic MDSC as single cells, ZY-, CD45+, $CD11b^{high}$, Ly6G+ gated to $CD11b^{high}$. Statistical significance was calculated by two-way ANOVA. Groups of mice consisted of 5 mice except for day 28, where 2 mice already died due to glioma growth in the PBS treated condition. Significant differences between two treatment conditions, PBS and nanovaccine, are indicated by asterisks: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. The symbols $ and # below the graph title indicate significant changes over time within the PBS treated and lysate treated populations respectively: $ or # $p < 0.05$; $$ or ## $p < 0.01$

Most probably only a little glimpse of the mechanism of action has been studied, but we can conclude that pre-tumor nanovaccine treatment shifts the cellular immune suppression present in the brain of glioma-bearing mice to one of immune activation. This immune response is driven both by $CD4^+$ and $CD8^+$ T cells. Importantly, the results might be slightly skewed by the decreased amount of animals in the mock treated group due to tumor growth, as these might be the animals with the lowest number of (active) T cells in the brain with higher amounts of Tregs.

Next to the brain immunocontexture, the peripheral immune response was studied for nanovaccine treated and PBS treated mice in the GL261 model. At 14 days post tumor inoculation, a time at which full immune activation is certainly expected, the spleen of pre-treated glioma-bearing mice was isolated. Following CFSE labelling of the splenocytes, these cells were co-cultured with *ex vivo* grown lysate-loaded mDCs in a ratio 10:1 during 5 days. Afterwards cells were stained for T cell surface markers and analysed by flow cytometry. Gating strategy for population analysis and employed antibodies for staining can be found in the "Addendum – flow cytometry information" and **Table 5** in the Materials and Methods section. Stimulation of splenocytes with mDCs resulted in a clear T cell expansion as shown by the decreased CFSE signal in **Figure 31**. In more detail, this expansion was nearly completely attributed to $CD8^+$ T cell proliferation (**Fig. 31A-C**). This limited functional testing of peripheral T cell populations supports the idea that a systemic immune response can also be generated by nanovaccine administration.

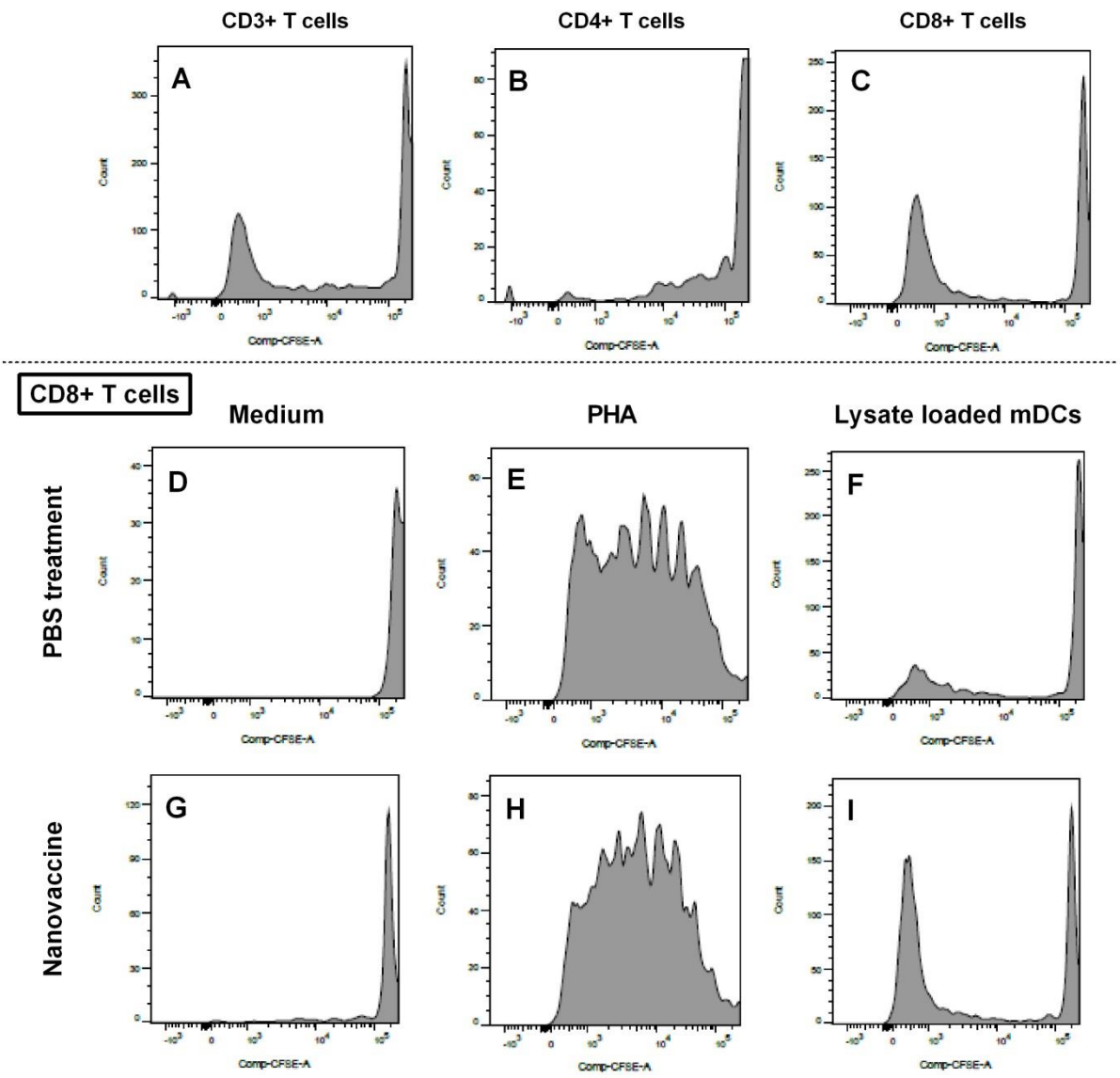


Figure 31: Systemic T cell proliferation in glioma-bearing mice pre-treated with nanovaccine. Treatment of mice was performed 14 and 7 days prior to tumor inoculation with 5 x 10^5 GL261 glioma cells. Splenocytes of pre-treated mice were isolated at day 14 post tumor inoculation and labelled with CFSE. CFSE-labelled splenocytes were left unstimulated in medium or stimulated with phytohaemagglutinin or lysate-loaded mature DCs at a responder:stimulator ratio of 10:1. After 5 days of stimulation, cells were surface stained for T cell markers CD3, CD4 and CD8. Graphs **(A)**,**(B)** and **(C)** represent the CFSE signal in different T cell populations. Graphs **(D-I)** focus on the $CD8^+$ T cell fraction of a PBS treated mouse in comparison to a nanovaccine treated animal. Data shown are representative for 8 different animals from two independent experiments.

4.2.9 Nanoparticle, lysate as well as nanoparticle-lysate conjugate injection develop a depot at the injection site and partly drain to local lymph nodes via dendritic cells

In order to assess the *in vivo* fate of nanovaccines following subcutaneous injection, non-glioma-bearing mice were used to study the biodistribution of NPs and lysate. For these experiments, mice were subcutaneously injected either with fluorescent NPs to follow these particles, FITC labelled lysate to follow lysate distribution, NP-lysate conjugates with fluorescent NPs to follow NPs as part of these conjugates and NP-lysate conjugates with FITC-lysate to follow lysate as part of the conjugates. Local lymph nodes (inguinal and axillary) as well as distant lymph nodes (mesenteric, cervical and auricular) were isolated 12 hours, 16 hours or 24 hours after injection.

In accordance to literature, subcutaneous injection of fluorescent NPs was rapidly followed by drainage to local lymph nodes. Measuring the fluorescent signal within $CD11c^+$ DCs from the inguinal and axillary lymph nodes, showed an increased amount of DCs that have internalised fluorescent particles already 16 hours post injection (**Fig. 32**). When non-fluorescent NPs were injected, only a background fluorescent signal was detected. Within the distant lymph nodes the background signal detected was maintained over the different test conditions (data not shown). As shown in **Figure 33**, it took a little bit more time for lysate and NP-lysate conjugates to be internalized by DCs and subsequently transported to the draining inguinal lymph nodes. In both of these conditions the $FITC^+CD11c^+$ DCs were only observed 24 hours following the injection of FITC labelled lysate and NP(GF)-lysate-FITC conjugates. The absolute number of $FITC^+CD11c^+$ DCs per lymph node showed a doubling of these cells after 24 hours as compared to a PBS injection negative control and the 12 hours incubation. Next to the isolation of lymph nodes, the injection place itself has also been studied. The subcutaneous region surrounding the injection place showed a visible accumulation of the fluorescent signal for over 14 days post injection (data not shown). Over time, a decrease in fluorescent signal was found starting from 24 hours after injection to 7 days and 14 days. Altogether these data support our hypothesis that whole tumor lysate conjugated to NPs can create a depot once injected subcutaneously. DCs that have internalized NP-lysate complexes can migrate to the draining lymph nodes where they can execute their functions.

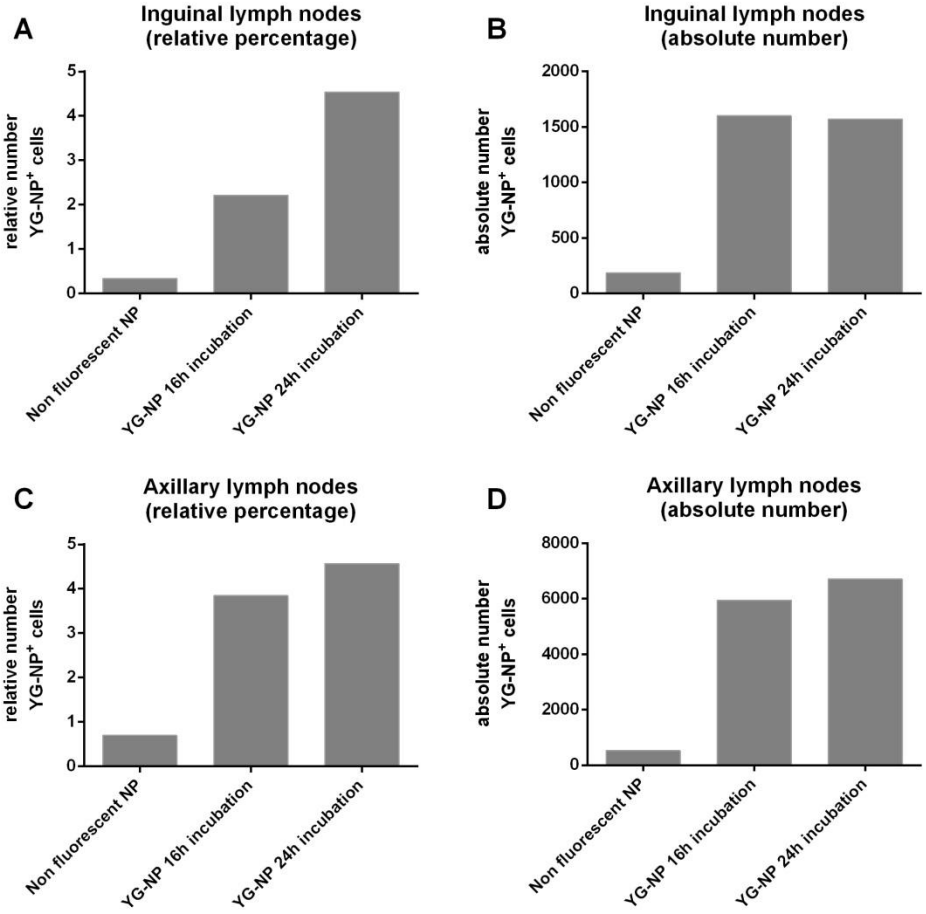


Figure 32: Fluorescent NPs observed in the draining lymph nodes following subcutaneous injection. Mice were subcutaneously injected in the abdominal wall, near the inguinal lymph node, with fluorescent polystyrene YG-NPs. After 16 hours and 24 hours, inguinal and axillary lymph nodes were isolated, processed to single cell suspensions and stained for the DC marker CD11c. Graphs **(A)** and **(C)** present the relative number of CD11c+YG-NP+ cells. The absolute number as presented in graphs **(B)** and **(D)**, is corrected for the number of lymph nodes that were isolated and can be considered the absolute number of double positive cells per lymph node.

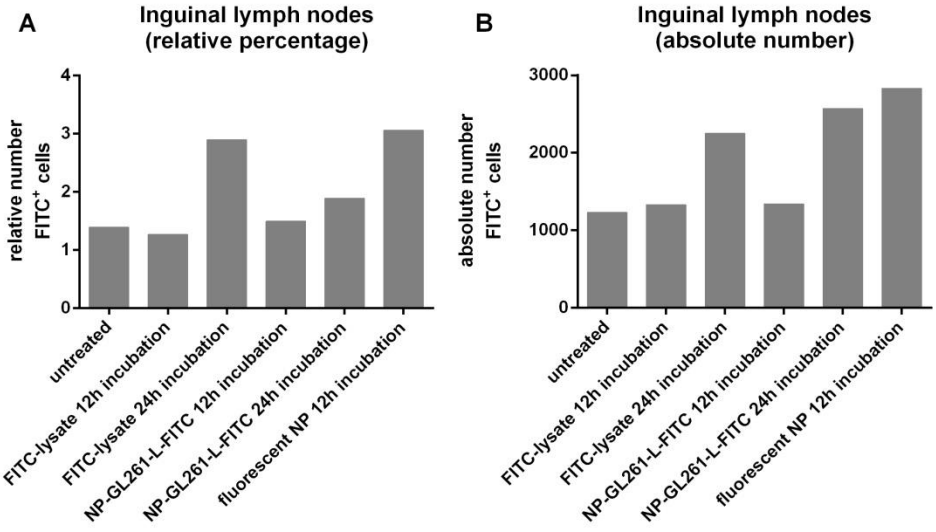
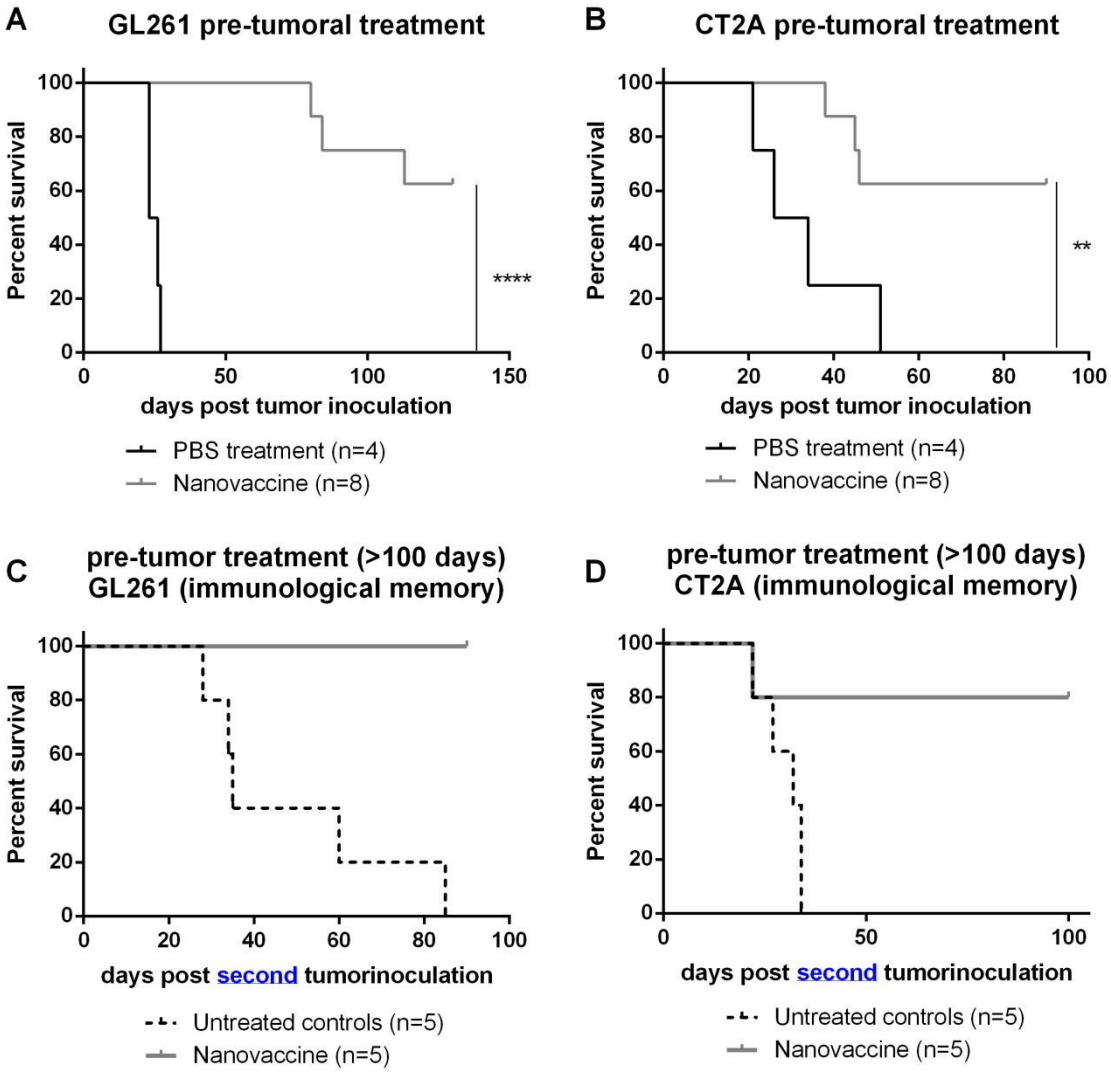


Figure 33: Fluorescent NPs observed in the draining lymph nodes following subcutaneous injection. Mice were subcutaneously injected in the abdominal wall, near the inguinal lymph node. Fluorescent NPs and FITC-labelled lysate were used to study the biodistribution of NPs, lysate and NP-lysate conjugates. Inguinal lymph nodes were isolated 12 hours and 24 hours after injection. Next the lymph nodes were processed to single cells and stained for the DC marker CD11c. Both relative amount and absolute number of $FITC^+CD11c^+$ cells are presented in graph **(A)** and **(B)** respectively. The absolute number presented is corrected for the number of lymph nodes that were isolated and can be considered the absolute number of double positive cells per lymph node.

4.2.10 Long-lasting immunological memory following pre-tumoral nanovaccine administration

Similar to the experiments performed in the autologous lysate treatment chapter, the presence of memory cells was checked by treating mice more than 100 days prior to tumor inoculation. Strong protection from tumor growth was observed following nanovaccine treatment in the GL261 as well as the CT2A glioma model (**Fig. 34, upper graphs**). Moreover, long-term survivors from the first tumor inoculation were re-inoculated with the same amount of tumor cells at the contralateral side of the brain. Only 1 out of 10 mice died within 100 days after the second tumor challenge as a consequence of tumor growth (**Fig. 34, lower graphs**). Finally, using subcutaneously injection with LLC cells, specificity of the immunological response and memory was shown for the CT2A glioma model. For this, another group of long term surviving mice was subcutaneously inoculated with either LLC cells or CT2A cells. Age matched untreated mice were used as controls for subcutaneous tumor growth. All controls except one in the CT2A group developed subcutaneous tumors. Nanovaccine treated long term survivors seemed to be protected from the development of subcutaneous CT2A tumors (**Fig. 34, table below**). However, long term surviving mice as well as age-matched controls inoculated with LLC tumor cells all developed subcutaneous tumors. In conclusion these data show a tumor-specific memory response as a result of pre-tumor nanovaccine treatment.



E

Tumor incidence in long term survivors and age-matched controls implanted subcutaneously with CT2A or LLC cells		
	Tumor inoculation age-matched controls	Rechallenge of long term-survivors
CT2A	9/10	1/7
LLC	10/10	8/8

Figure 34: Long-lasting and tumor-specific immunological memory following pre-tumor lysate treatment. In graphs **(A)** and **(B)**, mice were treated with nanovaccine > 100 days prior to tumor inoculation with 5 x 10^5 glioma cells of the corresponding cell line. In both glioma models, GL261 and CT2A, nanovaccine treated mice were compared to PBS treated control animals. **(C),(D)** Mice that survived the first tumor inoculation were rechallenged at the contralateral side of the brain with 5 x 10^5 glioma cells of the same tumor cell line as the first time without new treatment. New untreated controls were used as a control condition. **(E)** Tumor specificity was only tested in the CT2A glioma model and studied by inoculating other groups of long term survivors with LLC cells subcutaneously. Statistical significance was calculated by Log-rank test, ** $p < 0.01$; **** $p < 0.0001$

4.2.11 Peptide-loaded nanoparticles are able to treat

Leaving all possibilities open for nanovaccine treatment in GBM, we tried to provide evidence for a more commercial approach. Instead of using autologous lysate, tumor antigens can be used as an antigenic source for nanoimmunotherapy. The CD8 immunodominant peptides of gp100, TRP-2 and EphA2, three known onco-proteins for the GL261 glioma cell line, were used to study feasibility for this shape of nanoimmunotherapy. Conjugation of peptides to the polystyrene NPs was followed by dialysis of the conjugates to remove unbound peptides and exclude their contribution. Pre-tumoral nanovaccine treatment resulted in a statistically improved survival shifting median survival from 26 days to 37 days post tumor inoculation (**Fig. 35**). These data show that the development of an off-the-shelf nanovaccine product, using known tumor antigens, is achievable and can lead to a survival benefit following subcutaneous injection.

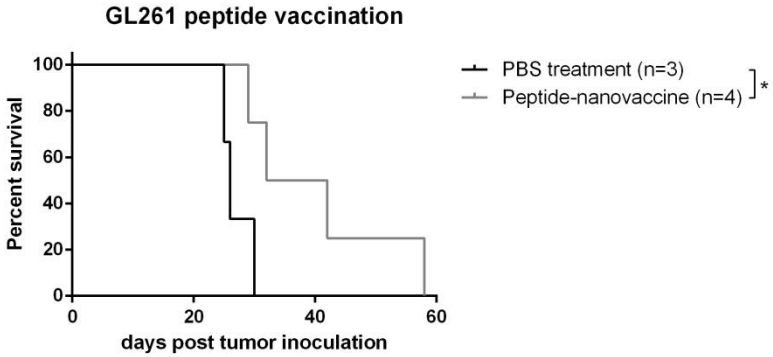


Figure 35: Subcutaneous, pre-tumor treatment with peptide-loaded nanoparticles results in a survival advantage as compared to mock treated animals in the GL261 glioma model. Mice were treated 14 and 7 days before tumor inoculation with 5 x 10^5 GL261 cells. Treatment consisted of a mixture of the CD8 immunodominant peptides of 3 different onco-proteins (gp100, TRP-2 and EphA2) conjugated to the surface of polystyrene NPs and injected subcutaneously. Statistical significance was calculated by Log-rank test, * $p < 0.05$

4.2.12 Therapeutic treatment by combining temozolomide and nanovaccine injections improves survival outcome in glioma-bearing mice

In order to create a more translational treatment strategy, a therapeutic nanovaccine therapy after tumor inoculation was pursued. Due to aggressive tumor growth and based on the results obtained in the autologous tumor lysate chapter, we hypothesised starting nanovaccine treatment alone, even early after tumor inoculation, to be ineffective. To address this problem, nanovaccine treatment was combined with another treatment modality to develop a successful therapeutic treatment strategy. The combination of nanovaccine and TMZ chemotherapy, a current standard drug for GBM patients, was pursued. Treatment was started after tumor inoculation, with six applications of TMZ 20 mg/kg bodyweight between day 5 and 16

followed by nanovaccine injections at day 21, 28 and 35. Although the combination therapy improved survival outcome by increasing median survival from 25 days post tumor inoculation for mock treated animals to 37 days, no difference in survival benefit was observed in comparison to TMZ monotherapy (**Fig. 36A**). In contrast to low dose TMZ applications, the use of an intermediate dosage of TMZ (40 mg/kg bodyweight) followed by nanovaccine administration did result in further improvement of median survival as compared to TMZ alone treatment (**Fig. 36B**). TMZ monotherapy in this experiment doubled the median survival in comparison to mock treated animals, but the mice in the treatment group TMZ + nanovaccine survived 22% longer than animals in the TMZ monotherapy group.

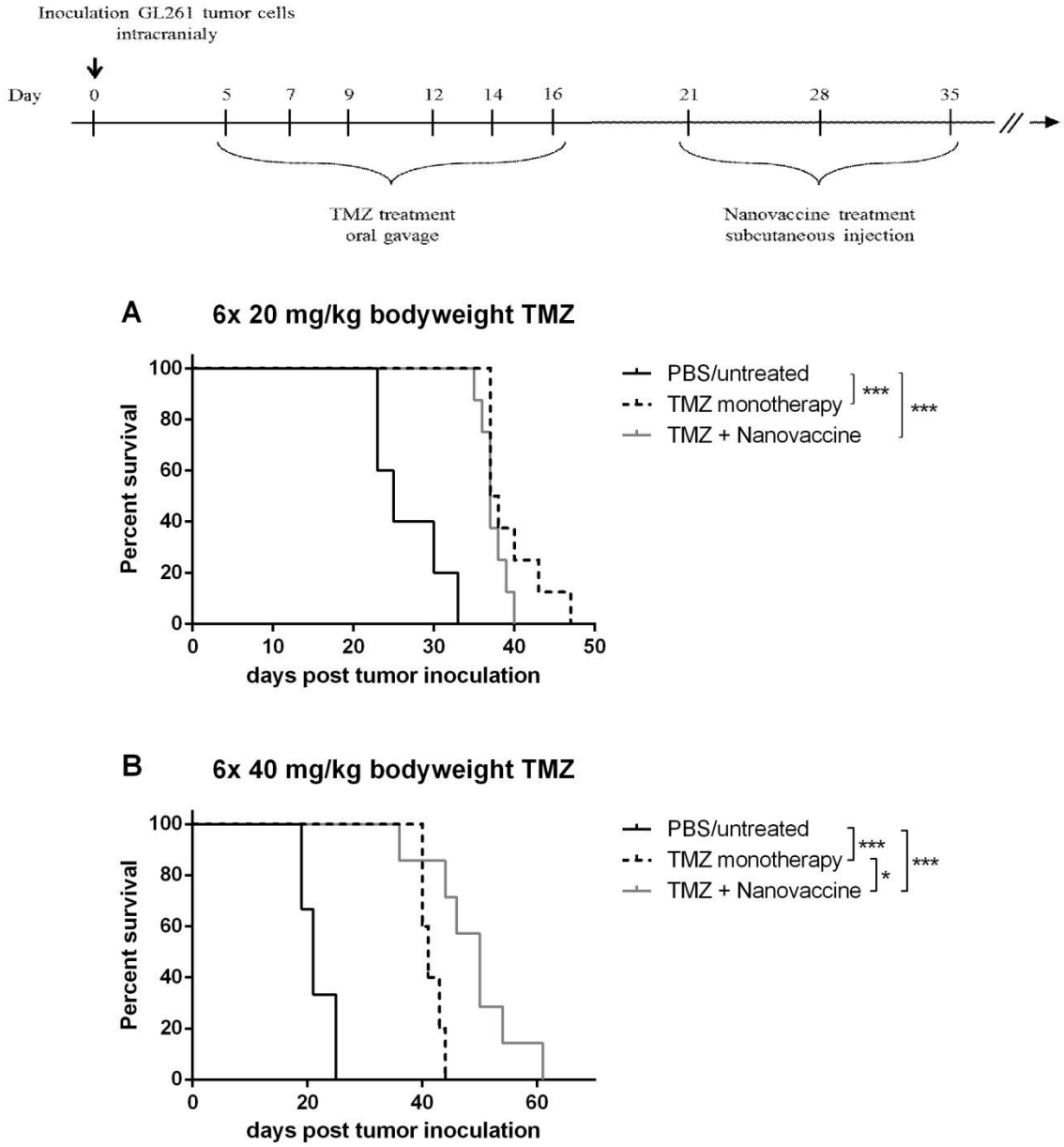


Figure 36: Combining temozolomide and nanovaccine injection in a therapeutic treatment improves median survival of glioma-bearing mice. To introduce a therapeutic treatment strategy, nanovaccine injection was combined with chemotherapy (temozolomide). First temozolomide was orally administered 6 times between day 5 and 16, followed by subcutaneous lysate injection at days 21, 28 and 35. Two different dosages of temozolomide were tested: a low dose of 20 mg/kg body weight **(A)** and an intermediate dose of 40 mg/kg body weight **(B)**. The combination of temozolomide and nanovaccine was compared to PBS treated controls and temozolomide monotherapy. In the PBS control group n=5 and in the experimental conditions n=8. Statistical significance was calculated by Log-rank test, * $p < 0.05$; *** $p < 0.001$

5 Discussion & Conclusion

Unfortunately, current treatment strategies in GBM result in limited survival benefit for patients. For long, gliomas were considered to be localized in an immune-privileged environment (270), but in the last years this view has changed. The brain is now accepted as a dynamic immunological environment (271), which changed the view that immunotherapeutic strategies might indeed improve the poor perspective of GBM patients. Amongst these strategies DC-based active immunotherapy, with either *ex vivo* grown DCs or *in vivo* targeting of these cells, is considered to be one of the contenders to end up as an adjuvant therapy next to the standard of care in GBM. The research described in this thesis aims to contribute to the knowledge that is gathered regarding DC immunotherapy in GBM and more specifically through *in vivo* targeting of these DCs. Therefore the potential of immunogenic autologous whole tumor lysate to induce an anti-tumor immune response was studied. Following proof of antigen internalisation by DCs *in vitro* and subsequent antigen processing and presentation, a positive survival effect in preclinical murine glioma models was observed by subcutaneous lysate injections. The immune response was characterized by a brain T cell influx, reduced immunosuppression and a tumor-specific immunological memory. Besides, combinatorial treatment strategies of autologous lysate vaccination with TMZ chemotherapy or PD-1 immune checkpoint blockade lead to ameliorated survival outcome in glioma-bearing mice. The second part builds upon the success of the first part while even attempting to improve it. For this, lysate was stably conjugated to the surface of NPs to create a nanovaccine. After an optimisation process judging size, antigen conjugation, *in vitro* DC internalisation and, in a limited manner, toxicity, the most ideal candidate was tested in two preclinical glioma mouse models. Comparable results as for autologous lysate treatment were observed, showing survival benefit of subcutaneous nanovaccine administrations with a long-lasting immunological memory. Again, brain T cell influx and diminished immune suppression were part of the immune response. Finally, a multimodal treatment strategy combining TMZ chemotherapy and nanovaccine injections resulted in a survival benefit as compared to monotherapeutic treatment.

The general aim of the performed research was to provide an alternative treatment strategy for GBM by using *in vivo* passive targeting of antigens to DCs instead of utilizing *ex vivo* grown DCs. The latter has been studied for nearly 2 decades and has not led to any major breakthroughs in GBM therapy. Survival benefit has been shown in multiple clinical trials, but in general it remains limited to a fraction of long term survivors and this despite the presence of objective immune responses like IFN-γ producing CTLs (112, 113). The motive for abandoning *ex vivo* loaded DC vaccines for this thesis can be found in both a potentially decreased potency of cultured DCs to induce an anti-tumor immune response and practical economic reasons. The already mentioned variable response rates can in part be assigned to a limited yield and viability of DCs *in vitro*, an incomplete uptake of antigens/lysate fragments, the potential absence of accompanying maturation stimuli and decreased homing to lymphoid

tissue once injected (139, 140). For example, it has been shown that injected DCs act indirectly through *in vivo* DCs. Moreover, *in vitro* DC culturing is a time-consuming, labour-intensive process requiring sterile handling and thus leading to an expensive vaccine. Besides, every step less in manipulation is a step further away from possible mistakes and contamination. A final incentive is the future possibility to create a broader applicable product which is not limited to an autologous setting. Still, moving from standard DC immunotherapy to tumor lysate vaccination is not an evident step. Some publications report the use of tumor lysate, but as a negative control condition (272-274), while other studies report on lysate treatment effect in different cancer types like melanoma, prostate cancer, glioma, *etc.* (272, 275, 276). Nevertheless, these latter studies nearly all make use of adjuvants added to the lysate e.g. cytokines such as GM-CSF and IL12 (273) HSPs (276) or CpG ODN (277). Investigating the potential of autologous tumor lysate treatment in GBM is further supported by the fact that in some preclinical and clinical studies testing DC immunotherapy, prime-boost treatment schedules are used where boosting is performed with lysate alone (146, 278). The only publication showing a positive effect of autologous lysate treatment, describes a human clinical trial for treating renal cell carcinoma after nephrectomy (279, 280). Autologous tumor cell vaccination in comparison to no additional treatment resulted in an improved survival in T3 staged patients. Importantly, lysate immunogenicity was improved by growing the isolated tumor cells in medium supplemented with IFN-γ and tocopherol acetate. The idea for this thesis to use autologous lysate in the absence of *ex vivo* cultured DCs and even an adjuvant, originally arose from our background knowledge in the immunogenicity of tumor lysate. Previous work within our research group stressed out the importance of lysate preparation and what impact it has on immunogenicity (137, 262). It was shown that both freeze/thawing tumor cells followed by X-ray irradiation and hypericin induced photodynamic therapy (Hyp-PDT) resulted in immunogenic lysate conditions. In general it is considered of great importance to induce oxidative stress of the ER which leads to the creation of OAMPs and thus generates immunogenic lysate (138). By irradiation of tumor cells, ROS are produced that can induce this required ER-stress in an indirect way. On the other hand, Hyp-PDT is a known ICD-inducer leading to ER-specific oxidative stress. For this reason lysate generated by Hyp-PDT is considered to have superior immunogenicity, while in the case of tumor cell irradiation, immunogenicity is considered collateral ‘damage’. However, Hyp-PDT treatment was not able to kill all tumor cells and this opposes the risk of subcutaneous cancer growth following subcutaneous lysate injection. Therefore, in the current research we started from the hypothesis that freeze/thaw lysate that was irradiated after lysing the cells contains enough activation molecules to load and mature the host’s DCs *in vivo*, as in this way it has been shown that among other things protein carbonylation occurs (75, 281).

With the rising of nanotechnology in the field of medicine, a renewed interest in tumor lysate vaccination was awoken. Targeting DCs *in vivo* is definitely a task NPs can undertake, hence it should not come as a surprise that nanoimmunotherapy would provide the continuation of the autologous lysate project. The main advantages for using NPs in active immunotherapy

are their variable physicochemical properties, cargo protection from degradation, multicomponent loading and their displaying of several adjuvant functions (282, 283). With the current research we aimed for a feasibility proof-of-concept study for using lysate-loaded NPs in the treatment of GBM that can be expanded in future. The restricted work employed easy to obtain polystyrene NPs surface-loaded with whole tumor lysate without the addition of targeting molecules nor adjuvants. Many reasons justify the use of these non-biodegradable, bio-inert polystyrene NPs for an explorative study implementing nanotechnology in the active immunotherapy studied within our lab at that time. Next to liposomes, polystyrene particles have already been used for over half a century in the exploration of cell uptake (284). Above all, these NPs were chosen because of commercial availability with a large variety of characteristics with the most important being diversity in size with the presence of fluorescent NPs within the same size, their uniform sizes within a batch (50 ± 3 nm for the particles used within this project) and several surface functionalisation groups. The NPs within the project were surface carboxylated, which enables the conjugation of lysate fragments to the particles using a stable covalent (peptide) bond in combination with the coupling molecules EDAC and NHS. These exact same polystyrene NPs were already shown to be able to conjugate OVA and this way create an *in vivo* immune response following intradermal injection which can protect from tumor growth and treat existing tumors (205-207). Liposomes, one of the most abundantly used NP groups, have an extra potential disadvantage as shown in an article of Herber *et al.* where they state that lipid accumulation within DCs abrogates the functional activity of these cells (285).

One of the most important factors determining cellular uptake is size of the cargo that needs to be internalized. As stated in literature, the optimal 'maximum' size for NP-lysate conjugates would need to be below 500 nm (197). Using polystyrene particles, Foged *et al.* reported optimal uptake by DCs of 0.5 μm particles, with an uptake predominantly occurring for particles of 0.5 μm and smaller. Indeed, within the size range of 200 to 500 nm DCs have been shown to have very high endocytic activity, while larger complexes are mainly scavenged by macrophages which very actively phagocyte larger particles (62, 201). Therefore, the first aim in our NP study was to develop NP-lysate conjugates within the stated size range. Only when gel filtration was implemented during the conjugation protocol, NP-lysate complexes of desirable size were generated. Moreover, an impact of gel filtration and possibly size was detected when DCs were cultured *in vitro* and loaded with NP-lysate conjugates. In the case no gel filtration was performed during the production of these conjugates, and thus much larger complexes were formed, most likely due to cross-linkage, a devastating effect was seen for the DCs with many cells showing signs of cell death according to microscopy. The importance of size can also be distilled out of the limited biodistribution data presented in this dissertation. Comparing the presence of a fluorescent signal in $CD11c^+$ DCs in the draining lymph nodes after subcutaneous injection of fluorescent NPs, FITC labelled lysate or NP(GF)-lysate-FITC complexes showed a difference in the earliest detection of this fluorescent signal. In the case fluorescent NPs were injected, the fluorescent

signal was already detected within 12 hours following the administration. On the other hand, larger payloads, FITC-lysate and NP(GF)-lysate-FITC conjugates, required more time to reach the local lymph nodes. This observation is also in line with the published results of other research groups that conclude DCs to prefer the incorporation of particulate antigens as compared to free antigens (67).

A fair question to ask is to what extent free lysate might affect the observations *in vitro* and *in vivo*. Although we have tried to limit this influence, we cannot exclude the presence of unbound lysate fragments in the NP-lysate conjugate conditions. Firstly, by working with a larger amount of NPs *in vitro* (10 mg/ml), nearly all of the protein originating antigens are conjugated to these particles. This idea was supported by the observation that nearly no signal was present in gel electrophoresis experiment when this amount of NPs was used to generate NP-lysate complexes. Utilizing EDAC-NHS coupling of amine groups from the lysate to carboxylated polystyrene NPs creates a stable covalent (peptide-) bond (286). This way, dissociation of antigens from the NPs can be excluded and thus the *in vitro* results of internalisation and MHC-I presentation can largely be assigned to NP-lysate conjugates. Secondly, the conjugation protocol does not allow all molecules of the lysate to be coupled to NPs and is largely limited to protein and peptide antigens. This does not necessarily have to be a disadvantage as many other molecules in the lysate such as mitochondrial DNA might have a positive effect as well, functioning as DAMPs for the induction of the immunological response (75).

Regrettably, due to the absence of an inherent adjuvant function of the polystyrene NPs within our hands, subcutaneous autologous lysate treatment and nanovaccine injections resulted in equivalent survival benefit. Nevertheless, the choice for these polystyrene NPs was in part based on the adjuvant function as described by Fifis *et al.* in literature (205). A possible reason for the ‘loss’ of this adjuvant function might be the proportion of NP size against that of lysate. In the article of Fifis *et al.* OVA was coupled to the NPs confinely increasing their size up to several ten nanometers, whereas in our research the same NPs were conjugated with lysate which increased the size to a few hundred nanometers. In one experiment, we investigated the co-injection of nanovaccine with an adjuvant. For this reason different TLR ligands (CpG ODN, R848 and Poly(I:C)) were tested individually in combination with nanovaccine treatment. The conclusion was unambiguously, at the concentrations used co-injection of our nanovaccine and one of the mentioned adjuvants was unsuccessful. Based on survival outcome mice that were administered the combination treatment did worse than nanovaccine treated animals and showed comparable survival as mock treated mice. In literature free TLR ligands have been described to induce systemic inflammation (287). Once injected these ligands can be drained to the local lymph nodes, where a massive cytokine release can take place. This cytokine storm is able to generate mature DCs and immunotolerant DCs which results in less antigen internalisation and thus antagonizes the nanovaccine activity (288). Besides, most TLR ligands can induce serious side effects when

administered in a high dose. As an example, repeated CpG ODN injections in mice can cause hepatic toxicity and severe damage to lymphoid tissue (289). In another study repeated TLR9 stimulation with CpG ODN has led to a macrophage activation syndrome in mice characterized by massive inflammation followed by multiple organ failure and eventually death (290). The loss of two mice out of six in the CpG treated group without any clear clinical symptoms of tumor growth, but also the rapid decease of the other animals in this treatment group might be explained in this way. In the other two conditions, using R848 and Poly(I:C), the antagonistic effect of TLR ligand injection to nanovaccine treatment might also be explained by the non-specific immune activation and/or toxicity that may result upon administration of those compounds. Particulate delivery of TLR ligands might offer a sustained release of these TLR ligands decreasing the negative effect due to repeated and high dose administrations (291). In mice, induction of T cell responses to OVA antigen was observed with a 10-100 fold lower dose of CpG ODN in comparison to free TLR ligand injection.

We consider the antigenic source that will generate the immune response to be of great importance in this project. As already stated in **Table 1** of the introduction, antigens of different origins can be used, each with its advantageous and disadvantageous. Using whole tumor cell lysate instead of one or more antigens in this research was a conscious choice because of the heterogeneity of GBM tumors, both interindividual as intraindividual (292, 293). Besides, loss of target-antigen expression by cancer cells, a known mechanism of tumors to escape an immune response, supports the use of whole tumor lysate instead of a certain amount of TSAs or TAAs (294). Although, the main focus in the project was on whole tumor cell lysate vaccination, the use of three tumor antigens in the form of $CD8^+$ immunodominant peptides was also tested. In contrast to literature, where free peptide injection is stated to be a far less effective antigen source as tumor lysate, pre-tumor peptide injection was able to improve survival of glioma-bearing mice as compared to mock treated animals. One study showing endogenous processing and presentation of antigens is far more efficient than presentation of free peptides concludes apoptotic cell presentation to DCs to be more efficient (even up to 50,000 times) as compared to free peptides (295). Next to the type of antigen used, the source of the antigenic material has also been described to be of significance. This is largely determined by the type of antigen used: whole tumor lysate treatment will always be executed in an autologous setting, while nearly all studies describing the use of TSAs or TAAs are performed in an allogenic setting. Unfortunately, we haven't found any study in literature directly comparing autologous and allogenic immunotherapy in GBM, it seems to be merely a deliberated choice of each research group. For whole tumor lysate vaccination, a third variable in the antigenic payload can be the origin of tumor cells from which lysate is generated. Cancer stem cells have gained tremendous interest for therapy targeting as well as an antigen source (28). As compared to non-stem cells, these glioma stem cells may express higher levels of TAAs and MHC molecules (296, 297). In one preclinical study using the GL261 glioma model, Pellegatta *et al.* have shown benefit of DC vaccination

with glioma stem cell lysate as compared to DC vaccination with non-stem cell lysate both on survival as on immunoreactivity (298).

Focussing on the nanoimmunotherapy project, it should be noted that NPs can affect every step of DC induced immunity (282). a) To begin, NPs can have a positive effect on DC maturation for example through co-delivery of antigen and adjuvant. Moreover, naked NPs have also been shown to increase the expression of DC maturation markers significantly. One research group in Osaka has developed and extensively studied biodegradable γ-PGA NPs which they have shown to induce both innate and adaptive immunity through TLR4 and MyD88 signalling pathway (299, 300). b) A second DC function affected by NPs is the homing capacity of these cells to lymphoid organs where the cells can localize closely to the T cells. One publication describes increased expression of CCR7 by *ex vivo* loaded DCs with gold NPs that co-deliver OVA antigen and CpG-ODN adjuvant (301). c) Moreover, NPs can enhance uptake, antigen processing and cross-presentation of 'transported' antigens. Foged *et al.* have shown superior capacity of NPs to induce cross-presentation, up till a 1000-fold lower antigen concentrations as compared to free antigens (302). d) Finally, DC induced T cell differentiation can be affected by NPs. This way NPs are able to polarize the T cell response to a Th1, a Th2 or even a Th17 response (303). Other advantages of antigen presentation to DCs in a particulate form over soluble antigen presentation are prolonged presence of antigenic material and improvement of the strength of the immune response by delivery of multiple TSAs and/or TAAs. Although the ideal source of multi-antigenic material would be represented by whole tumor lysate, studies showing real efficacy are limited due to the presence of large amounts of interfering molecules such as: housekeeping proteins, carbohydrates, nucleic acids, lipids, and other intracellular contents (304). Unfortunately, the use of whole tumor lysate introduced important drawbacks into our nanoimmunotherapy study. First, due to the use of tumor lysate, we were unable to quantitate the amount of lysate conjugated to the NPs. Therefore an indirect measurement of the amount of free lysate was performed using SDS-PAGE and Coomassie blue staining. The amount of free protein fragments was hypothesized to be correlated to the amount of free lysate fragments. Less free protein fragments in the gel for NP(GF)-L conjugates as compared to lysate alone gave a confined qualitative idea of conjugation. Due to practical reasons, explained above, lysate loaded NPs are harder to use than single or multiple antigen loaded NPs. One solution to this is the use of lysate encapsulation within nanocarriers and therefore most literature describing lysate loaded NPs handles lysate encapsulated NPs (236-238). In the setting of nanoimmunotherapy, we therefore hypothesized the concentration/amount of lysate necessary to induce an immune response can be further decreased as compared to autologous lysate vaccination. This was not the case, although in the CT2A glioma model an insignificant survival improvement at half the concentration was observed (**Fig. 27B**). Perhaps with some improvements of the nanovaccine, this effect at lower concentration can be exploited. Moreover, in future, when more TSA are to be discovered, we can move away from the necessity of lysate. Secondly, due to the choice of working with whole tumor lysate, we were

not able to implement a purification step of NP-lysate conjugates at the end of the conjugation process. Some research groups are able to use e.g. dialysis or centrifugation to remove non-coupled antigen fragments from their NP-antigen complexes (254, 255, 286). Due to the very variable size of tumor lysate (data not shown), it was impossible to separate free lysate fragments from NP-lysate conjugates.

In the current study we used two fully immunocompetent glioma mouse models. Both the GL261 model and the CT2A model are syngeneic cell lines for C57BL/6 mice, but they originate from different cells: the former resembling more of an ependymoblastoma and the latter being more of an anaplastic astrocytoma (245). The murine GL261 glioma model is the standard model to test immunotherapy in GBM (264). CT2A cells resemble more the GBM phenotype due to an invasive nature, high mitotic index and cell density (252). Comparing the survival benefit of autologous lysate treatment in the GL261 and the CT2A glioma models in **Figure 9** shows a better protection in the CT2A glioma model. Our hypothesis is that CT2A tumor cells might be more immunogenic as compared to GL261 cells because they have a more stem cell like phenotype. CT2A cells have been described to express stem cell markers like CD133 and Nestin, and are characterized by a more invasive and proliferative *in vivo* growth (252). As earlier described in this discussion, many authors designate cancer stem cells to be the ideal cells for tumor lysate generation (298). Another possible explanation for the better protection of autologous lysate vaccination in the CT2A model, might be the inevitable variability between the *in vivo* experiments. For the *in vivo* experiments of this dissertation, we aimed to keep the variability as small as possible by standardizing as much handlings as possible. However, miniscule differences between the tumor cells used for each experiment might explain the difference between comparable control conditions over the various *in vivo* experiments. For each *in vivo* experiment new tumor cells were cultured. Although all cells originate from the same mother batch and having the same passage number, small differences in *in vitro* cell growth were observed. This observation might be translated to the *in vivo* growth and hence explain the difference seen between comparable control conditions. As a consequence, for the comparison of different experiments, it might be important to check whether the PBS treated control mice show a comparable survival before drawing any conclusions.

Within our therapeutic treatment experiments, TMZ treatment was used as an anti-tumor therapy and followed by immunotherapeutic autologous lysate or nanovaccine injections. On the one hand, TMZ administration can be considered a tool to obtain minimal residual disease when immunotherapy is started. On the other hand, numerous times combining immunotherapy with chemotherapy has been described in literature. Frazier *et al.* published a review summarizing the outcome of different preclinical and clinical combinations of active, specific immunotherapy with chemotherapy for a range of cancers, with amongst them brain tumors (305). Clinical relevance for the given treatment combination is also provided by Shore *et al.* who describe the positive effect of combining both anti-tumor therapy and

immunotherapy in prostate cancer (306). Starting therapy with the anti-tumor effects of chemotherapy diminishes the tumor burden after which the tumor might recur. Lower tumor burden might also result in a tumor more susceptible to immunotherapy and thus immunotherapy can increase patient survival. It is clear from the results in **Figures 17 and 36B** that the combination of TMZ chemotherapy and lysate or nanovaccine injection respectively, creates a potential therapeutic treatment strategy in the GL261 glioma model. Therefore, active immunotherapy with autologous lysate or nanovaccine in our GL261 glioma mouse model can at least be considered to be additive, and although not entirely correct due to the absence of a treatment effect by therapeutic autologous lysate monotherapy, even synergistic to TMZ chemotherapy. As already stated by our therapeutic treatment experiment as well as in literature, the strength of cancer immunotherapy lies in combining different treatment strategies (28). In this context, another treatment combination studied in this dissertation was autologous lysate treatment and anti-PD-1 immune checkpoint blockade. Blocking the negative costimulatory receptor PD-1, showed to be complementary with tumor vaccination in a glioma mouse model (56). Within these mice, increased levels of T cell activity, and especially $CD8^+$ T cells, were observed while Treg suppression was attenuated. Moreover, immune checkpoint blockade therapies can mediate their effect through reactivation of neo-antigen specific T cells (307). Likewise, clinical data suggest an anti-tumor effect due to reactivation of existing neo-epitope specific T cells (308, 309). Our results, depicted in **Figure 18**, support the observations of Antonios *et al.* concerning survival. Although, lysate vaccination in combination with anti-PD-1 mAb administration improved median survival by ±25%, it was clearly less effective than lysate vaccination following TMZ administration (±80% median survival improvement). Unfortunately, the anti-PD-1 monotherapy was not able to improve survival of glioma-bearing mice and thus no statement can be made about potential additive or synergistic effect. This complete immunotherapeutic combination strategy is backed up by numerous clinical trials as depicted in Table 1 of the "Addendum – immunotherapeutic treatment in glioma clinical trials". Taken together, this work as well as literature shows the importance of combining therapeutic strategies in the treatment of gliomas.

Originally, $CD8^+$ T cells were considered the main component of the cellular immune response against cancer (310). Likewise, their contribution in nanoimmunotherapy was considered to be of great importance (**Fig. 37**). One essential step to elicit this $CD8^+$ T cell response is cross-presentation of tumor antigens delivered by the functional nanomaterials, which in his turn requires endosomal escape of the antigens after internalisation by DCs (80, 311). The role of $CD4^+$ T cells was presumed to be limited to enhancing and sustaining the $CD8^+$ T cell and B cell responses and regulation of the immune response acting as Treg cells (105, 310). More recently $CD4^+$ T cells have been pointed out to have more direct roles in cancer immunity with the description of tumor-reactive, cytotoxic $CD4^+$ T cells (312-315). Moreover, some studies like the one performed by Murphy *et al.* describe a $CD8^+$ T cell-independent anti-tumor immune response following tumor lysate vaccination (142, 143).

These observations were obtained in the GL261 glioma mouse model for animals treated with intradermal autologous tumor lysate co-administered with CpG ODN in combination with intraperitoneal OX40 ligand immunoglobuline protein fusion. The anti-tumor response and following prolonged survival, were shown to be $CD4^+$ T cell-, NK cell- and B cell-dependent. Their conclusion is, at least in part, supported on a remaining positive treatment effect within CD8 knock-out animals. A possible explanation for this observation might be assigned to the adjuvants used in the described study, as it is shown that adjuvants are able to direct the immune response in a certain direction (316).

Within our studies, the results of the brain immunocontexture in both the lysate project and the nanoimmunotherapeutic project were largely comparable and for that reason will next be discussed in general. The early observed brain T cell influx at day 7 was described to originate from increased proportions of $CD4^+$ T cells. Moreover a clear decrease in Treg cells and a less pronounced decrease in immunosuppressive MDSCs was observed. Although we have no direct proof, $CD4^+$ T cells in our model could be involved both in sustaining $CD8^+$ T cells as well as having direct cytotoxic effects. Using the kinetic brain immune-contexture study, we were able to detect changes over time with the biggest difference between treated and untreated animals as early as day 7. Although no significant difference in $CD8^+$ T cells was detected between lysate- or nanovaccine-treated *versus* mock-treated mice at any time point, an increase over time of (activated) $CD8^+$ T cells could be observed. We consider this immune influx in the brain of PBS treated mice a tumor response. We hypothesize that the combination of the lower amount of Tregs and MDSCs in combination with the activation of $CD8^+$ T cells later on, is able to attack the tumor in lysate- or nanovaccine-treated mice. Importantly, we should mention a bias in our brain immune cell experiments. In the experiments to study the immunocontexture, not all mice survived up till the moment of analysis: 40-60% of the animals died in the mock-treated group and 20% in the lysate group, whereas all mice in the nanovaccine group survived up till the day of analysis. This might result in slightly skewing of the data as these mice might have had the lowest numbers of (active) T cells in the brain with higher amounts of Tregs. Based on the presented results, we cannot exclude the contribution of B cells to the immune response in the glioma mouse models. Due to the presence of the blood-brain barrier, antibody permeability is restricted and therefore the humoral response is largely ignored in brain tumor immunotherapy literature (317). This contrasts for example with haematological malignancies, in which antibody responses are considered to have an important part in cancer treatment (318).

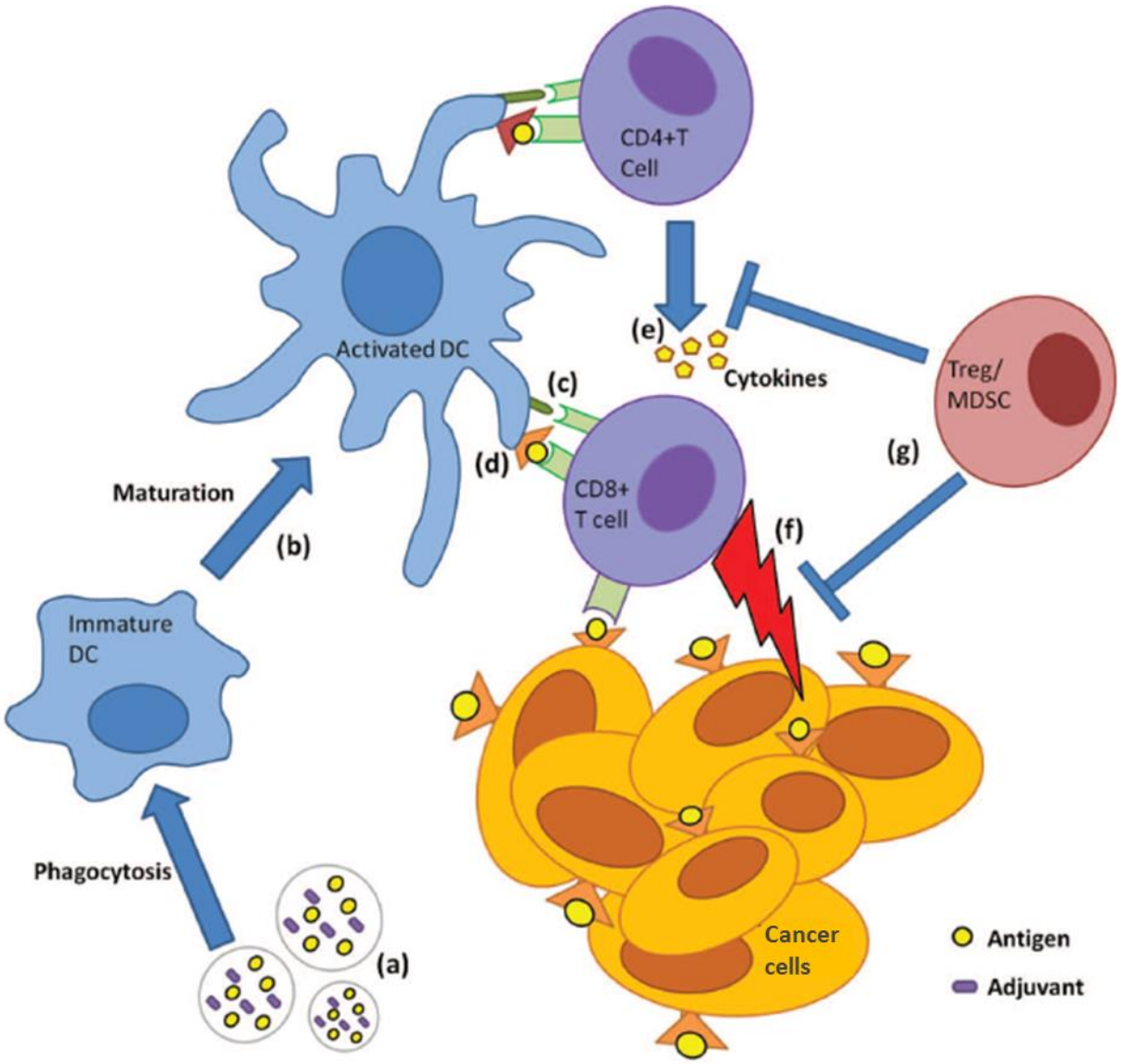


Figure 37. Schematic representation of the induction of a CD8$^+$ T cell response by antigen-loaded nanoparticles. Following internalisation of NPs that co-deliver antigen and an adjuvant **(a)** by DCs, maturation of these cells takes place **(b)**. Co-stimulatory molecules **(c)** as well as MHC class I molecules cross-presenting the antigen **(d)** are expressed at the surface of DCs and interact with CD8$^+$ T cells which become activated, cytotoxic T cells under impulse of cytokines secreted by activated CD4$^+$ T cells **(e)**. Cytotoxic T cells travel from lymphoid tissue to tumor sites and eradicate antigen expressing cancerous cells **(f)**. To be more complete, suppressor cells like MDSC and Tregs are able to dampen the generated immune response by secretion of inhibitory cytokines that prevent effector T cell function.
(Adapted and modified with permission from Joshi et al. 2013 (319))

The administration route can be of great importance for immunotherapeutic treatment strategies and especially for active immunotherapy targeting DCs. DCs in particular reside more in the dermis and subdermis, and especially DCs that are described to have good cross-presentation characteristics such as Langerhans cells (320, 321). For this reason lysate

vaccination and nanovaccine administration were performed subcutaneously, to passively reach these skin resident DCs. Another possibility would have been to vaccinate intradermally, but in mice it is very difficult to distinct between subcutaneous and intradermal injection (322). In these small rodent it is even that hard that the scientific community considers these two administration routes to be comparable, with the exception of footpad injection, which is stated to be the only 100% intradermal injection (323). In the nanovaccine project, next to the administration route, the size can determine transportation of NPs following intradermal or subcutaneous injection. Several literature articles have studied this size-dependent 'transportation' of NPs with the main conclusion being small NPs (<100 nm) to drain passively to local lymph nodes via lymphatics, while larger particles can be internalized by DCs and are actively transported to lymph nodes (199, 201). Of major importance is the observation by multiple research groups that retention of nanovaccines in the skin occurs and can be perceived as a bump at the site of injection (324-327). The generated depot effect can work in favour of the induction of an immune response against antigens delivered in this way. The skin retention can be considered a place of sustained release and thus an adjuvant property of nanovaccine injection. However, within our hands, the subcutaneous injection of tumor lysate also resulted in a bump that remained for a few days an can serve as a depot for antigen uptake by DCs. Therefore we concluded that for both stories, the lysate alone injection and the NP-lysate conjugates, a potential depot effect might play a very important role (**Fig. 38**). Unfortunately, we were only able to show this depot effect up to the uptake of DCs.

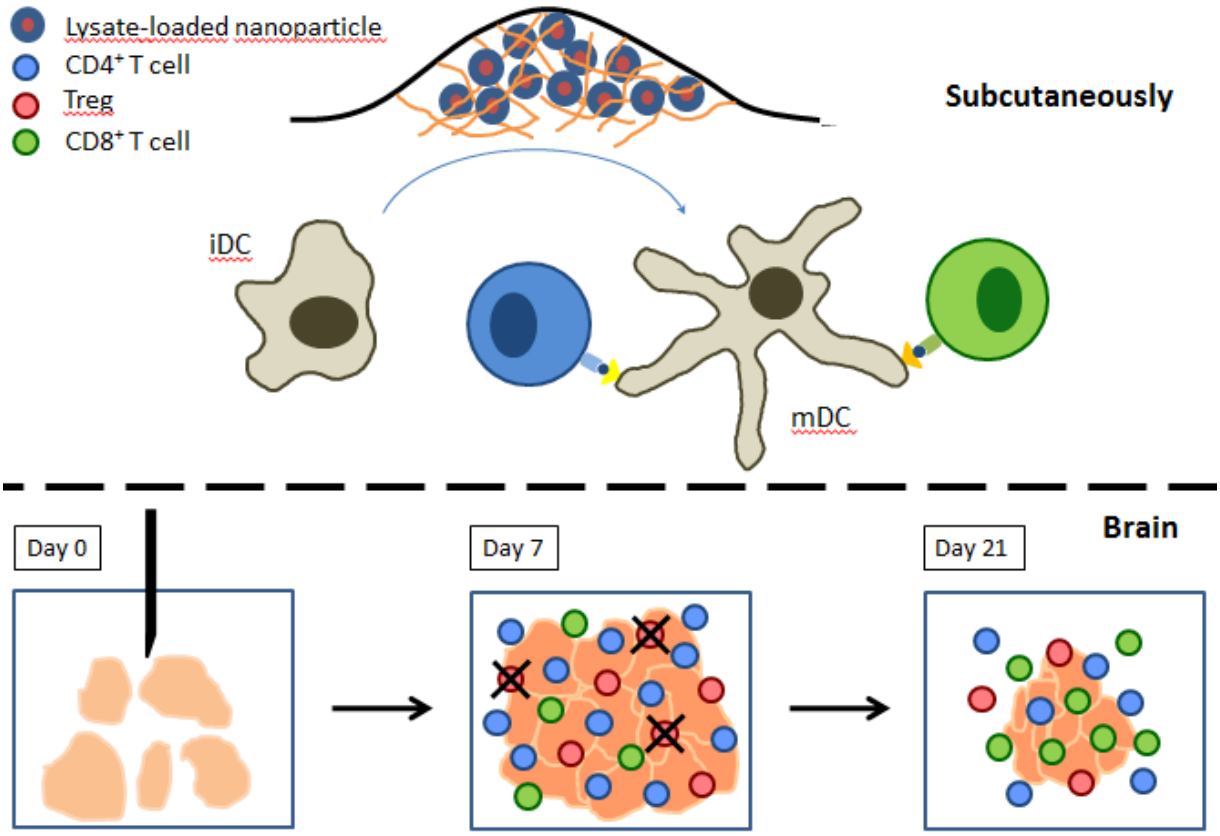


Figure 38. Hypothetical mechanism of action for nanovaccine treatment in glioma-bearing mice. (upper part) Subcutaneous injection of lysate-loaded NPs leads to the generation of a depot because nanovaccine particles, due to their size, are not able to freely move through the extracellular matrix. Immature DCs have the opportunity to sample antigen-loaded particles at the depot in the skin and differentiate to mature DCs which can activate $CD4^+$ T cells as well as $CD8^+$ T cells. These T cells can move to the tumor site to carry out their function. Importantly, the same hypothesis can be considered for lysate treatment. **(lower part)** At day 0, tumor cells are injected into the brain of pre-treated mice where these cells will create a tumor within a few days. Already 7 days after tumor inoculation treated animals show an increase in T cells (especially $CD4^+$ T cells) in combination with a decrease in Treg cells. Over time, moving to day 21, the population of CD8+ T cells becomes more abundantly present.

Finally, we are aware of some general drawbacks of the studies described in this dissertation. A first drawback is the absence of the investigation of histopathological sections of the tumor site in the brain and the injection site in the skin. Although flow cytometry indicates attraction of immune cells to the tumor, the possibility exists that they remain in the peri-tumoral environment without infiltrating the tumor itself. However, the study of tumor infiltrating cells results in more quantitative information and is generally accepted (56, 137, 262, 328). Our data are compatible with T cell activation as demonstrated here by IFN-γ production. A second drawback of our study is that we focus on murine survival which gives no complete insight in the mechanistical events underlying the survival advantage. However, we have clear indication of T cell activation and given the strong differences in murine survival depending on tumor lysate injection and nanovaccine administration, both in pre-tumor implementation

and in post-tumor implantation settings (when combined with TMZ), we consider these findings strong enough to potentially find their way into clinical setting. Another shortage in this work is the very limited focus on toxicity of NPs. We are aware that polystyrene NPs can induce certain toxicity problems. Comparable polystyrene NPs, although with another surface functional group (amine instead of carboxyl), were tested *in vitro* by Anguissola *et al.* and could not show detrimental effects on different organ cell lines (329). However, NPs may act in a different way within an organism than *in vitro*, showing the necessity for future toxicology studies within this project. Considering all the *in vivo* experiments performed during this research and limiting our observations to visual irritation at the injection site, none of the nanovaccine-treated animals showed toxicity except for the mice treated with the co-injection of nanovaccine and adjuvants. In these animals irritation was clearly present as the animals started scratching extensively at the injection site during grooming. The latter observation might also be caused by the high concentration of these adjuvants. Nevertheless, the first next step in the nanovaccine project would be to convert to biodegradable NPs. People who would like to gain more information about nanotoxicology are referred to literature from Oberdörster *et al.* (330-332).

6 Global conclusion and future perspectives

The presented work demonstrated that active immunotherapy using autologous tumor lysate and targeting DCs *in vivo* can dose-dependently initiate an immune response that suppresses tumor growth of orthotopic gliomas. Therefore immunogenic lysate was produced by combining freeze/thawing of tumor cells with high dose gamma irradiation and was injected subcutaneously. The effectiveness of lysate treatment for gliomas offers a time and cost-effective approach in comparison to DC therapy, and thus it should be considered as a potential adjuvant treatment for glioma. Moreover, we aimed at refinement of this treatment strategy and to improve therapeutic effects on gliomas by implementation of NPs. Nanoimmunotherapy forms an attractive path for combining lysate delivery and adjuvant properties. Therefore the immunogenic lysate was stably conjugated to the surface of polystyrene NPs. Passive targeting of these NP-lysate conjugates was aimed for by means of size as DCs have been described to preferably internalise particulate antigens with a size below 500 nm. Unfortunately, within our hands, no improvement in survival was observed of nanovaccine administration as compared to tumor lysate vaccination. A major advantage of nanocarriers is the possibility of multicomponent loading. This way the immunostimulatory function and endocytosis by DCs can be improved by the addition of immunomodulatory (e.g. adjuvants) and targeting molecules (e.g. DEC205 ligands) respectively to the NPs (316, 333). Importantly, before in human use is possible, NPs will have to be biodegradable. Currently a biodegradable nanocarrier system is being investigated by another PhD student as a continuation of the current project. By development of mesoporous silica NPs with the ability to incorporate lysate within the particles, these NPs can transport lysate fragments to DCs. The large amount of advantages that NPs entail definitely make this nanoimmunotherapy strategy in glioma worthwhile to investigate. To stress out the strength of the nanovaccine project, once more common tumor associated antigens are known for GBM, the possibility exists to use antigen loading instead of whole tumor lysate. This way a universal off-the-shelve product can be created which would be impossible for DC therapy because of the necessity of autologous cells. Besides, it is hypothesised that smaller amounts of lysate/antigen could be used in case they are delivered to DCs *in vivo* by means of NPs that are optimised to generate a strong anti-tumor immune response.

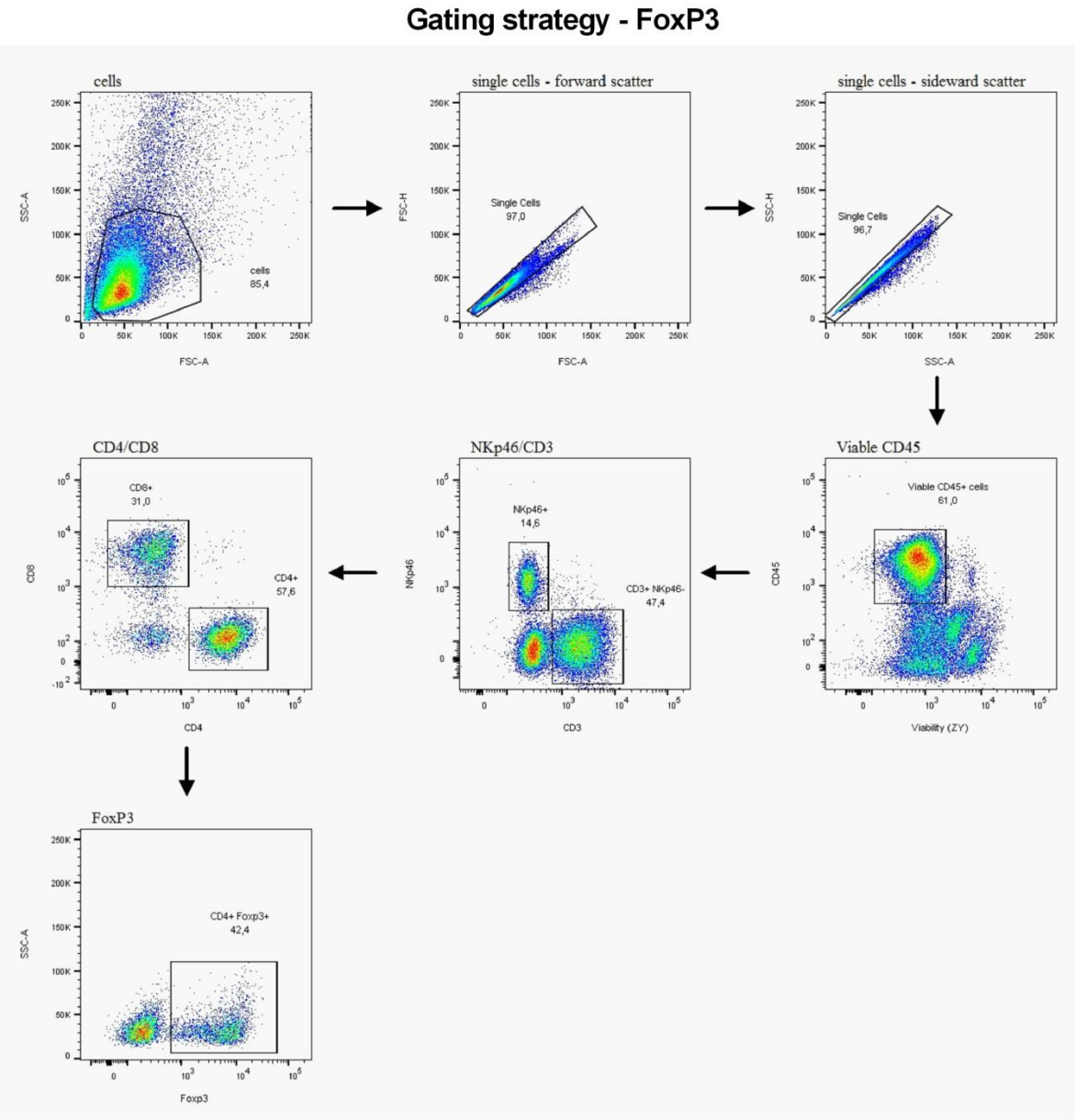
Addendum – immunotherapeutic treatment in glioma clinical trials

Addendum Table 1. Summary of clinical studies using immunotherapeutic therapies in glioblastoma that are discussed in this thesis.

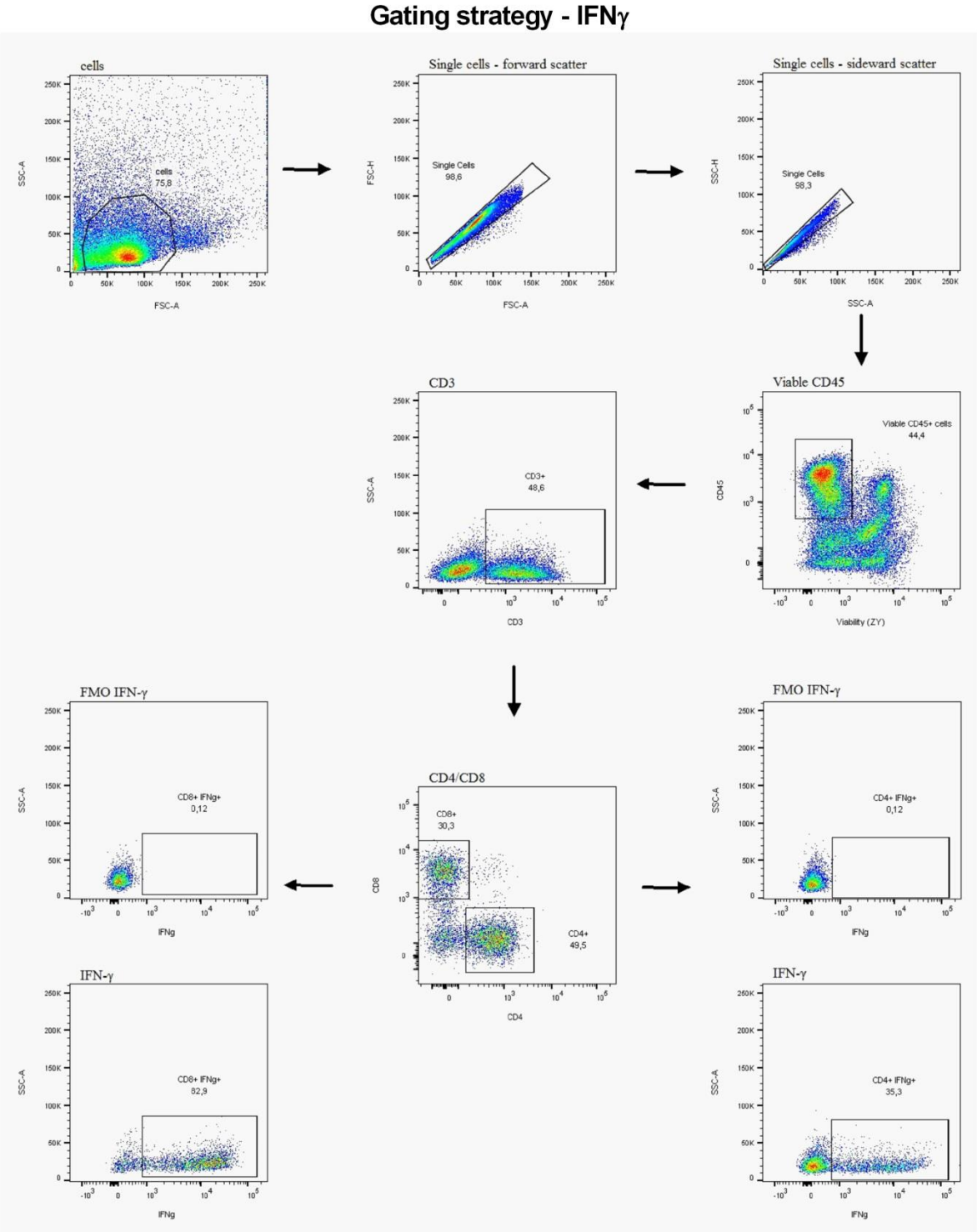
Intervention	Phase	Clinical trial number	Condition
EGFRvIII-directed CAR T cells	Phase I	NCT02209376	Recurrent, EGFRvIII$^+$ GBM
Nivolumab, nivolumab + ipilimumab or bevacizumab	Phase III	NCT02017717	Recurrent GBM
Additive role of nivolumab to radiotherapy	Phase III	NCT02617589	Newly diagnosed GBM (unmethylated MGMT)
Additive role of nivolumab to radio- and chemotherapy	Phase III	NCT02667587	Newly diagnosed GBM (MGMT-methylated)
DC vaccination + nivolumab	Phase II	NCT03014804	Recurrent GBM
DC vaccination + nivolumab	Phase I	NCT02529072	Recurrent malignant gliomas
Rindopepimut + dalizumab	Phase I	NCT00626015	Surgical removed GBM
Rindopepimut + bevacizumab	Phase II	NCT01498328	Recurrent, EGFRvIII$^+$ GBM
DCVax-L (DC vaccination)	Phase III	NCT00045968	Newly diagnosed GBM
ICT-107 (DC vaccination)	Phase III	NCT02546102	Newly diagnosed GBM

CAR T cell, chimeric antigen receptor T cell; DC, dendritic cell; EGFRvIII, epidermal growth factor receptor variant 3; GBM, glioblastoma; MGMT, O^6-methylguanine methyltransferase;

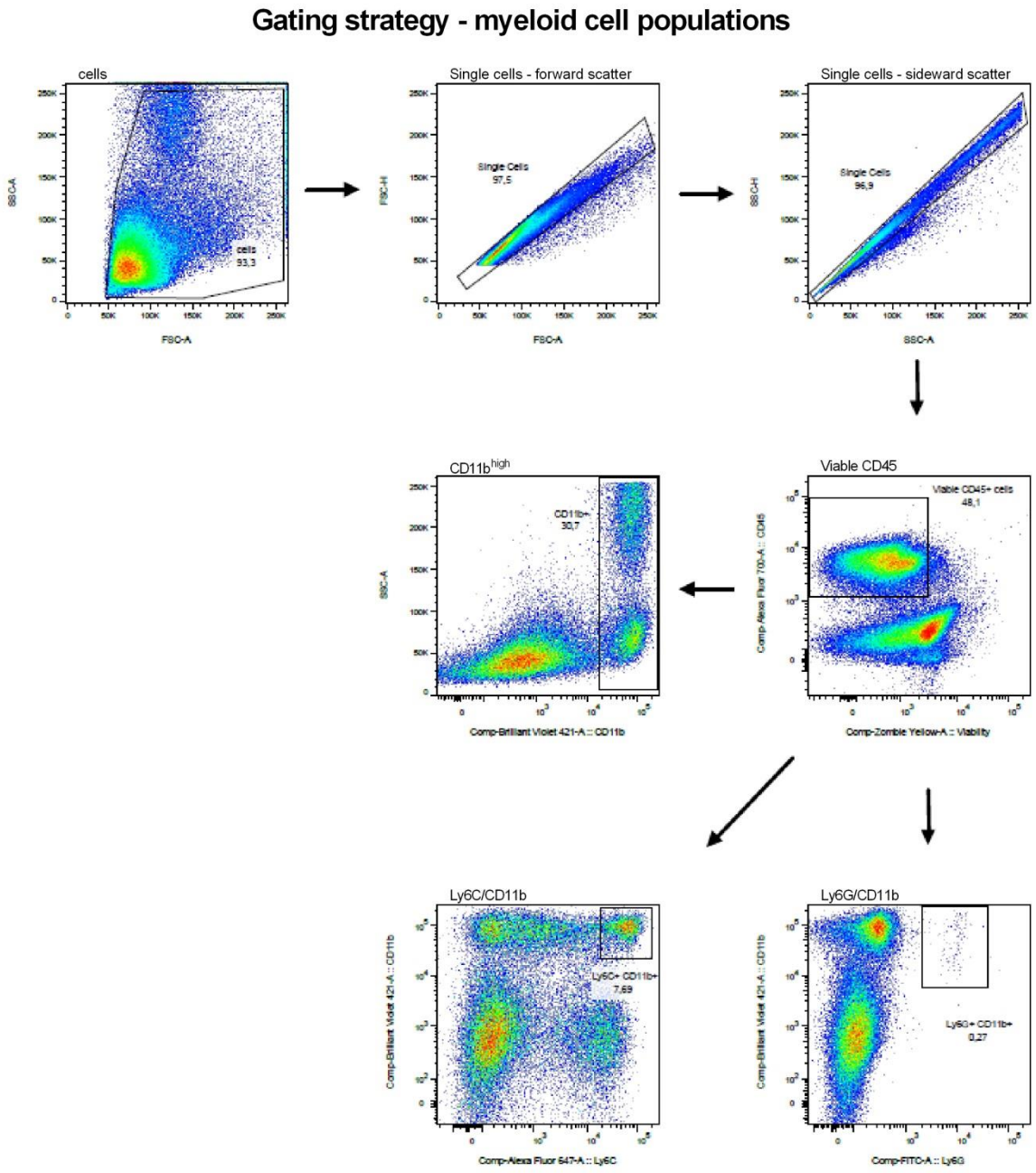
Addendum – flow cytometry information



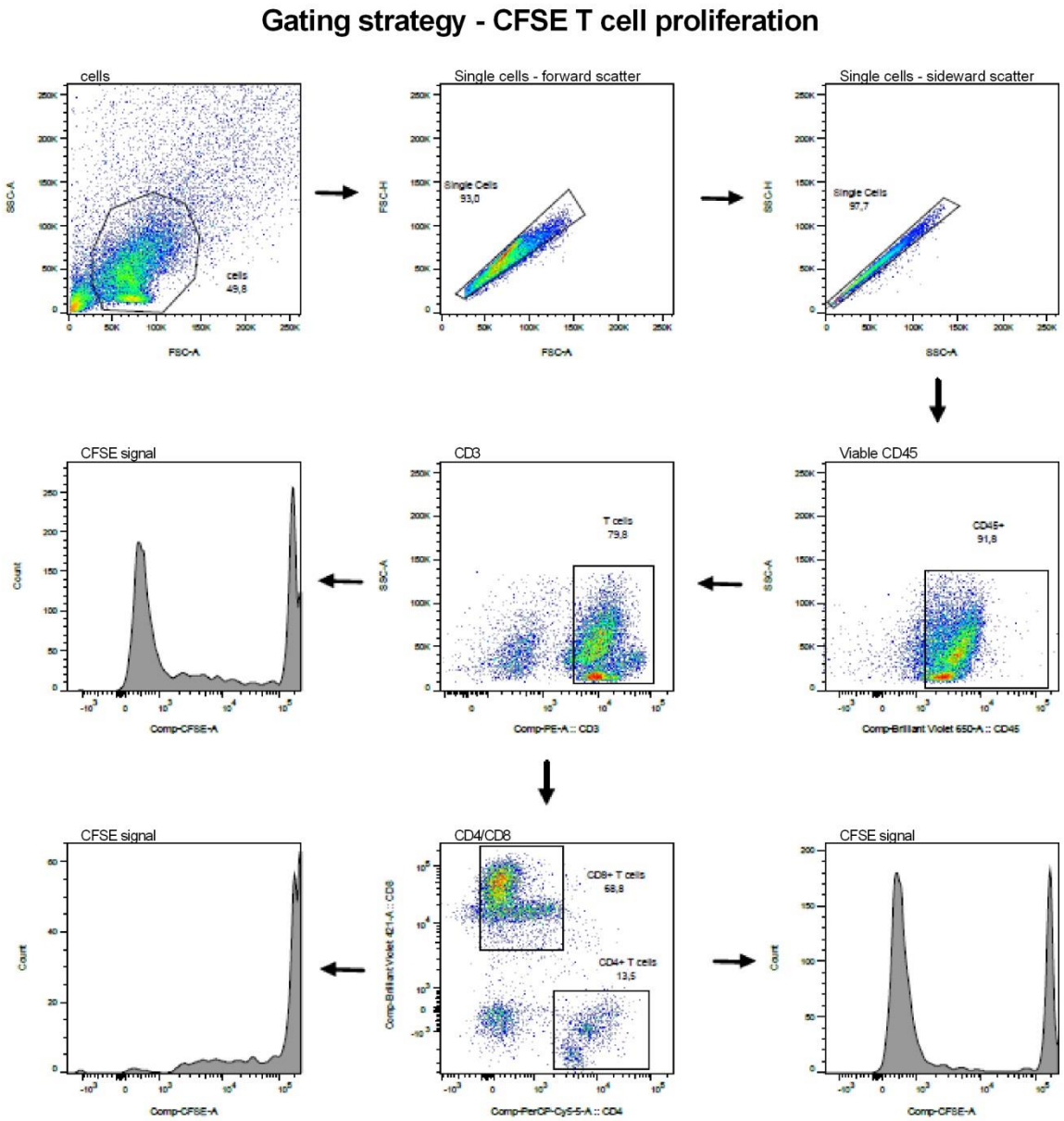
Addendum Figure 1: Gating strategy for flow cytometry of brain regulatory T cell influx. Flow cytometry was performed on brain infiltrating cells of pre-treated mice and gated for cells, to remove debris, single cells (via SSC and FSC), and viable leukocytes (CD45+ and ZY-). Next, the $CD3^{+}NKp46^{-}$ lymphocytes were selected and further subdivided in $CD4^{+}$ and $CD8^{+}$ T cells. Finally, the expression of FoxP3 within the $CD4^{+}$ gate was monitored.



Addendum Figure 2: Flow cytometric gating strategy of IFN-γ producing brain immune cells. Flow cytometry was performed on isolated mononuclear brain infiltrating cells of pre-treated mice and gated for cells (to remove debris), single cells (via SSC and FSC), viable leukocytes (CD45+ ZY-), and $CD3^+$ lymphocytes. Subsequently, we looked into IFN-γ production of CD4 and CD8 T cells. Therefore we used FMO (fluorescence minus one) to determine the proper IFN-γ gating strategy.



Addendum Figure 3: Flow cytometric gating strategy for myeloid derived suppressor cells in the brain of pre-treated glioma-bearing mice. Flow cytometry was performed on isolated mononuclear brain infiltrating cells of pre-treated mice and gated for cells (to remove debris), single cells (via SSC and FSC), viable leukocytes (CD45+ ZY-), and CD11b+ lymphocytes. Subsequently, we looked into MDSC cell populations using Ly6C for monocytic MDSCs and Ly6G for granulocytic MDSCs.



Addendum Figure 4: Flow cytometric gating strategy for CFSE labelled T cell populations. Flow cytometry was performed on spleen cells of pre-treated mice and gated for cells (to remove debris), single cells (via SSC and FSC) and viable leukocytes (CD45+ ZY-). Next, the $CD3^+$ lymphocytes were selected and further subdivided in $CD4^+$ and $CD8^+$ T cells. CFSE labelling was studied in CD3+ lymphocytes as well as CD4+ and CD8+ T cells.

Abstract

Immunotherapeutic strategies for glioblastoma (GBM), the most frequent malignant primary brain tumor, aim to improve its disastrous consequences. On top of the standard treatment, one strategy uses T cell activation by autologous dendritic cells (DC) *ex vivo* loaded with tumor lysate to attack remaining cancer cells. Wondering whether 'targeting' *in vivo* DCs could replace these *ex vivo* ones, immunogenic autologous tumor lysate was used to treat glioma-inoculated mice in the absence of *ex vivo* loaded DCs. Moreover, in an attempt to refine autologous lysate treatment, nanoparticles (NPs) were implemented in this treatment strategy. Therefore, the immunogenic lysate was stably conjugated to the surface of polystyrene NPs, creating a nanovaccine able to passively target DCs by means of its size. Importantly, nanovaccine development was followed by showing the *in vitro* presence of cross-presentation and the potency to stimulate/activate anti-tumoral T cells.

Potential immune mechanisms for both experimental treatment strategies were studied in two orthotopic, immunocompetent murine glioma models. Pre-tumoral subcutaneous lysate injection as well as nanovaccine treatment resulted in a survival benefit comparable to subcutaneous DC therapy. Subcutaneous nanovaccine injection was at least partly followed by drainage to local lymph nodes. Focussing on the immune response, glioma T cell infiltration was observed in parallel with decreased amounts of immunosuppressive cell populations such as regulatory T cells and myeloid derived suppressive cells. Moreover, in both treatment conditions, these results were accompanied by the presence of a strong tumor-specific immunological memory, as shown by complete survival of a second glioblastoma tumor, inoculated 100 days after the first one. Finally, in combination with temozolomide, survival of established gliomas in mice could be increased. Unfortunately, within our hands, no improvement in survival was observed of nanovaccine administration as compared to autologous tumor lysate vaccination.

We hypothesize that the generation of a depot site following subcutaneous injection has an important role to play in the potential mechanism of action for both treatment options. Unfortunately, we were only able to show this depot effect up to the uptake of DCs.

Our results show the potential of immunogenic autologous tumor lysate used to treat murine glioblastoma, which will be worthwhile to study in clinical trials as it has potential as a cost-efficient adjuvant treatment strategy for gliomas. Moreover, using a non-biodegradable nanocarrier system, this work further shows proof of principle to exploit NPs in a nanovaccine treatment for GBM. Bearing in mind the potential advances of using NPs, implementation in DC immunotherapy seems to be a potential future path for active immunotherapy in the treatment of glioma patients.

Summary

Being diagnosed with glioblastoma (GBM) is related to a grim future, with patients showing a median overall survival of less than 15 months despite intensive radio- and chemotherapy. As a consequence, the scientific community is searching for adjuvant treatment modalities that might improve the outcome of this devastating disease. In this regard, immunotherapy has gained great interest. One immunotherapeutic strategy uses dendritic cells (DCs) to generate an anti-tumor response induced by the patient's own immune system. This DC-based active immunotherapy consists of two groups using either *ex vivo* grown DCs or *in vivo* targeting of these cells. The latter condition might be preferred because of efficiency, variable response rates of *ex vivo* grown DCs plus limited migration to lymphoid tissue, as well as practical and economic reasons (time-consuming, labour-intensive process requiring sterile handling leading to an expensive vaccine). In order to induce a broader immune response against the cancer cells and because of the strong heterogeneity of these tumors, whole tumor cell lysate is being preferred as an antigenic source in glioma. Moreover, we were wondering if the tumor lysate vaccination could benefit from the implementation of nanoparticles (NPs) in this treatment. These small particles are able to improve DC immunotherapy for example by their variable physicochemical properties, by combining antigen and adjuvant internalisation and by rendering the opportunity to target DCs *in vivo*. To achieve this goal, a nanovaccine was generated by surface loading polystyrene NPs with whole tumor lysate and this way aiming for a passive targeting of DCs following subcutaneous injection. Based on the knowledge our research group obtained concerning the strength of the immunogenic signature of our lysate product, we investigated the potential effect and immune mechanisms of naked autologous immunogenic tumor lysate and lysate-NP conjugates, without the addition of DCs or adjuvants, to treat GBM in a preclinical mouse model.

Following proof of antigen internalisation by DCs *in vitro* and subsequent antigen processing and presentation, a positive survival effect in preclinical murine glioma models was observed by subcutaneous lysate injections. Next, the process of nanovaccine generation was started by stably conjugating lysate to the surface of NPs. Following an optimisation process which judged size, antigen conjugation, *in vitro* DC internalisation and, in a limited manner, toxicity, the most ideal nanovaccine composition was tested in the glioma mouse models. Comparable results as for autologous lysate treatment were observed, showing survival benefit of subcutaneous nanovaccine administrations. In both treatment modalities, the immune response was characterized by an increase of T cells and a diminished immune suppression in the brain, with the induction of a tumor-specific immunological memory. Finally, combinatorial treatment strategies of autologous lysate vaccination or nanovaccine with TMZ chemotherapy lead to ameliorated survival outcome in glioma-bearing mice.

The presented work demonstrated that subcutaneous injection of autologous whole tumor lysate can dose-dependently initiate an immune response that suppresses tumor growth of gliomas. The effectiveness of lysate treatment for gliomas offers a time and cost-effective

approach in comparison to DC therapy, and thus it should be considered as a potential adjuvant treatment for glioma. With the nanovaccine research we aimed for a feasibility proof-of-concept study for using lysate-loaded NPs in the treatment of GBM that can be expanded in future. Further optimisation of a nanovaccine e.g. by co-delivery of antigens and immune adjuvants or active targeting of DCs, might be the future for autologous lysate vaccination.

Nederlandstalige samenvatting

Tot op de dag van vandaag is de standaard behandeling voor kwaadaardige gliomen en meer specifiek glioblastoma, de meest agressieve hersentumor, onvoldoende om patiënten te genezen. Daarom wordt reeds vele jaren onderzoek gevoerd naar bijkomstige behandelingsmethoden die bovenop de huidige standaard behandeling, een verbetering kunnen geven in de overleving van patiënten. In dit opzicht werd binnen ons labo een immuuntherapie in de vorm van tumorvaccinatie onderzocht als mogelijke piste. Hierbij werd getracht het eigen immuunsysteem te stimuleren om de resterende tumorcellen na standaard behandeling te bestrijden. Initieel was het onderzoek gericht op het creëren van een dendritische cel (DC) vaccinatie waarbij patiënt-specifieke witte bloedcellen dienen opgekweekt te worden tot DCs, een proces dat plaats moet vinden in een laboratorium-omgeving, onder zeer strikte en gecontroleerde omstandigheden. Dit leidt tot een zeer kostelijk vaccin waarvan de kwaliteit bovendien ook varieert afhankelijk van de toestand van de patiënt zijn/haar bloedcellen. In deel één van het huidig onderzoek werd in een proefdiermodel nagegaan of het mogelijk zou zijn om lichaamseigen dendritische cellen, de cellen die een immuunreactie kunnen dirigeren, te gebruiken om het immuunsysteem te activeren tegen de tumor en zo de kostelijke celcultuur over te slaan. Hierbij werd bestudeerd of het onderhuids injecteren van zeer kleine stukjes van de eigen tumor, een lysaat, door de lichaamseigen DCs kan gebruikt worden om een anti-tumor respons te induceren. Het tumorlysaat bevat onder andere de tumor antigenen, een soort unieke vingerafdruk van tumorcellen die kan gebruikt worden om een deel van het immuunsysteem specifiek tegen deze tumorcellen te richten. In een tweede deel van het project werd het tumorlysaat voor injectie nog gebonden aan nanodeeltjes, bolletjes 200 miljoen keer kleiner dan een voetbal. Een mogelijk voordeel van het nanovaccin dat daarbij wordt gecreëerd, is dat DCs de voorkeurcellen zijn om deeltjes binnen de nanometer grootorde op te nemen. Hierbij is het theoretisch mogelijk om na onderhuidse injectie op passieve wijze het tumorlysaat te sturen naar een opname door de plaatselijke DCs. De werking van beide concepten, onderhuids inspuiten van eigen tumorlysaat en het nanovaccin, werd aangetoond in twee verschillende muismodellen van maligne glioma.

Om te beginnen werd in muis celculturen aangetoond dat DCs de antigenen kunnen opnemen, verwerken en presenteren aan immuuncellen, waarna bij het testen van de lysaat injecties in de maligne glioma muismodellen een verbeterde overleving voor de behandelde dieren werd geobserveerd. Vervolgens werd het nanovaccin ontwikkeld door tumorlysaat stabiel te binden aan de oppervlakte van nanodeeltjes. Na een optimalisatie waarbij grootte, antigen koppeling aan de deeltjes, opname en verwerking door DCs in celcultuur en op beperkte wijze toxiciteit werden bestudeerd, werd het meest ideale nanovaccin geselecteerd om in de muismodellen te testen. Onderhuidse nanovaccin injectie leidde tot een vergelijkbaar overlevingsvoordeel in de glioma muismodellen als waargenomen bij lysaat vaccinatie. Bovendien werd in beide experimentele behandelingsmethoden de immuunrespons bestudeerd. Hierbij werd een

stijging van het aantal T immuuncellen en een vermindering van het aantal immuunsysteem onderdrukkende cellen in de hersenen geobserveerd, en dit in combinatie met het ontwikkelen van een tumor-specifiek immunologisch geheugen. Tenslotte werd een combinatie-behandeling met temozolomide chemotherapie uitgewerkt die in staat was om een verbeterde overleving te bieden voor muizen die reeds een hersentumor hadden.

In deze thesis onderzochten we of de mogelijkheid bestaat om een goedkoper alternatief voor DC-vaccinatie in de behandeling van kwaadaardige gliomen uit te werken. Hiervoor werd eigen tumorlysaat onderhuids ingespoten, wat leidde tot de opbouw van een immuunrespons die de tumorgroei kon onderdrukken. Bovendien werd met het onderzoek binnen het nanovaccin project een mogelijke basis gelegd voor het gebruik van nanodeeltjes beladen met stukjes tumor in de behandeling van glioblastoma in de verre toekomst. Een mogelijk voordeel is dat op deze manier meer gestandaardiseerde vaccinaties kunnen geproduceerd worden op een goedkopere manier en in grotere hoeveelheden.

List of references

1. Bernard W. Stewart CPW. World cancer report 2014. Lyon, France: International Agency for Research on Cancer (IARC); 2014.
2. Burnet NG, Jefferies SJ, Benson RJ, Hunt DP, Treasure FP. Years of life lost (YLL) from cancer is an important measure of population burden--and should be considered when allocating research funds. Br J Cancer. 2005;92(2):241-5.
3. Rouse C, Gittleman H, Ostrom QT, Kruchko C, Barnholtz-Sloan JS. Years of potential life lost for brain and CNS tumors relative to other cancers in adults in the United States, 2010. Neuro Oncol. 2016;18(1):70-7.
4. Ostrom QT, Bauchet L, Davis FG, Deltour I, Fisher JL, Langer CE, et al. The epidemiology of glioma in adults: a "state of the science" review. Neuro Oncol. 2014;16(7):896-913.
5. Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, et al. The 2007 WHO classification of tumours of the central nervous system. Acta Neuropathol. 2007;114(2):97-109.
6. Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK, et al. The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. Acta Neuropathol. 2016;131(6):803-20.
7. Ohgaki H, Kleihues P. The definition of primary and secondary glioblastoma. Clin Cancer Res. 2013;19(4):764-72.
8. Ohgaki H, Kleihues P. Genetic pathways to primary and secondary glioblastoma. Am J Pathol. 2007;170(5):1445-53.
9. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med. 2005;352(10):987-96.
10. Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC, et al. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. Lancet Oncol. 2009;10(5):459-66.
11. Greenspoon JN, Sharieff W, Hirte H, Overholt A, Devillers R, Gunnarsson T, et al. Fractionated stereotactic radiosurgery with concurrent temozolomide chemotherapy for locally recurrent glioblastoma multiforme: a prospective cohort study. Onco Targets Ther. 2014;7:485-90.
12. Hervey-Jumper SL, Berger MS. Maximizing safe resection of low- and high-grade glioma. J Neurooncol. 2016;130(2):269-82.
13. Villano JL, Seery TE, Bressler LR. Temozolomide in malignant gliomas: current use and future targets. Cancer Chemother Pharmacol. 2009;64(4):647-55.
14. Hegi ME, Diserens AC, Gorlia T, Hamou MF, de Tribolet N, Weller M, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med. 2005;352(10):997-1003.
15. Bell EH, Zhang P, Fisher BJ, Macdonald DR, McElroy JP, Lesser GJ, et al. Association of MGMT Promoter Methylation Status With Survival Outcomes in Patients With High-Risk Glioma Treated With Radiotherapy and Temozolomide: An Analysis From the NRG Oncology/RTOG 0424 Trial. JAMA Oncol. 2018;4(10):1405-9.
16. Curry RC, Dahiya S, Alva Venur V, Raizer JJ, Ahluwalia MS. Bevacizumab in high-grade gliomas: past, present, and future. Expert Rev Anticancer Ther. 2015;15(4):387-97.

17. Gilbert MR, Dignam JJ, Armstrong TS, Wefel JS, Blumenthal DT, Vogelbaum MA, et al. A randomized trial of bevacizumab for newly diagnosed glioblastoma. N Engl J Med. 2014;370(8):699-708.
18. Chinot OL, Wick W, Mason W, Henriksson R, Saran F, Nishikawa R, et al. Bevacizumab plus radiotherapy-temozolomide for newly diagnosed glioblastoma. N Engl J Med. 2014;370(8):709-22.
19. Polivka J, Jr., Polivka J, Holubec L, Kubikova T, Priban V, Hes O, et al. Advances in Experimental Targeted Therapy and Immunotherapy for Patients with Glioblastoma Multiforme. Anticancer Res. 2017;37(1):21-33.
20. Anjum K, Shagufta BI, Abbas SQ, Patel S, Khan I, Shah SAA, et al. Current status and future therapeutic perspectives of glioblastoma multiforme (GBM) therapy: A review. Biomed Pharmacother. 2017;92:681-9.
21. Touat M, Idbaih A, Sanson M, Ligon KL. Glioblastoma targeted therapy: updated approaches from recent biological insights. Ann Oncol. 2017;28(7):1457-72.
22. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100(1):57-70.
23. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.
24. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol. 2002;3(11):991-8.
25. Burnet M. Cancer; a biological approach. I. The processes of control. Br Med J. 1957;1(5022):779-86.
26. Dunn GP, Fecci PE, Curry WT. Cancer immunoediting in malignant glioma. Neurosurgery. 2012;71(2):201-22; discussion 22-3.
27. Kim R, Emi M, Tanabe K. Cancer immunoediting from immune surveillance to immune escape. Immunology. 2007;121(1):1-14.
28. Reardon DA, Freeman G, Wu C, Chiocca EA, Wucherpfennig KW, Wen PY, et al. Immunotherapy advances for glioblastoma. Neuro Oncol. 2014;16(11):1441-58.
29. Weller RO, Djuanda E, Yow HY, Carare RO. Lymphatic drainage of the brain and the pathophysiology of neurological disease. Acta Neuropathol. 2009;117(1):1-14.
30. Liu KK, Dorovini-Zis K. Regulation of CXCL12 and CXCR4 expression by human brain endothelial cells and their role in CD4+ and CD8+ T cell adhesion and transendothelial migration. J Neuroimmunol. 2009;215(1-2):49-64.
31. Calzascia T, Masson F, Di Berardino-Besson W, Contassot E, Wilmotte R, Aurrand-Lions M, et al. Homing phenotypes of tumor-specific CD8 T cells are predetermined at the tumor site by crosspresenting APCs. Immunity. 2005;22(2):175-84.
32. Mittal D, Gubin MM, Schreiber RD, Smyth MJ. New insights into cancer immunoediting and its three component phases--elimination, equilibrium and escape. Curr Opin Immunol. 2014;27:16-25.
33. Zou W. Immunosuppressive networks in the tumour environment and their therapeutic relevance. Nat Rev Cancer. 2005;5(4):263-74.
34. Lohr J, Ratliff T, Huppertz A, Ge Y, Dictus C, Ahmadi R, et al. Effector T-cell infiltration positively impacts survival of glioblastoma patients and is impaired by tumor-derived TGF-beta. Clin Cancer Res. 2011;17(13):4296-308.
35. Mostafa H, Pala A, Hogel J, Hlavac M, Dietrich E, Westhoff MA, et al. Immune phenotypes predict survival in patients with glioblastoma multiforme. J Hematol Oncol. 2016;9(1):77.
36. Heimberger AB, Abou-Ghazal M, Reina-Ortiz C, Yang DS, Sun W, Qiao W, et al. Incidence and prognostic impact of FoxP3+ regulatory T cells in human gliomas. Clin Cancer Res. 2008;14(16):5166-72.

37. Raychaudhuri B, Rayman P, Ireland J, Ko J, Rini B, Borden EC, et al. Myeloid-derived suppressor cell accumulation and function in patients with newly diagnosed glioblastoma. Neuro Oncol. 2011;13(6):591-9.
38. Gielen PR, Schulte BM, Kers-Rebel ED, Verrijp K, Petersen-Baltussen HM, ter Laan M, et al. Increase in both CD14-positive and CD15-positive myeloid-derived suppressor cell subpopulations in the blood of patients with glioma but predominance of CD15-positive myeloid-derived suppressor cells in glioma tissue. J Neuropathol Exp Neurol. 2015;74(5):390-400.
39. Dix AR, Brooks WH, Roszman TL, Morford LA. Immune defects observed in patients with primary malignant brain tumors. J Neuroimmunol. 1999;100(1-2):216-32.
40. Saas P, Walker PR, Hahne M, Quiquerez AL, Schnuriger V, Perrin G, et al. Fas ligand expression by astrocytoma in vivo: maintaining immune privilege in the brain? J Clin Invest. 1997;99(6):1173-8.
41. Badie B, Schartner J, Prabakaran S, Paul J, Vorpahl J. Expression of Fas ligand by microglia: possible role in glioma immune evasion. J Neuroimmunol. 2001;120(1-2):19-24.
42. Couzin-Frankel J. Breakthrough of the year 2013. Cancer immunotherapy. Science. 2013;342(6165):1432-3.
43. Kamran N, Calinescu A, Candolfi M, Chandran M, Mineharu Y, Asad AS, et al. Recent advances and future of immunotherapy for glioblastoma. Expert Opin Biol Ther. 2016;16(10):1245-64.
44. Fecci PE, Heimberger AB, Sampson JH. Immunotherapy for primary brain tumors: no longer a matter of privilege. Clin Cancer Res. 2014;20(22):5620-9.
45. Weathers SP, Gilbert MR. Current challenges in designing GBM trials for immunotherapy. J Neurooncol. 2015;123(3):331-7.
46. Johnson LA, Scholler J, Ohkuri T, Kosaka A, Patel PR, McGettigan SE, et al. Rational development and characterization of humanized anti-EGFR variant III chimeric antigen receptor T cells for glioblastoma. Sci Transl Med. 2015;7(275):275ra22.
47. Heimberger AB, Hlatky R, Suki D, Yang D, Weinberg J, Gilbert M, et al. Prognostic effect of epidermal growth factor receptor and EGFRvIII in glioblastoma multiforme patients. Clin Cancer Res. 2005;11(4):1462-6.
48. Migliorini D, Dietrich PY, Stupp R, Linette GP, Posey AD, Jr., June CH. CAR T-Cell Therapies in Glioblastoma: A First Look. Clin Cancer Res. 2018;24(3):535-40.
49. O'Rourke DM, Nasrallah MP, Desai A, Melenhorst JJ, Mansfield K, Morrissette JJD, et al. A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma. Sci Transl Med. 2017;9(399).
50. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer. 2012;12(4):252-64.
51. Margolin K, Ernstoff MS, Hamid O, Lawrence D, McDermott D, Puzanov I, et al. Ipilimumab in patients with melanoma and brain metastases: an open-label, phase 2 trial. Lancet Oncol. 2012;13(5):459-65.
52. Yao Y, Tao R, Wang X, Wang Y, Mao Y, Zhou LF. B7-H1 is correlated with malignancy-grade gliomas but is not expressed exclusively on tumor stem-like cells. Neuro Oncol. 2009;11(6):757-66.
53. Jacobs JF, Idema AJ, Bol KF, Nierkens S, Grauer OM, Wesseling P, et al. Regulatory T cells and the PD-L1/PD-1 pathway mediate immune suppression in malignant human brain tumors. Neuro Oncol. 2009;11(4):394-402.
54. Filley AC, Henriquez M, Dey M. Recurrent glioma clinical trial, CheckMate-143: the game is not over yet. Oncotarget. 2017;8(53):91779-94.

55. Reardon DA, Lassman AB, van den Bent M, Kumthekar P, Merrell R, Scott AM, et al. Efficacy and safety results of ABT-414 in combination with radiation and temozolomide in newly diagnosed glioblastoma. Neuro Oncol. 2017;19(7):965-75.
56. Antonios JP, Soto H, Everson RG, Orpilla J, Moughon D, Shin N, et al. PD-1 blockade enhances the vaccination-induced immune response in glioma. JCI Insight. 2016;1(10).
57. Steinman RM, Adams JC, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. IV. Identification and distribution in mouse spleen. J Exp Med. 1975;141(4):804-20.
58. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998;392(6673):245-52.
59. Collin M, Bigley V. Human dendritic cell subsets: an update. Immunology. 2018;154(1):3-20.
60. Inaba K, Inaba M. Antigen recognition and presentation by dendritic cells. Int J Hematol. 2005;81(3):181-7.
61. Lipscomb MF, Masten BJ. Dendritic cells: immune regulators in health and disease. Physiol Rev. 2002;82(1):97-130.
62. Trombetta ES, Mellman I. Cell biology of antigen processing in vitro and in vivo. Annu Rev Immunol. 2005;23:975-1028.
63. Stuart LM, Ezekowitz RA. Phagocytosis: elegant complexity. Immunity. 2005;22(5):539-50.
64. Lim JP, Gleeson PA. Macropinocytosis: an endocytic pathway for internalising large gulps. Immunol Cell Biol. 2011;89(8):836-43.
65. Liu Z, Roche PA. Macropinocytosis in phagocytes: regulation of MHC class-II-restricted antigen presentation in dendritic cells. Front Physiol. 2015;6:1.
66. Burgdorf S, Kurts C. Endocytosis mechanisms and the cell biology of antigen presentation. Curr Opin Immunol. 2008;20(1):89-95.
67. Hillaireau H, Couvreur P. Nanocarriers' entry into the cell: relevance to drug delivery. Cell Mol Life Sci. 2009;66(17):2873-96.
68. Bonifaz L, Bonnyay D, Mahnke K, Rivera M, Nussenzweig MC, Steinman RM. Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. J Exp Med. 2002;196(12):1627-38.
69. Schwartz RH. T cell anergy. Annu Rev Immunol. 2003;21:305-34.
70. Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. J Leukoc Biol. 2007;81(1):1-5.
71. Pulendran B, Palucka K, Banchereau J. Sensing pathogens and tuning immune responses. Science. 2001;293(5528):253-6.
72. Achek A, Yesudhas D, Choi S. Toll-like receptors: promising therapeutic targets for inflammatory diseases. Arch Pharm Res. 2016;39(8):1032-49.
73. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol. 2010;11(5):373-84.
74. O'Neill LA, Bowie AG. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. Nat Rev Immunol. 2007;7(5):353-64.
75. Schaefer L. Complexity of danger: the diverse nature of damage-associated molecular patterns. J Biol Chem. 2014;289(51):35237-45.
76. Chen GY, Nunez G. Sterile inflammation: sensing and reacting to damage. Nat Rev Immunol. 2010;10(12):826-37.
77. Blum JS, Wearsch PA, Cresswell P. Pathways of antigen processing. Annu Rev Immunol. 2013;31:443-73.

78. Neefjes J, Jongsma ML, Paul P, Bakke O. Towards a systems understanding of MHC class I and MHC class II antigen presentation. Nat Rev Immunol. 2011;11(12):823-36.
79. Vyas JM, Van der Veen AG, Ploegh HL. The known unknowns of antigen processing and presentation. Nat Rev Immunol. 2008;8(8):607-18.
80. Joffre OP, Segura E, Savina A, Amigorena S. Cross-presentation by dendritic cells. Nat Rev Immunol. 2012;12(8):557-69.
81. Flinsenberg TW, Compeer EB, Boelens JJ, Boes M. Antigen cross-presentation: extending recent laboratory findings to therapeutic intervention. Clin Exp Immunol. 2011;165(1):8-18.
82. Rock KL, Shen L. Cross-presentation: underlying mechanisms and role in immune surveillance. Immunol Rev. 2005;207:166-83.
83. Jung S, Unutmaz D, Wong P, Sano G, De los Santos K, Sparwasser T, et al. In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens. Immunity. 2002;17(2):211-20.
84. Schnorrer P, Behrens GM, Wilson NS, Pooley JL, Smith CM, El-Sukkari D, et al. The dominant role of CD8+ dendritic cells in cross-presentation is not dictated by antigen capture. Proc Natl Acad Sci U S A. 2006;103(28):10729-34.
85. Savina A, Peres A, Cebrian I, Carmo N, Moita C, Hacohen N, et al. The small GTPase Rac2 controls phagosomal alkalinization and antigen crosspresentation selectively in CD8(+) dendritic cells. Immunity. 2009;30(4):544-55.
86. Delamarre L, Pack M, Chang H, Mellman I, Trombetta ES. Differential lysosomal proteolysis in antigen-presenting cells determines antigen fate. Science. 2005;307(5715):1630-4.
87. Trombetta ES, Ebersold M, Garrett W, Pypaert M, Mellman I. Activation of lysosomal function during dendritic cell maturation. Science. 2003;299(5611):1400-3.
88. Duan F, Srivastava PK. An invariant road to cross-presentation. Nat Immunol. 2012;13(3):207-8.
89. Shen L, Sigal LJ, Boes M, Rock KL. Important role of cathepsin S in generating peptides for TAP-independent MHC class I crosspresentation in vivo. Immunity. 2004;21(2):155-65.
90. Kovacsovics-Bankowski M, Rock KL. A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. Science. 1995;267(5195):243-6.
91. Ackerman AL, Giodini A, Cresswell P. A role for the endoplasmic reticulum protein retrotranslocation machinery during crosspresentation by dendritic cells. Immunity. 2006;25(4):607-17.
92. Wang H, Yu X, Guo C, Zuo D, Fisher PB, Subjeck JR, et al. Enhanced endoplasmic reticulum entry of tumor antigen is crucial for cross-presentation induced by dendritic cell-targeted vaccination. J Immunol. 2013;191(12):6010-21.
93. Houde M, Bertholet S, Gagnon E, Brunet S, Goyette G, Laplante A, et al. Phagosomes are competent organelles for antigen cross-presentation. Nature. 2003;425(6956):402-6.
94. Basha G, Omilusik K, Chavez-Steenbock A, Reinicke AT, Lack N, Choi KB, et al. A CD74-dependent MHC class I endolysosomal cross-presentation pathway. Nat Immunol. 2012;13(3):237-45.
95. De Temmerman ML, Rejman J, Demeester J, Irvine DJ, Gander B, De Smedt SC. Particulate vaccines: on the quest for optimal delivery and immune response. Drug Discov Today. 2011;16(13-14):569-82.
96. Iezzi G, Karjalainen K, Lanzavecchia A. The duration of antigenic stimulation determines the fate of naive and effector T cells. Immunity. 1998;8(1):89-95.
97. Lanzavecchia A, Iezzi G, Viola A. From TCR engagement to T cell activation: a kinetic view of T cell behavior. Cell. 1999;96(1):1-4.

98. Lanzavecchia A, Sallusto F. Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells. Science. 2000;290(5489):92-7.
99. Grakoui A, Bromley SK, Sumen C, Davis MM, Shaw AS, Allen PM, et al. The immunological synapse: a molecular machine controlling T cell activation. Science. 1999;285(5425):221-7.
100. Stock A, Napolitani G, Cerundolo V. Intestinal DC in migrational imprinting of immune cells. Immunol Cell Biol. 2013;91(3):240-9.
101. Mikhak Z, Strassner JP, Luster AD. Lung dendritic cells imprint T cell lung homing and promote lung immunity through the chemokine receptor CCR4. J Exp Med. 2013;210(9):1855-69.
102. Kurts C, Robinson BW, Knolle PA. Cross-priming in health and disease. Nat Rev Immunol. 2010;10(6):403-14.
103. Thaiss CA, Semmling V, Franken L, Wagner H, Kurts C. Chemokines: a new dendritic cell signal for T cell activation. Front Immunol. 2011;2:31.
104. Reardon DA, Wucherpfennig KW, Freeman G, Wu CJ, Chiocca EA, Wen PY, et al. An update on vaccine therapy and other immunotherapeutic approaches for glioblastoma. Expert Rev Vaccines. 2013;12(6):597-615.
105. Palucka K, Banchereau J. Cancer immunotherapy via dendritic cells. Nat Rev Cancer. 2012;12(4):265-77.
106. Ophir E, Bobisse S, Coukos G, Harari A, Kandalaft LE. Personalized approaches to active immunotherapy in cancer. Biochim Biophys Acta. 2016;1865(1):72-82.
107. Anguille S, Smits EL, Lion E, van Tendeloo VF, Berneman ZN. Clinical use of dendritic cells for cancer therapy. Lancet Oncol. 2014;15(7):e257-67.
108. Eyrich M, Rachor J, Schreiber SC, Wolfl M, Schlegel PG. Dendritic cell vaccination in pediatric gliomas: lessons learnt and future perspectives. Front Pediatr. 2013;1:12.
109. Van Gool SW. Brain Tumor Immunotherapy: What have We Learned so Far? Front Oncol. 2015;5:98.
110. Cheever MA, Higano CS. PROVENGE (Sipuleucel-T) in prostate cancer: the first FDA-approved therapeutic cancer vaccine. Clin Cancer Res. 2011;17(11):3520-6.
111. Huber ML, Haynes L, Parker C, Iversen P. Interdisciplinary critique of sipuleucel-T as immunotherapy in castration-resistant prostate cancer. J Natl Cancer Inst. 2012;104(4):273-9.
112. Cao JX, Zhang XY, Liu JL, Li D, Li JL, Liu YS, et al. Clinical efficacy of tumor antigen-pulsed DC treatment for high-grade glioma patients: evidence from a meta-analysis. PLoS One. 2014;9(9):e107173.
113. Bregy A, Wong TM, Shah AH, Goldberg JM, Komotar RJ. Active immunotherapy using dendritic cells in the treatment of glioblastoma multiforme. Cancer Treat Rev. 2013;39(8):891-907.
114. Wang X, Zhao HY, Zhang FC, Sun Y, Xiong ZY, Jiang XB. Dendritic cell-based vaccine for the treatment of malignant glioma: a systematic review. Cancer Invest. 2014;32(9):451-7.
115. Eagles ME, Nassiri F, Badhiwala JH, Suppiah S, Almenawer SA, Zadeh G, et al. Dendritic cell vaccines for high-grade gliomas. Ther Clin Risk Manag. 2018;14:1299-313.
116. Hdeib A, Sloan AE. Dendritic cell immunotherapy for solid tumors: evaluation of the DCVax(R) platform in the treatment of glioblastoma multiforme. CNS Oncol. 2015;4(2):63-9.
117. Polyzoidis S, Ashkan K. DCVax(R)-L--developed by Northwest Biotherapeutics. Hum Vaccin Immunother. 2014;10(11):3139-45.
118. Liau LM, Ashkan K, Tran DD, Campian JL, Trusheim JE, Cobbs CS, et al. First results on survival from a large Phase 3 clinical trial of an autologous dendritic cell vaccine in newly diagnosed glioblastoma. J Transl Med. 2018;16(1):142.

119. Phuphanich S, Wheeler CJ, Rudnick JD, Mazer M, Wang H, Nuno MA, et al. Phase I trial of a multi-epitope-pulsed dendritic cell vaccine for patients with newly diagnosed glioblastoma. Cancer Immunol Immunother. 2013;62(1):125-35.
120. Wen PY, Reardon DA, Phuphanich S, Aiken R, Landolfi JC, Curry WT, et al. A randomized, doubleblind, placebo-controlled phase 2 trial of dendritic cell (DC) vaccination with ICT-107 in newly diagnosed glioblastoma (GBM) patients [abstract]. J Clin Oncol. 2014;32.
121. Jongbloed SL, Kassianos AJ, McDonald KJ, Clark GJ, Ju X, Angel CE, et al. Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. J Exp Med. 2010;207(6):1247-60.
122. Wimmers F, Schreibelt G, Skold AE, Figdor CG, De Vries IJ. Paradigm Shift in Dendritic Cell-Based Immunotherapy: From in vitro Generated Monocyte-Derived DCs to Naturally Circulating DC Subsets. Front Immunol. 2014;5:165.
123. Laoui D, Keirsse J, Morias Y, Van Overmeire E, Geeraerts X, Elkrim Y, et al. The tumour microenvironment harbours ontogenically distinct dendritic cell populations with opposing effects on tumour immunity. Nat Commun. 2016;7:13720.
124. Bol KF, Schreibelt G, Gerritsen WR, de Vries IJ, Figdor CG. Dendritic Cell-Based Immunotherapy: State of the Art and Beyond. Clin Cancer Res. 2016;22(8):1897-906.
125. McNamara MA, Nair SK, Holl EK. RNA-Based Vaccines in Cancer Immunotherapy. J Immunol Res. 2015;2015:794528.
126. Kajihara M, Takakura K, Ohkusa T, Koido S. The impact of dendritic cell-tumor fusion cells on cancer vaccines - past progress and future strategies. Immunotherapy. 2015;7(10):1111-22.
127. Gu X, Erb U, Buchler MW, Zoller M. Improved vaccine efficacy of tumor exosome compared to tumor lysate loaded dendritic cells in mice. Int J Cancer. 2015;136(4):E74-84.
128. Neller MA, Lopez JA, Schmidt CW. Antigens for cancer immunotherapy. Semin Immunol. 2008;20(5):286-95.
129. Prins RM, Wang X, Soto H, Young E, Lisiero DN, Fong B, et al. Comparison of glioma-associated antigen peptide-loaded versus autologous tumor lysate-loaded dendritic cell vaccination in malignant glioma patients. J Immunother. 2013;36(2):152-7.
130. Rapp M, Grauer OM, Kamp M, Sevens N, Zotz N, Sabel M, et al. A randomized controlled phase II trial of vaccination with lysate-loaded, mature dendritic cells integrated into standard radiochemotherapy of newly diagnosed glioblastoma (GlioVax): study protocol for a randomized controlled trial. Trials. 2018;19(1):293.
131. Inoges S, Tejada S, de Cerio AL, Gallego Perez-Larraya J, Espinos J, Idoate MA, et al. A phase II trial of autologous dendritic cell vaccination and radiochemotherapy following fluorescence-guided surgery in newly diagnosed glioblastoma patients. J Transl Med. 2017;15(1):104.
132. Eyrich M, Schreiber SC, Rachor J, Krauss J, Pauwels F, Hain J, et al. Development and validation of a fully GMP-compliant production process of autologous, tumor-lysate-pulsed dendritic cells. Cytotherapy. 2014;16(7):946-64.
133. Nava S, Dossena M, Pogliani S, Pellegatta S, Antozzi C, Baggi F, et al. An optimized method for manufacturing a clinical scale dendritic cell-based vaccine for the treatment of glioblastoma. PLoS One. 2012;7(12):e52301.
134. Winters M, Dabir B, Yu M, Kohn EC. Constitution and quantity of lysis buffer alters outcome of reverse phase protein microarrays. Proteomics. 2007;7(22):4066-8.
135. Garg AD, Galluzzi L, Apetoh L, Baert T, Birge RB, Bravo-San Pedro JM, et al. Molecular and Translational Classifications of DAMPs in Immunogenic Cell Death. Front Immunol. 2015;6:588.

136. Vandenberk L, Belmans J, Van Woensel M, Riva M, Van Gool SW. Exploiting the Immunogenic Potential of Cancer Cells for Improved Dendritic Cell Vaccines. Front Immunol. 2015;6:663.
137. Vandenberk L, Garg AD, Verschuere T, Koks C, Belmans J, Beullens M, et al. Irradiation of necrotic cancer cells, employed for pulsing dendritic cells (DCs), potentiates DC vaccine-induced antitumor immunity against high-grade glioma. Oncoimmunology. 2016;5(2):e1083669.
138. Krysko DV, Garg AD, Kaczmarek A, Krysko O, Agostinis P, Vandenabeele P. Immunogenic cell death and DAMPs in cancer therapy. Nat Rev Cancer. 2012;12(12):860-75.
139. De Vries IJ, Krooshoop DJ, Scharenborg NM, Lesterhuis WJ, Diepstra JH, Van Muijen GN, et al. Effective migration of antigen-pulsed dendritic cells to lymph nodes in melanoma patients is determined by their maturation state. Cancer Res. 2003;63(1):12-7.
140. Allan RS, Waithman J, Bedoui S, Jones CM, Villadangos JA, Zhan Y, et al. Migratory dendritic cells transfer antigen to a lymph node-resident dendritic cell population for efficient CTL priming. Immunity. 2006;25(1):153-62.
141. Wu A, Oh S, Gharagozlou S, Vedi RN, Ericson K, Low WC, et al. In vivo vaccination with tumor cell lysate plus CpG oligodeoxynucleotides eradicates murine glioblastoma. J Immunother. 2007;30(8):789-97.
142. Murphy KA, Lechner MG, Popescu FE, Bedi J, Decker SA, Hu P, et al. An in vivo immunotherapy screen of costimulatory molecules identifies Fc-OX40L as a potent reagent for the treatment of established murine gliomas. Clin Cancer Res. 2012;18(17):4657-68.
143. Murphy KA, Erickson JR, Johnson CS, Seiler CE, Bedi J, Hu P, et al. CD8+ T cell-independent tumor regression induced by Fc-OX40L and therapeutic vaccination in a mouse model of glioma. J Immunol. 2014;192(1):224-33.
144. Stathopoulos A, Samuelson C, Milbouw G, Hermanne JP, Schijns VE, Chen TC. Therapeutic vaccination against malignant gliomas based on allorecognition and syngeneic tumor antigens: proof of principle in two strains of rat. Vaccine. 2008;26(14):1764-72.
145. Bota DA, Chung J, Dandekar M, Carrillo JA, Kong XT, Fu BD, et al. Phase II study of ERC1671 plus bevacizumab versus bevacizumab plus placebo in recurrent glioblastoma: interim results and correlations with CD4(+) T-lymphocyte counts. CNS Oncol. 2018;7(3):CNS22.
146. Jouanneau E, Poujol D, Gulia S, Le Mercier I, Blay JY, Belin MF, et al. Dendritic cells are essential for priming but inefficient for boosting antitumour immune response in an orthotopic murine glioma model. Cancer Immunol Immunother. 2006;55(3):254-67.
147. Bloom HJ, Peckham MJ, Richardson AE, Alexander PA, Payne PM. Glioblastoma multiforme: a controlled trial to assess the value of specific active immunotherapy in patients treated by radical surgery and radiotherapy. Br J Cancer. 1973;27(3):253-67.
148. Schneider T, Gerhards R, Kirches E, Firsching R. Preliminary results of active specific immunization with modified tumor cell vaccine in glioblastoma multiforme. J Neurooncol. 2001;53(1):39-46.
149. Steiner HH, Bonsanto MM, Beckhove P, Brysch M, Geletneky K, Ahmadi R, et al. Antitumor vaccination of patients with glioblastoma multiforme: a pilot study to assess feasibility, safety, and clinical benefit. J Clin Oncol. 2004;22(21):4272-81.
150. Ishikawa E, Tsuboi K, Yamamoto T, Muroi A, Takano S, Enomoto T, et al. Clinical trial of autologous formalin-fixed tumor vaccine for glioblastoma multiforme patients. Cancer Sci. 2007;98(8):1226-33.
151. Clavreul A, Piard N, Tanguy JY, Gamelin E, Rousselet MC, Leynia P, et al. Autologous tumor cell vaccination plus infusion of GM-CSF by a programmable pump in the treatment of recurrent malignant gliomas. J Clin Neurosci. 2010;17(7):842-8.

152. Mahaley MS, Jr., Bigner DD, Dudka LF, Wilds PR, Williams DH, Bouldin TW, et al. Immunobiology of primary intracranial tumors. Part 7: Active immunization of patients with anaplastic human glioma cells: a pilot study. J Neurosurg. 1983;59(2):201-7.
153. Sobol RE, Fakhrai H, Shawler D, Gjerset R, Dorigo O, Carson C, et al. Interleukin-2 gene therapy in a patient with glioblastoma. Gene Ther. 1995;2(2):164-7.
154. Okada H, Lieberman FS, Edington HD, Witham TF, Wargo MJ, Cai Q, et al. Autologous glioma cell vaccine admixed with interleukin-4 gene transfected fibroblasts in the treatment of recurrent glioblastoma: preliminary observations in a patient with a favorable response to therapy. J Neurooncol. 2003;64(1-2):13-20.
155. Parney IF, Chang LJ, Farr-Jones MA, Hao C, Smylie M, Petruk KC. Technical hurdles in a pilot clinical trial of combined B7-2 and GM-CSF immunogene therapy for glioblastomas and melanomas. J Neurooncol. 2006;78(1):71-80.
156. Kreuter J. Nanoparticles and microparticles for drug and vaccine delivery. J Anat. 1996;189 (Pt 3):503-5.
157. Kiessling F, Mertens ME, Grimm J, Lammers T. Nanoparticles for imaging: top or flop? Radiology. 2014;273(1):10-28.
158. Zhang L, Gu FX, Chan JM, Wang AZ, Langer RS, Farokhzad OC. Nanoparticles in medicine: therapeutic applications and developments. Clin Pharmacol Ther. 2008;83(5):761-9.
159. Xu X, Ho W, Zhang X, Bertrand N, Farokhzad O. Cancer nanomedicine: from targeted delivery to combination therapy. Trends Mol Med. 2015;21(4):223-32.
160. Jindal AB. The effect of particle shape on cellular interaction and drug delivery applications of micro- and nanoparticles. Int J Pharm. 2017;532(1):450-65.
161. Silva JM, Videira M, Gaspar R, Preat V, Florindo HF. Immune system targeting by biodegradable nanoparticles for cancer vaccines. J Control Release. 2013;168(2):179-99.
162. Kim BY, Rutka JT, Chan WC. Nanomedicine. N Engl J Med. 2010;363(25):2434-43.
163. Matsumura Y, Maeda H. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. Cancer Res. 1986;46(12 Pt 1):6387-92.
164. Gerlowski LE, Jain RK. Microvascular permeability of normal and neoplastic tissues. Microvasc Res. 1986;31(3):288-305.
165. Fang J, Nakamura H, Maeda H. The EPR effect: Unique features of tumor blood vessels for drug delivery, factors involved, and limitations and augmentation of the effect. Adv Drug Deliv Rev. 2011;63(3):136-51.
166. Barenholz Y. Doxil(R)--the first FDA-approved nano-drug: lessons learned. J Control Release. 2012;160(2):117-34.
167. Mir M, Ishtiaq S, Rabia S, Khatoon M, Zeb A, Khan GM, et al. Nanotechnology: from In Vivo Imaging System to Controlled Drug Delivery. Nanoscale Res Lett. 2017;12(1):500.
168. Tang J, Huang N, Zhang X, Zhou T, Tan Y, Pi J, et al. Aptamer-conjugated PEGylated quantum dots targeting epidermal growth factor receptor variant III for fluorescence imaging of glioma. Int J Nanomedicine. 2017;12:3899-911.
169. Martinkova P, Brtnicky M, Kynicky J, Pohanka M. Iron Oxide Nanoparticles: Innovative Tool in Cancer Diagnosis and Therapy. Adv Healthc Mater. 2017.
170. Thorek DL, Chen AK, Czupryna J, Tsourkas A. Superparamagnetic iron oxide nanoparticle probes for molecular imaging. Ann Biomed Eng. 2006;34(1):23-38.
171. Choudhury H, Pandey M, Chin PX, Phang YL, Cheah JY, Ooi SC, et al. Transferrin receptors-targeting nanocarriers for efficient targeted delivery and transcytosis of drugs into the brain tumors: a review of recent advancements and emerging trends. Drug Deliv Transl Res. 2018;8(5):1545-63.

172. Fang C, Wang K, Stephen ZR, Mu Q, Kievit FM, Chiu DT, et al. Temozolomide nanoparticles for targeted glioblastoma therapy. ACS Appl Mater Interfaces. 2015;7(12):6674-82.
173. Jain DS, Athawale RB, Bajaj AN, Shrikhande SS, Goel PN, Nikam Y, et al. Unraveling the cytotoxic potential of Temozolomide loaded into PLGA nanoparticles. Daru. 2014;22(1):18.
174. Huo H, Gao Y, Wang Y, Zhang J, Wang ZY, Jiang T, et al. Polyion complex micelles composed of pegylated polyasparthydrazide derivatives for siRNA delivery to the brain. J Colloid Interface Sci. 2015;447:8-15.
175. Li XY, Zhao Y, Sun MG, Shi JF, Ju RJ, Zhang CX, et al. Multifunctional liposomes loaded with paclitaxel and artemether for treatment of invasive brain glioma. Biomaterials. 2014;35(21):5591-604.
176. Guo J, Rahme K, He Y, Li LL, Holmes JD, O'Driscoll CM. Gold nanoparticles enlighten the future of cancer theranostics. Int J Nanomedicine. 2017;12:6131-52.
177. Maier-Hauff K, Ulrich F, Nestler D, Niehoff H, Wust P, Thiesen B, et al. Efficacy and safety of intratumoral thermotherapy using magnetic iron-oxide nanoparticles combined with external beam radiotherapy on patients with recurrent glioblastoma multiforme. J Neurooncol. 2011;103(2):317-24.
178. Li M, Zhang F, Su Y, Zhou J, Wang W. Nanoparticles designed to regulate tumor microenvironment for cancer therapy. Life Sci. 2018;201:37-44.
179. Van Woensel M, Wauthoz N, Rosiere R, Mathieu V, Kiss R, Lefranc F, et al. Development of siRNA-loaded chitosan nanoparticles targeting Galectin-1 for the treatment of glioblastoma multiforme via intranasal administration. J Control Release. 2016;227:71-81.
180. Van Woensel M, Mathivet T, Wauthoz N, Rosiere R, Garg AD, Agostinis P, et al. Sensitization of glioblastoma tumor micro-environment to chemo- and immunotherapy by Galectin-1 intranasal knock-down strategy. Sci Rep. 2017;7(1):1217.
181. Li W, Wei H, Li H, Gao J, Feng SS, Guo Y. Cancer nanoimmunotherapy using advanced pharmaceutical nanotechnology. Nanomedicine (Lond). 2014;9(16):2587-605.
182. Serda RE. Particle platforms for cancer immunotherapy. Int J Nanomedicine. 2013;8:1683-96.
183. Moon JJ, Huang B, Irvine DJ. Engineering nano- and microparticles to tune immunity. Adv Mater. 2012;24(28):3724-46.
184. Shao K, Singha S, Clemente-Casares X, Tsai S, Yang Y, Santamaria P. Nanoparticle-based immunotherapy for cancer. ACS Nano. 2015;9(1):16-30.
185. Fang RH, Kroll AV, Zhang L. Nanoparticle-Based Manipulation of Antigen-Presenting Cells for Cancer Immunotherapy. Small. 2015;11(41):5483-96.
186. Bareford LM, Swaan PW. Endocytic mechanisms for targeted drug delivery. Adv Drug Deliv Rev. 2007;59(8):748-58.
187. Pelkmans L, Kartenbeck J, Helenius A. Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. Nat Cell Biol. 2001;3(5):473-83.
188. Song C, Noh YW, Lim YT. Polymer nanoparticles for cross-presentation of exogenous antigens and enhanced cytotoxic T-lymphocyte immune response. Int J Nanomedicine. 2016;11:3753-64.
189. Chakraborty A, Jana NR. Clathrin to Lipid Raft-Endocytosis via Controlled Surface Chemistry and Efficient Perinuclear Targeting of Nanoparticle. J Phys Chem Lett. 2015;6(18):3688-97.
190. Nel AE, Madler L, Velegol D, Xia T, Hoek EM, Somasundaran P, et al. Understanding biophysicochemical interactions at the nano-bio interface. Nat Mater. 2009;8(7):543-57.

191. Lavin Y, Mortha A, Rahman A, Merad M. Regulation of macrophage development and function in peripheral tissues. Nat Rev Immunol. 2015;15(12):731-44.
192. Chong CS, Cao M, Wong WW, Fischer KP, Addison WR, Kwon GS, et al. Enhancement of T helper type 1 immune responses against hepatitis B virus core antigen by PLGA nanoparticle vaccine delivery. J Control Release. 2005;102(1):85-99.
193. Jewell CM, Lopez SC, Irvine DJ. In situ engineering of the lymph node microenvironment via intranodal injection of adjuvant-releasing polymer particles. Proc Natl Acad Sci U S A. 2011;108(38):15745-50.
194. Hamdy S, Haddadi A, Hung RW, Lavasanifar A. Targeting dendritic cells with nano-particulate PLGA cancer vaccine formulations. Adv Drug Deliv Rev. 2011;63(10-11):943-55.
195. Bachmann MF, Jennings GT. Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. Nat Rev Immunol. 2010;10(11):787-96.
196. Xiang SD, Scholzen A, Minigo G, David C, Apostolopoulos V, Mottram PL, et al. Pathogen recognition and development of particulate vaccines: does size matter? Methods. 2006;40(1):1-9.
197. Foged C, Brodin B, Frokjaer S, Sundblad A. Particle size and surface charge affect particle uptake by human dendritic cells in an in vitro model. Int J Pharm. 2005;298(2):315-22.
198. Reddy ST, Rehor A, Schmoekel HG, Hubbell JA, Swartz MA. In vivo targeting of dendritic cells in lymph nodes with poly(propylene sulfide) nanoparticles. J Control Release. 2006;112(1):26-34.
199. Reddy ST, van der Vlies AJ, Simeoni E, Angeli V, Randolph GJ, O'Neil CP, et al. Exploiting lymphatic transport and complement activation in nanoparticle vaccines. Nat Biotechnol. 2007;25(10):1159-64.
200. Cubas R, Zhang S, Kwon S, Sevick-Muraca EM, Li M, Chen C, et al. Virus-like particle (VLP) lymphatic trafficking and immune response generation after immunization by different routes. J Immunother. 2009;32(2):118-28.
201. Manolova V, Flace A, Bauer M, Schwarz K, Saudan P, Bachmann MF. Nanoparticles target distinct dendritic cell populations according to their size. Eur J Immunol. 2008;38(5):1404-13.
202. Shima F, Uto T, Akagi T, Baba M, Akashi M. Size effect of amphiphilic poly(gamma-glutamic acid) nanoparticles on cellular uptake and maturation of dendritic cells in vivo. Acta Biomater. 2013;9(11):8894-901.
203. Kumar S, Anselmo AC, Banerjee A, Zakrewsky M, Mitragotri S. Shape and size-dependent immune response to antigen-carrying nanoparticles. J Control Release. 2015;220(Pt A):141-8.
204. Minigo G, Scholzen A, Tang CK, Hanley JC, Kalkanidis M, Pietersz GA, et al. Poly-L-lysine-coated nanoparticles: a potent delivery system to enhance DNA vaccine efficacy. Vaccine. 2007;25(7):1316-27.
205. Fifis T, Gamvrellis A, Crimeen-Irwin B, Pietersz GA, Li J, Mottram PL, et al. Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors. J Immunol. 2004;173(5):3148-54.
206. Fifis T, Mottram P, Bogdanoska V, Hanley J, Plebanski M. Short peptide sequences containing MHC class I and/or class II epitopes linked to nano-beads induce strong immunity and inhibition of growth of antigen-specific tumour challenge in mice. Vaccine. 2004;23(2):258-66.
207. Scheerlinck JP, Gloster S, Gamvrellis A, Mottram PL, Plebanski M. Systemic immune responses in sheep, induced by a novel nano-bead adjuvant. Vaccine. 2006;24(8):1124-31.

208. Mottram PL, Leong D, Crimeen-Irwin B, Gloster S, Xiang SD, Meanger J, et al. Type 1 and 2 immunity following vaccination is influenced by nanoparticle size: formulation of a model vaccine for respiratory syncytial virus. Mol Pharm. 2007;4(1):73-84.
209. Mathaes R, Winter G, Siahaan TJ, Besheer A, Engert J. Influence of particle size, an elongated particle geometry, and adjuvants on dendritic cell activation. Eur J Pharm Biopharm. 2015;94:542-9.
210. Champion JA, Mitragotri S. Role of target geometry in phagocytosis. Proc Natl Acad Sci U S A. 2006;103(13):4930-4.
211. Luo M, Samandi LZ, Wang Z, Chen ZJ, Gao J. Synthetic nanovaccines for immunotherapy. J Control Release. 2017;263:200-10.
212. van den Berg JH, Oosterhuis K, Hennink WE, Storm G, van der Aa LJ, Engbersen JF, et al. Shielding the cationic charge of nanoparticle-formulated dermal DNA vaccines is essential for antigen expression and immunogenicity. J Control Release. 2010;141(2):234-40.
213. Uto T, Toyama M, Nishi Y, Akagi T, Shima F, Akashi M, et al. Uptake of biodegradable poly(gamma-glutamic acid) nanoparticles and antigen presentation by dendritic cells in vivo. Results Immunol. 2013;3:1-9.
214. Mukai Y, Yoshinaga T, Yoshikawa M, Matsuo K, Yoshikawa T, Matsuo K, et al. Induction of endoplasmic reticulum-endosome fusion for antigen cross-presentation induced by poly (gamma-glutamic acid) nanoparticles. J Immunol. 2011;187(12):6249-55.
215. Shen H, Ackerman AL, Cody V, Giodini A, Hinson ER, Cresswell P, et al. Enhanced and prolonged cross-presentation following endosomal escape of exogenous antigens encapsulated in biodegradable nanoparticles. Immunology. 2006;117(1):78-88.
216. Hirosue S, Kourtis IC, van der Vlies AJ, Hubbell JA, Swartz MA. Antigen delivery to dendritic cells by poly(propylene sulfide) nanoparticles with disulfide conjugated peptides: Cross-presentation and T cell activation. Vaccine. 2010;28(50):7897-906.
217. Dong H, Wen ZF, Chen L, Zhou N, Liu H, Dong S, et al. Polyethyleneimine modification of aluminum hydroxide nanoparticle enhances antigen transportation and cross-presentation of dendritic cells. Int J Nanomedicine. 2018;13:3353-65.
218. Rakoff-Nahoum S, Medzhitov R. Toll-like receptors and cancer. Nat Rev Cancer. 2009;9(1):57-63.
219. Hamdy S, Elamanchili P, Alshamsan A, Molavi O, Satou T, Samuel J. Enhanced antigen-specific primary CD4+ and CD8+ responses by codelivery of ovalbumin and toll-like receptor ligand monophosphoryl lipid A in poly(D,L-lactic-co-glycolic acid) nanoparticles. J Biomed Mater Res A. 2007;81(3):652-62.
220. Xu Z, Ramishetti S, Tseng YC, Guo S, Wang Y, Huang L. Multifunctional nanoparticles co-delivering Trp2 peptide and CpG adjuvant induce potent cytotoxic T-lymphocyte response against melanoma and its lung metastasis. J Control Release. 2013;172(1):259-65.
221. Tacken PJ, Figdor CG. Targeted antigen delivery and activation of dendritic cells in vivo: steps towards cost effective vaccines. Semin Immunol. 2011;23(1):12-20.
222. Kawamura K, Kadowaki N, Suzuki R, Udagawa S, Kasaoka S, Utoguchi N, et al. Dendritic cells that endocytosed antigen-containing IgG-liposomes elicit effective antitumor immunity. J Immunother. 2006;29(2):165-74.
223. Figdor CG, van Kooyk Y, Adema GJ. C-type lectin receptors on dendritic cells and Langerhans cells. Nat Rev Immunol. 2002;2(2):77-84.
224. Silva JM, Zupancic E, Vandermeulen G, Oliveira VG, Salgado A, Videira M, et al. In vivo delivery of peptides and Toll-like receptor ligands by mannose-functionalized polymeric nanoparticles induces prophylactic and therapeutic anti-tumor immune responses in a melanoma model. J Control Release. 2015;198:91-103.

225. Cruz LJ, Tacken PJ, Fokkink R, Figdor CG. The influence of PEG chain length and targeting moiety on antibody-mediated delivery of nanoparticle vaccines to human dendritic cells. Biomaterials. 2011;32(28):6791-803.
226. Caminschi I, Proietto AI, Ahmet F, Kitsoulis S, Shin Teh J, Lo JC, et al. The dendritic cell subtype-restricted C-type lectin Clec9A is a target for vaccine enhancement. Blood. 2008;112(8):3264-73.
227. van Broekhoven CL, Parish CR, Demangel C, Britton WJ, Altin JG. Targeting dendritic cells with antigen-containing liposomes: a highly effective procedure for induction of antitumor immunity and for tumor immunotherapy. Cancer Res. 2004;64(12):4357-65.
228. Ueno H, Palucka AK, Banchereau J. The expanding family of dendritic cell subsets. Nat Biotechnol. 2010;28(8):813-5.
229. Idoyaga J, Lubkin A, Fiorese C, Lahoud MH, Caminschi I, Huang Y, et al. Comparable T helper 1 (Th1) and CD8 T-cell immunity by targeting HIV gag p24 to CD8 dendritic cells within antibodies to Langerin, DEC205, and Clec9A. Proc Natl Acad Sci U S A. 2011;108(6):2384-9.
230. Tel J, Sittig SP, Blom RA, Cruz LJ, Schreibelt G, Figdor CG, et al. Targeting uptake receptors on human plasmacytoid dendritic cells triggers antigen cross-presentation and robust type I IFN secretion. J Immunol. 2013;191(10):5005-12.
231. Hanlon DJ, Aldo PB, Devine L, Alvero AB, Engberg AK, Edelson R, et al. Enhanced stimulation of anti-ovarian cancer CD8(+) T cells by dendritic cells loaded with nanoparticle encapsulated tumor antigen. Am J Reprod Immunol. 2011;65(6):597-609.
232. Mueller M, Reichardt W, Koerner J, Groettrup M. Coencapsulation of tumor lysate and CpG-ODN in PLGA-microspheres enables successful immunotherapy of prostate carcinoma in TRAMP mice. J Control Release. 2012;162(1):159-66.
233. Sapin A, Garcion E, Clavreul A, Lagarce F, Benoit JP, Menei P. Development of new polymer-based particulate systems for anti-glioma vaccination. Int J Pharm. 2006;309(1-2):1-5.
234. Sapin A, Clavreul A, Garcion E, Benoit JP, Menei P. Evaluation of particulate systems supporting tumor cell fractions in a preventive vaccination against intracranial rat glioma. J Neurosurg. 2006;105(5):745-52.
235. Gross BP, Wongrakpanich A, Francis MB, Salem AK, Norian LA. A therapeutic microparticle-based tumor lysate vaccine reduces spontaneous metastases in murine breast cancer. AAPS J. 2014;16(6):1194-203.
236. Prasad S, Cody V, Saucier-Sawyer JK, Saltzman WM, Sasaki CT, Edelson RL, et al. Polymer nanoparticles containing tumor lysates as antigen delivery vehicles for dendritic cell-based antitumor immunotherapy. Nanomedicine. 2011;7(1):1-10.
237. Prasad S, Cody V, Saucier-Sawyer JK, Fadel TR, Edelson RL, Birchall MA, et al. Optimization of stability, encapsulation, release, and cross-priming of tumor antigen-containing PLGA nanoparticles. Pharm Res. 2012;29(9):2565-77.
238. Solbrig CM, Saucier-Sawyer JK, Cody V, Saltzman WM, Hanlon DJ. Polymer nanoparticles for immunotherapy from encapsulated tumor-associated antigens and whole tumor cells. Mol Pharm. 2007;4(1):47-57.
239. Goforth R, Salem AK, Zhu X, Miles S, Zhang XQ, Lee JH, et al. Immune stimulatory antigen loaded particles combined with depletion of regulatory T-cells induce potent tumor specific immunity in a mouse model of melanoma. Cancer Immunol Immunother. 2009;58(4):517-30.
240. Ali OA, Emerich D, Dranoff G, Mooney DJ. In situ regulation of DC subsets and T cells mediates tumor regression in mice. Sci Transl Med. 2009;1(8):8ra19.

241. Tawde SA, Chablani L, Akalkotkar A, D'Souza C, Chiriva-Internati M, Selvaraj P, et al. Formulation and evaluation of oral microparticulate ovarian cancer vaccines. Vaccine. 2012;30(38):5675-81.
242. Yoshikawa T, Okada N, Tsujino M, Gao JQ, Hayashi A, Tsutsumi Y, et al. Vaccine efficacy of fusogenic liposomes containing tumor cell-lysate against murine B16BL6 melanoma. Biol Pharm Bull. 2006;29(1):100-4.
243. Bhargava A, Mishra DK, Jain SK, Srivastava RK, Lohiya NK, Mishra PK. Comparative assessment of lipid based nano-carrier systems for dendritic cell based targeting of tumor re-initiating cells in gynecological cancers. Mol Immunol. 2016;79:98-112.
244. Meng J, Meng J, Duan J, Kong H, Li L, Wang C, et al. Carbon nanotubes conjugated to tumor lysate protein enhance the efficacy of an antitumor immunotherapy. Small. 2008;4(9):1364-70.
245. Oh T, Fakurnejad S, Sayegh ET, Clark AJ, Ivan ME, Sun MZ, et al. Immunocompetent murine models for the study of glioblastoma immunotherapy. J Transl Med. 2014;12:107.
246. Seligman AM, Shear M. Studies in carcinogenesis: VIII. Experimental production of brain tumors in mice with methylcholanthrene. Am J Cancer. 1939;37:364-95.
247. Gould SE, Junttila MR, de Sauvage FJ. Translational value of mouse models in oncology drug development. Nat Med. 2015;21(5):431-9.
248. Szatmari T, Lumniczky K, Desaknai S, Trajcevski S, Hidvegi EJ, Hamada H, et al. Detailed characterization of the mouse glioma 261 tumor model for experimental glioblastoma therapy. Cancer Sci. 2006;97(6):546-53.
249. Ausman JI, Shapiro WR, Rall DP. Studies on the chemotherapy of experimental brain tumors: development of an experimental model. Cancer Res. 1970;30(9):2394-400.
250. Hatano M, Kuwashima N, Tatsumi T, Dusak JE, Nishimura F, Reilly KM, et al. Vaccination with EphA2-derived T cell-epitopes promotes immunity against both EphA2-expressing and EphA2-negative tumors. J Transl Med. 2004;2(1):40.
251. Seyfried TN, el-Abbadi M, Roy ML. Ganglioside distribution in murine neural tumors. Mol Chem Neuropathol. 1992;17(2):147-67.
252. Binello E, Qadeer ZA, Kothari HP, Emdad L, Germano IM. Stemness of the CT-2A Immunocompetent Mouse Brain Tumor Model: Characterization In Vitro. J Cancer. 2012;3:166-74.
253. De Vleeschouwer S, Arredouani M, Ade M, Cadot P, Vermassen E, Ceuppens JL, et al. Uptake and presentation of malignant glioma tumor cell lysates by monocyte-derived dendritic cells. Cancer Immunol Immunother. 2005;54(4):372-82.
254. Kalkanidis M, Pietersz GA, Xiang SD, Mottram PL, Crimeen-Irwin B, Ardipradja K, et al. Methods for nano-particle based vaccine formulation and evaluation of their immunogenicity. Methods. 2006;40(1):20-9.
255. Xiang SD, Wilson K, Day S, Fuchsberger M, Plebanski M. Methods of effective conjugation of antigens to nanoparticles as non-inflammatory vaccine carriers. Methods. 2013;60(3):232-41.
256. Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, et al. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J Exp Med. 1992;176(6):1693-702.
257. Lutz MB, Kukutsch N, Ogilvie AL, Rossner S, Koch F, Romani N, et al. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J Immunol Methods. 1999;223(1):77-92.
258. Okada H, Tahara H, Shurin MR, Attanucci J, Giezeman-Smits KM, Fellows WK, et al. Bone marrow-derived dendritic cells pulsed with a tumor-specific peptide elicit effective anti-tumor immunity against intracranial neoplasms. Int J Cancer. 1998;78(2):196-201.

259. Loike JD, Silverstein SC. A fluorescence quenching technique using trypan blue to differentiate between attached and ingested glutaraldehyde-fixed red blood cells in phagocytosing murine macrophages. J Immunol Methods. 1983;57(1-3):373-9.
260. Maes W, Rosas GG, Verbinnen B, Boon L, De Vleeschouwer S, Ceuppens JL, et al. DC vaccination with anti-CD25 treatment leads to long-term immunity against experimental glioma. Neuro Oncol. 2009;11(5):529-42.
261. Prins RM, Odesa SK, Liau LM. Immunotherapeutic targeting of shared melanoma-associated antigens in a murine glioma model. Cancer Res. 2003;63(23):8487-91.
262. Garg AD, Vandenberk L, Koks C, Verschuere T, Boon L, Van Gool SW, et al. Dendritic cell vaccines based on immunogenic cell death elicit danger signals and T cell-driven rejection of high-grade glioma. Sci Transl Med. 2016;8(328):328ra27.
263. Price JG, Idoyaga J, Salmon H, Hogstad B, Bigarella CL, Ghaffari S, et al. CDKN1A regulates Langerhans cell survival and promotes Treg cell generation upon exposure to ionizing irradiation. Nat Immunol. 2015;16(10):1060-8.
264. Maes W, Van Gool SW. Experimental immunotherapy for malignant glioma: lessons from two decades of research in the GL261 model. Cancer Immunol Immunother. 2011;60(2):153-60.
265. Heath WR, Carbone FR. The skin-resident and migratory immune system in steady state and memory: innate lymphocytes, dendritic cells and T cells. Nat Immunol. 2013;14(10):978-85.
266. Sondak VK, Sosman JA. Results of clinical trials with an allogenic melanoma tumor cell lysate vaccine: Melacine. Semin Cancer Biol. 2003;13(6):409-15.
267. Litterman AJ, Zellmer DM, Grinnen KL, Hunt MA, Dudek AZ, Salazar AM, et al. Profound impairment of adaptive immune responses by alkylating chemotherapy. J Immunol. 2013;190(12):6259-68.
268. Walker JM. The Protein Protocols Handbook. Third Edition. Third ed. New York (NY): Springer-Verlag; 2009.
269. Hardy CL, Lemasurier JS, Mohamud R, Yao J, Xiang SD, Rolland JM, et al. Differential uptake of nanoparticles and microparticles by pulmonary APC subsets induces discrete immunological imprints. J Immunol. 2013;191(10):5278-90.
270. Weller RO, Engelhardt B, Phillips MJ. Lymphocyte targeting of the central nervous system: a review of afferent and efferent CNS-immune pathways. Brain Pathol. 1996;6(3):275-88.
271. Ameratunga M, Coleman N, Welsh L, Saran F, Lopez J. CNS cancer immunity cycle and strategies to target this for glioblastoma. Oncotarget. 2018;9(32):22802-16.
272. Small EJ, Sacks N, Nemunaitis J, Urba WJ, Dula E, Centeno AS, et al. Granulocyte macrophage colony-stimulating factor--secreting allogeneic cellular immunotherapy for hormone-refractory prostate cancer. Clin Cancer Res. 2007;13(13):3883-91.
273. Chen JC, Chen Y, Wu JM, Su YH, Tai KF, Tseng SH. Effects of irradiated tumor vaccine and infusion of granulocyte-macrophage colony-stimulating factor and interleukin-12 on established gliomas in rats. Cancer Immunol Immunother. 2006;55(7):873-83.
274. Goldstein MJ, Varghese B, Brody JD, Rajapaksa R, Kohrt H, Czerwinski DK, et al. A CpG-loaded tumor cell vaccine induces antitumor CD4+ T cells that are effective in adoptive therapy for large and established tumors. Blood. 2011;117(1):118-27.
275. Baars A, Claessen AM, van den Eertwegh AJ, Gall HE, Stam AG, Meijer S, et al. Skin tests predict survival after autologous tumor cell vaccination in metastatic melanoma: experience in 81 patients. Ann Oncol. 2000;11(8):965-70.
276. Crane CA, Han SJ, Ahn B, Oehlke J, Kivett V, Fedoroff A, et al. Individual patient-specific immunity against high-grade glioma after vaccination with autologous tumor derived peptides bound to the 96 KD chaperone protein. Clin Cancer Res. 2013;19(1):205-14.

277. Olin MR, Andersen BM, Zellmer DM, Grogan PT, Popescu FE, Xiong Z, et al. Superior efficacy of tumor cell vaccines grown in physiologic oxygen. Clin Cancer Res. 2010;16(19):4800-8.
278. Ardon H, Van Gool SW, Verschuere T, Maes W, Fieuws S, Sciot R, et al. Integration of autologous dendritic cell-based immunotherapy in the standard of care treatment for patients with newly diagnosed glioblastoma: results of the HGG-2006 phase I/II trial. Cancer Immunol Immunother. 2012;61(11):2033-44.
279. Jocham D, Richter A, Hoffmann L, Iwig K, Fahlenkamp D, Zakrzewski G, et al. Adjuvant autologous renal tumour cell vaccine and risk of tumour progression in patients with renal-cell carcinoma after radical nephrectomy: phase III, randomised controlled trial. Lancet. 2004;363(9409):594-9.
280. May M, Brookman-May S, Hoschke B, Gilfrich C, Kendel F, Baxmann S, et al. Ten-year survival analysis for renal carcinoma patients treated with an autologous tumour lysate vaccine in an adjuvant setting. Cancer Immunol Immunother. 2010;59(5):687-95.
281. Carrasco-Marin E, Paz-Miguel JE, Lopez-Mato P, Alvarez-Dominguez C, Leyva-Cobian F. Oxidation of defined antigens allows protein unfolding and increases both proteolytic processing and exposes peptide epitopes which are recognized by specific T cells. Immunology. 1998;95(3):314-21.
282. Jia J, Zhang Y, Xin Y, Jiang C, Yan B, Zhai S. Interactions Between Nanoparticles and Dendritic Cells: From the Perspective of Cancer Immunotherapy. Front Oncol. 2018;8:404.
283. Tran TH, Tran TTP, Nguyen HT, Phung CD, Jeong JH, Stenzel MH, et al. Nanoparticles for dendritic cell-based immunotherapy. Int J Pharm. 2018;542(1-2):253-65.
284. Sanders E, Ashworth CT. A study of particulate intestinal absorption and hepatocellular uptake. Use of polystyrene latex particles. Exp Cell Res. 1961;22:137-45.
285. Herber DL, Cao W, Nefedova Y, Novitskiy SV, Nagaraj S, Tyurin VA, et al. Lipid accumulation and dendritic cell dysfunction in cancer. Nat Med. 2010;16(8):880-6.
286. Aubin-Tam ME, Hamad-Schifferli K. Structure and function of nanoparticle-protein conjugates. Biomed Mater. 2008;3(3):034001.
287. Cohn L, Delamarre L. Dendritic cell-targeted vaccines. Front Immunol. 2014;5:255.
288. Yang Y, Huang CT, Huang X, Pardoll DM. Persistent Toll-like receptor signals are required for reversal of regulatory T cell-mediated CD8 tolerance. Nat Immunol. 2004;5(5):508-15.
289. Heikenwalder M, Polymenidou M, Junt T, Sigurdson C, Wagner H, Akira S, et al. Lymphoid follicle destruction and immunosuppression after repeated CpG oligodeoxynucleotide administration. Nat Med. 2004;10(2):187-92.
290. Behrens EM, Canna SW, Slade K, Rao S, Kreiger PA, Paessler M, et al. Repeated TLR9 stimulation results in macrophage activation syndrome-like disease in mice. J Clin Invest. 2011;121(6):2264-77.
291. Diwan M, Elamanchili P, Cao M, Samuel J. Dose sparing of CpG oligodeoxynucleotide vaccine adjuvants by nanoparticle delivery. Curr Drug Deliv. 2004;1(4):405-12.
292. Sottoriva A, Spiteri I, Piccirillo SG, Touloumis A, Collins VP, Marioni JC, et al. Intratumor heterogeneity in human glioblastoma reflects cancer evolutionary dynamics. Proc Natl Acad Sci U S A. 2013;110(10):4009-14.
293. Patel AP, Tirosh I, Trombetta JJ, Shalek AK, Gillespie SM, Wakimoto H, et al. Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. Science. 2014;344(6190):1396-401.
294. Khong HT, Restifo NP. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. Nat Immunol. 2002;3(11):999-1005.

295. Li M, Davey GM, Sutherland RM, Kurts C, Lew AM, Hirst C, et al. Cell-associated ovalbumin is cross-presented much more efficiently than soluble ovalbumin in vivo. J Immunol. 2001;166(10):6099-103.
296. Xu Q, Liu G, Yuan X, Xu M, Wang H, Ji J, et al. Antigen-specific T-cell response from dendritic cell vaccination using cancer stem-like cell-associated antigens. Stem Cells. 2009;27(8):1734-40.
297. Di Tomaso T, Mazzoleni S, Wang E, Sovena G, Clavenna D, Franzin A, et al. Immunobiological characterization of cancer stem cells isolated from glioblastoma patients. Clin Cancer Res. 2010;16(3):800-13.
298. Pellegatta S, Poliani PL, Corno D, Menghi F, Ghielmetti F, Suarez-Merino B, et al. Neurospheres enriched in cancer stem-like cells are highly effective in eliciting a dendritic cell-mediated immune response against malignant gliomas. Cancer Res. 2006;66(21):10247-52.
299. Uto T, Akagi T, Yoshinaga K, Toyama M, Akashi M, Baba M. The induction of innate and adaptive immunity by biodegradable poly(gamma-glutamic acid) nanoparticles via a TLR4 and MyD88 signaling pathway. Biomaterials. 2011;32(22):5206-12.
300. Kim H, Uto T, Akagi T, Baba M, Akashi M. Amphiphilic poly (amoino acid) nanoparticles induce size-dependent dendritic cell maturation. Adv Funct Mater. 2010;20:3925-31.
301. Zhou Q, Zhang Y, Du J, Li Y, Zhou Y, Fu Q, et al. Different-Sized Gold Nanoparticle Activator/Antigen Increases Dendritic Cells Accumulation in Liver-Draining Lymph Nodes and CD8+ T Cell Responses. ACS Nano. 2016;10(2):2678-92.
302. Foged C, Hansen J, Agger EM. License to kill: Formulation requirements for optimal priming of CD8(+) CTL responses with particulate vaccine delivery systems. Eur J Pharm Sci. 2012;45(4):482-91.
303. Tomic S, Ethokic J, Vasilijic S, Ogrinc N, Rudolf R, Pelicon P, et al. Size-dependent effects of gold nanoparticles uptake on maturation and antitumor functions of human dendritic cells in vitro. PLoS One. 2014;9(5):e96584.
304. Lokhov PG, Balashova EE. Cellular cancer vaccines: an update on the development of vaccines generated from cell surface antigens. J Cancer. 2010;1:230-41.
305. Frazier JL, Han JE, Lim M, Olivi A. Immunotherapy combined with chemotherapy in the treatment of tumors. Neurosurg Clin N Am. 2010;21(1):187-94.
306. Shore ND. Advances in the understanding of cancer immunotherapy. BJU Int. 2015;116(3):321-9.
307. Gubin MM, Zhang X, Schuster H, Caron E, Ward JP, Noguchi T, et al. Checkpoint blockade cancer immunotherapy targets tumour-specific mutant antigens. Nature. 2014;515(7528):577-81.
308. Snyder A, Wolchok JD, Chan TA. Genetic basis for clinical response to CTLA-4 blockade. N Engl J Med. 2015;372(8):783.
309. Rizvi NA, Hellmann MD, Snyder A, Kvistborg P, Makarov V, Havel JJ, et al. Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. Science. 2015;348(6230):124-8.
310. Pardoll DM, Topalian SL. The role of CD4+ T cell responses in antitumor immunity. Curr Opin Immunol. 1998;10(5):588-94.
311. Fan Y, Moon JJ. Nanoparticle Drug Delivery Systems Designed to Improve Cancer Vaccines and Immunotherapy. Vaccines (Basel). 2015;3(3):662-85.
312. Perez-Diez A, Joncker NT, Choi K, Chan WF, Anderson CC, Lantz O, et al. CD4 cells can be more efficient at tumor rejection than CD8 cells. Blood. 2007;109(12):5346-54.

313. Ladekarl M, Agger R, Fleischer CC, Hokland M, Hulgaard EF, Kirkin A, et al. Detection of circulating tumor lysate-reactive CD4+ T cells in melanoma patients. Cancer Immunol Immunother. 2004;53(6):560-6.
314. Quezada SA, Peggs KS. Tumor-reactive CD4+ T cells: plasticity beyond helper and regulatory activities. Immunotherapy. 2011;3(8):915-7.
315. Zanetti M. Tapping CD4 T cells for cancer immunotherapy: the choice of personalized genomics. J Immunol. 2015;194(5):2049-56.
316. Brito LA, O'Hagan DT. Designing and building the next generation of improved vaccine adjuvants. J Control Release. 2014;190:563-79.
317. Pluhar GE, Grogan PT, Seiler C, Goulart M, Santacruz KS, Carlson C, et al. Anti-tumor immune response correlates with neurological symptoms in a dog with spontaneous astrocytoma treated by gene and vaccine therapy. Vaccine. 2010;28(19):3371-8.
318. Le Pogam C, Patel S, Gorombei P, Guerenne L, Krief P, Omidvar N, et al. DNA-mediated adjuvant immunotherapy extends survival in two different mouse models of myeloid malignancies. Oncotarget. 2015;6(32):32494-508.
319. Joshi VB, Geary SM, Salem AK. Biodegradable particles as vaccine antigen delivery systems for stimulating cellular immune responses. Hum Vaccin Immunother. 2013;9(12):2584-90.
320. Malissen B, Tamoutounour S, Henri S. The origins and functions of dendritic cells and macrophages in the skin. Nat Rev Immunol. 2014;14(6):417-28.
321. Sparber F, Tripp CH, Hermann M, Romani N, Stoitzner P. Langerhans cells and dermal dendritic cells capture protein antigens in the skin: possible targets for vaccination through the skin. Immunobiology. 2010;215(9-10):770-9.
322. Turner PV, Brabb T, Pekow C, Vasbinder MA. Administration of substances to laboratory animals: routes of administration and factors to consider. J Am Assoc Lab Anim Sci. 2011;50(5):600-13.
323. Morton DB, Jennings M, Buckwell A, Ewbank R, Godfrey C, Holgate B, et al. Refining procedures for the administration of substances. Report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement. British Veterinary Association Animal Welfare Foundation/Fund for the Replacement of Animals in Medical Experiments/Royal Society for the Prevention of Cruelty to Animals/Universities Federation for Animal Welfare. Lab Anim. 2001;35(1):1-41.
324. Zhu S, Li X, Lansakara PD, Kumar A, Cui Z. A nanoparticle depot formulation of 4-(N)-stearoyl gemcitabine shows a strong anti-tumour activity. J Pharm Pharmacol. 2013;65(2):236-42.
325. Zhang W, Wang L, Liu Y, Chen X, Liu Q, Jia J, et al. Immune responses to vaccines involving a combined antigen-nanoparticle mixture and nanoparticle-encapsulated antigen formulation. Biomaterials. 2014;35(23):6086-97.
326. Guo Y, Wang D, Song Q, Wu T, Zhuang X, Bao Y, et al. Erythrocyte Membrane-Enveloped Polymeric Nanoparticles as Nanovaccine for Induction of Antitumor Immunity against Melanoma. ACS Nano. 2015;9(7):6918-33.
327. Jahan ST, Sadat SMA, Yarahmadi M, Haddadi A. Potentiating Antigen Specific Immune Response by Targeted Delivery of the PLGA-Based Model Cancer Vaccine. Mol Pharm. 2019;16(2):498-509.
328. Kim JE, Patel MA, Mangraviti A, Kim ES, Theodros D, Velarde E, et al. Combination Therapy with Anti-PD-1, Anti-TIM-3, and Focal Radiation Results in Regression of Murine Gliomas. Clin Cancer Res. 2017;23(1):124-36.
329. Anguissola S, Garry D, Salvati A, O'Brien PJ, Dawson KA. High content analysis provides mechanistic insights on the pathways of toxicity induced by amine-modified polystyrene nanoparticles. PLoS One. 2014;9(9):e108025.

330. Oberdorster G, Maynard A, Donaldson K, Castranova V, Fitzpatrick J, Ausman K, et al. Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy. Part Fibre Toxicol. 2005;2:8.
331. Oberdorster G, Oberdorster E, Oberdorster J. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environ Health Perspect. 2005;113(7):823-39.
332. Oberdorster G. Safety assessment for nanotechnology and nanomedicine: concepts of nanotoxicology. J Intern Med. 2010;267(1):89-105.
333. Kastenmuller W, Kastenmuller K, Kurts C, Seder RA. Dendritic cell-targeted vaccines--hope or hype? Nat Rev Immunol. 2014;14(10):705-11.

Acknowledgements, personal contributions and conflicts of interest

Acknowledgements

The research leading to the results of this project has mainly been supported by the Olivia Fund (http://www.olivia.be) and to a lesser extent by the James E. Kearney Memorial Foundation (http://www.jekfoundation.org). Jochen Belmans received a research grant for this project by the Belgian Brain Tumor Support (www.bbts.be).

We wish to thank the Cell Imaging Core at the KU Leuven for the availability of their fluorescence and confocal microscopes. We also thank Brecht Creyns, Matthias van Woensel and Joost Dejaegher for their help with the immune cell isolations and cell stainings, Jonathan Cremer for his advice and technical support in flow cytometry experiments, Lieve Coorevits, Ellen Dilissen and Anaïs Van Hoylandt for their technical support in acquisition for flow cytometry, and Bert de Roo and Stephanie Seré for their help with the size measurements of nanoparticles and nanoparticle-lysate conjugates.

Personal contributions

All experiments were designed by Jochen Belmans, with essential input from Prof. Dr. Stefaan Van Gool, Prof. Dr. Dominique Bullens and Prof. Dr. Jean-Pierre Locquet.

Fluorescence and confocal microscopy was performed by Jochen Belmans at the Cell Imaging Core, KU Leuven, under supervision of Prof. Dr. Pieter Vanden Berghe.

Dynamic light scattering measurements were conducted at the Functional Nanosystems group by Jochen Belmans, with the help of Bert de Roo and Stephanie Seré, and the guidance of Prof. Dr. Jean-Pierre Locquet.

Jochen Belmans performed all murine experiments with the help of Brecht Creyns, Dr. Matthias Van Woensel and Dr. Dr. Joost Dejaegher. The anti-PD-1 antibody was developed and purified by Dr. Louis Boon.

Immune cell isolations and cell stainings were done by Jochen Belmans, with the help of Dr. Matthias Van Woensel, Brecht Creyns, Dr. Dr. Joost Dejaegher and Dr. Brecht Steelant. Antibody panels were designed by Jochen Belmans and Dr. Tina Verschuere with technical support from Jonathan Cremer. Acquisition for flow cytometry was performed by Jonathan Cremer, Lieve Coorevits, Ellen Dilissen and Anaïs Van Hoylandt. All flow cytometry analyses were done by Jochen Belmans, with support of Jonathan Cremer.

Statistical analysis was done by Jochen Belmans, under guidance of Prof. Dr. Stefaan Van Gool and Prof. Dr. Dominique Bullens

All figures and tables in this manuscript were made by Jochen Belmans.

This dissertation was written by Jochen Belmans, critically reviewed by Prof. Dr. Dominique Bullens, Prof. Dr. Stefaan Van Gool and Prof. Dr. Jean-Pierre Locquet.

Conflicts of interest

There are no conflicts of interest.

Curriculum Vitae

Jochen Belmans was born in Herentals (Belgium) on September 23rd, in 1988. He graduated from secondary school in 2006 at Vrije Sint-Lambertusschool in Westerlo with a major in Science-Mathematics. In 2006 he started his studies in Biomedical Sciences at the KU Leuven. During this study he performed internships in the Research Unit for Lung Toxicology, at the Stem Cell Institute Leuven and in the Laboratory of Auditory Neurophysiology. In 2010 he started his master thesis work under the supervision of Prof. Dr. Peter Hoet, Prof. Dr. Jeroen Vanoirbeek and Dr. Vanessa De Vooght in the Research Unit of Lung Toxicology. In 2011 he graduated cum laude as Master in the Biomedical Sciences with a major in Biomedical Research and a minor in Research in Biomedical Sciences. Subsequently, he started his PhD in the laboratory of Pediatric Immunology under supervision of Prof. Dr. Stefaan Van Gool and Prof. Dr. Dominique Bullens, and under guidance of Prof. Dr. Jean-Pierre Locquet of the Division of Functional Nanosystems of the KU Leuven Research and Development.

Bibliography and awards

Belmans J, Van Woensel M, Creyns B, Dejaegher J, Bullens DM, Van Gool SW. Immunotherapy with subcutaneous immunogenic autologous tumor lysate increases murine glioblastoma survival. Sci Rep. 2017;7(1):13902.

Van Woensel M, Mathivet T, Wauthoz N, Rosiere R, Garg AD, Agostinis P, Mathieu V, Kiss R, Lefranc F, Boon L, **Belmans J**, Van Gool SW, Gerhardt H, Amighi K, De Vleeschouwer S. Sensitization of glioblastoma tumor micro-environment to chemo- and immunotherapy by Galectin-1 intranasal knock-down strategy. Sci Rep. 2017;7(1):1217.

Garg AD, Vandenberk L, Van Woensel M, **Belmans J**, Schaaf M, Boon L, De Vleeschouwer S, Agostinis P. Preclinical efficacy of immune-checkpoint monotherapy does not recapitulate corresponding biomarkers-based clinical predictions in glioblastoma. Oncoimmunology. 2017;6(4):e1295903.

Vandenberk L, Garg AD, Verschuere T, Koks C, **Belmans J**, Beullens M, Agostinis P, De Vleeschouwer S, Van Gool SW. Irradiation of necrotic cancer cells, employed for pulsing dendritic cells (DCs), potentiates DC vaccine-induced antitumor immunity against high-grade glioma. Oncoimmunology. 2016;5(2):e1083669.

Vandenberk L, **Belmans J**, Van Woensel M, Riva M, Van Gool SW. Exploiting the Immunogenic Potential of Cancer Cells for Improved Dendritic Cell Vaccines. Front Immunol. 2015;6:663.

Mathivet T, Bouleti C, Van Woensel M, Stanchi F, Verschuere T, Phng LK, Dejaegher J, Balcer M, Matsumoto K, Georgieva PB, **Belmans J**, Sciot R, Stockmann C, Mazzone M, De Vleeschouwer S, Gerhardt H. Dynamic stroma reorganization drives blood vessel dysmorphia during glioma growth. EMBO Mol Med. 2017;9(12):1629-45.

Steelant B, Farre R, Wawrzyniak P, **Belmans J**, Dekimpe E, Vanheel H, Van Gerven L, Kortekaas Krohn I, Bullens DM, Ceuppens JL, Akdis CA, Boeckxstaens G, Seys SF, Hellings PW. Impaired barrier function in patients with house dust mite-induced allergic rhinitis is accompanied by decreased occludin and zonula occludens-1 expression. J Allergy Clin Immunol. 2016;137(4):1043-53 e1-5.

De Vooght V, Smulders S, Haenen S, **Belmans J**, Opdenakker G, Verbeken E, Nemery B, Hout PH, Vanoirbeek JA. Neutrophil and eosinophil granulocytes as key players in a mouse model of chemical-induced asthma. Toxicol Sci. 2013;131(2):406-18.

Award

Belgian Brain Tumor Support (BBTS) prize for the results obtained in my PhD project (December 2014)

Posters at scientific meetings

Oncoforum 2013, KU Leuven, Belgium.
Title: Tumor protein loaded nanoparticles in the treatment of Glioblastoma Multiforme

Oncoforum 2014, KU Leuven, Belgium.
Title: Nanoparticles conjugated with autologous tumor lysate are taken up by dendritic cells

LKI symposium - Tumor Immunology and Immunotherapy, September 2016, Leuven.
Title: Immunotherapy with subcutaneous autologous tumor lysate increases glioblastoma survival in mice

Presentations at scientific meetings

7th Rostock symposium for Tumor Immunology 2013, Rostock, Germany.
Title: Tumor protein loaded nanoparticles in the treatment of glioblastoma multiforme

8th Rostock symposium for Tumor Immunology 2014, Rostock, Germany.
Title: Nanotechnology in cancer immunotherapy - Tumor protein loaded nanoparticles in the treatment of glioblastoma multiforme

Immunology Summer School, September 2014, Leuven, Belgium.
Title: Nanotechnology in cancer immunotherapy - Tumor protein loaded nanoparticles in the treatment of glioblastoma multiforme

12th HGG-Immuno meeting, November 2014, Würzburg, Germany.
Title: Can nanotechnology help immunotherapy in glioblastoma multiforme? Tumor protein loaded nanoparticles in the treatment of glioblastoma multiforme

9th Rostock symposium for Tumor Immunology 2015, Rostock, Germany.
Title: Can nanotechnology help immunotherapy in glioblastoma multiforme? Tumor protein loaded nanoparticles

BVK – SBP congress 2015, Liège, Belgium.
Title: Can nanotechnology help immunotherapy in glioblastoma multiforme? Use of tumor protein loaded nanoparticles

6th International congress on Nanotechnology in medicine and biology, 08-10/04/2015, Medical university of Graz, Austria
Title: Can nanotechnology help immunotherapy in glioblastoma multiforme? Use of tumor protein loaded nanoparticles