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DESIGN OF IMMUNE-MODULATING POLYMERIC MICROPARTICLES IN VIEW OF CELL-DERIVED CANCER VACCINATION

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It’s our choices that show what we truly are,
far more than our abilities.

Harry Potter and the Chamber of Secrets - J.K Rowling

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LIST OF ABBREVIATIONS

1-MT	1-methyl-D,L-tryptophan
ACT	Adoptive cell transfer
ACVA	4,4’-azobis(4-cyanovaleric acid)
ADCC	Antibody-dependent cell-mediated cytotoxicity
AE	Adverse events
AICD	Activation-induced cell death
APC	Antigen presenting cell
APMA	N-(3-aminopropyl)methacrylamide
Arg1	Arginase 1
ATP	Adenosine triphosphate
ATRA	All-trans retinoic acid
Bcl-2	B-cell lymphoma-2
Bcl-XL	B-cell lymphoma-extra large
bmDC	Bone marrow-derived dendritic cells
BSA	Bovine serum albumin
BTLA	B-and T-lymphocyte attenuator protein
$CaCl_2$	Calcium chloride
$CaCO_3$	Calcium carbonate
CAF	Carcinoma-associated fibroblast
C-AM	Calcein acetoxymethyl

LIST OF ABBREVIATIONS

CAR	Chimeric antigen receptor
CCL	CC chemokine ligand
CD	Cluster of differentiation
CDC	Complement-dependent cytotoxicity
CLIP	Class II-associated invariant-chain peptide
CLR	C-type lectin receptor
CpG	Unmethylated cytosine guanine
CRS	Cytokine release syndrome
CRT	Calreticulin
CT5.3-eGFP	Colon tumor-derived cell line expressing eGFP
CTB	Cholera toxin subunit B
CTL	Cytotoxic T-lymphocyte
CTLA-4	Cytotoxic T-lymphocyte antigen-4
CTP	4-cyanovaleric acid dithiobenzoate
CXCL	CXC chemokine ligand
D_2O	Deuterium oxide
DAMP	Damage-associated molecular patterns
DC	Dendritic cell
DEXS	Dextran sulfate
DLS	Dynamic light scattering
DMAc	Dimethylacetamide
DMEM	Dulbecco's Modified Eagle Medium

LIST OF ABBREVIATIONS

DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DMTMM	4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium Chloride
DNA	Deoxyribonucleic acid
DP	Degree of polymerization
DPDS	Dipyridyldisulfide
dsRNA	Double-stranded ribonucleic acid
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EGF	Epidermal growth factor
eGFP	Enhanced green fluorescent protein
EM	Electron microscopy
ER	Endoplasmic reticulum
ESI-MS	Electron spray ionization mass spectrometry
FasL	Fas ligand
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FLIP	Fas-associated death domain (FADD)-like interleuking-1β-converting enzyme (FLICE)-like inhibitory protein
FSC	Forward scatter
G-CSF	Granulocyte colony-stimulating factor
GITR	Glucocorticoid-induced tumor necrosis factor receptor

LIST OF ABBREVIATIONS

GM-CSF	Granulocyte-macrophage colony-stimulating factor
HCl	Hydrogen chloride
HIF-1α	Hypoxia-inducible factor-1α
HLA	Human leucocyte antigen
HMGB1	High mobility group box-1
HPMA	N-(2-hydroxypropyl)methacrylamide
HSP	Heat shock protein
ICD	Immunogenic cell death
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ii	Invariant chain
IL	Interleukin
ITAM	Immuno-receptor tyrosine-based activating motifs
KIR	Killer-cell immunoglobulin-like receptor
LAG-3	Lymphocyte activation gene-3
LAL	Limulus amebocyte lysate
L-arg	L-arginine
LbL	Layer-by-layer
L-Cys	L-cystein
LiBr	Lithium bromide
LLC	Lewis Lung cancer
LMP	Low-molecular-weight protein

LIST OF ABBREVIATIONS

LOX	Lysyl oxidase
LPS	Lipopolysaccharide
L-Trp	L-tryptophan
mAb	Monoclonal antibody
MDSC	Myeloid-derived suppressor cell
MHC	Major Histocompatibility complex
MPLA	Monophosphoryl lipid A
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVD	Maximum valid dilution
MWCO	Molecular weight cut off
Na_2CO_3	Sodium carbonate
Na_2EDTA	Disodium ethylenediaminetetraacetic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NK	Natural killer
NKG2D	Natural killer group 2 member D
NLR	NOD-like receptor
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
Nos-2	Nitric oxide synthase-2

LIST OF ABBREVIATIONS

Nox-2	NADPH oxidase-2
NRF-2	Nf-E2-related factor-2
OVA	Ovalbumin
OVA-AF488	Ovalbumin Alexa Fluor 488
PAMP	Pathogen-associated molecular patterns
PAP	Prostatic acid phosphase
PBS	Phosphate buffered saline
PD-1	Programmed-death receptor-1
PDE-5	Phosphodiesterase-5
PDL1	Programmed-death ligand-1
PDPA	3-(2-pyridyldithio)-propanoic acid
PDS	Pyridyldisulfide
PFA	Paraformaldehyde
PGE-2	Prostaglandin E2
PI	Propidium iodide
P_LARG	Poly-L-arginine
PLC	Peptide loading complex
PMMA	Polymethylmethacrylate
PRR	Pathogen recognition receptor
PVP	Poly(vinylpyrrolidone)
RAFT	Reversible addition-fragmentation chain-transfer
RID	Refractor index detector

LIST OF ABBREVIATIONS

RIG	Retinoic acid-inducible gene
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SATP	S-acetylthiopropionate N-succinimidyl ester
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodevyl sulfate polyacrylamide gel electrophoresis
SEAP	Secreted embryonic alkaline phosphatase
SEC	Size exclusion chromatography
SEM	Scanning electron microscopy
SMIP	Small molecule immune potentiators
SSC	Side scatter
ssRNA	Single-stranded ribonucleic acid
STAT	Signal transducer and activator of transcription
TA	Tannic acid
TAA	Tumor-associated antigens
TAM	Tumor-associated macrophage
TAP	Transporter associated with antigen processing
TCR	T-cell receptor
TEM	Transmission electron microscopy
TGF-β	Transforming growth factor-β

LIST OF ABBREVIATIONS

TH cells	T-helper cells
TIL	Tumor-infiltrating lymphocytes
TIM-3	T-cell immunoglobulin- and mucin-containing protein-3
TLR	Toll-like receptor
TME	Tumor microenvironment
TNBSA	Trinitrobenzene sulfonic acid
TNM classification	Tumor burden – lymph node – metastasis classification
tol-DC	Tolerogenic dendritic cell
Treg	Regulatory T-cell
TSA	Tumor-specific antigens
T-VEC	Talimogene laherparepvec
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
VLS	Vascular leak syndrome

OUTLINE AND AIM OF THESIS

Anti-tumor immune-therapy has gathered major attention in the search for innovative strategies to fight cancer in a more specific manner. It merges the field of oncology with immunology into immuno-oncology, an exciting path that has the potential to dramatically improve treatment efficacy and specificity whilst evoking immunological memory and prolonged protection against malignancies. A promising immunotherapeutic strategy is cancer vaccination which aims to educate dendritic cells at priming cytotoxic T-cell responses that can specifically recognize and eliminate tumor cells.

This thesis focuses on the design of immunogenic vaccine microparticles that encapsulate cancer cell lysates in view of personalized anti-cancer vaccination based on tumor-associated and tumor-specific antigens. The majority of current vaccine approaches involve tumor-associated antigens and face several drawbacks such as limited applicability and treatment failure due to thymic tolerance, mutation or lack of expression. Personalized cancer vaccines that contain cancer cell material collected from biopsy or surgery are therefore an interesting approach to circumvent these drawbacks and hold potential to evoke more robust immune responses specifically tailored to the patient's unique mutanome.

In total four different strategies are developed that allow for the formulation of cancer cell lysate (Chapter 3 and 4) and whole cancer cells (Chapter 5 and 6) under mild conditions into microparticles. In addition, efforts are also devoted to enhance the immunogenicity of the microparticles to enable not only efficient internalization by dendritic cells but also activation of the latter which is required for optimal priming of robust T-cell responses.

Chapter 1 provides a brief introduction of the innate immune system and the adaptive immune system that protect the host against invading pathogens.

Chapter 2 describes the dual and complex role of the immune system in tumor development and tumor eradication, thereby raising the potential of immune-therapy in anti-cancer treatment. In addition, an overview is given of the current approaches for anti-cancer immune-therapy.

Chapter 3 and Chapter 4 involve the formulation of soluble cancer cell lysates.

Chapter 3 deals with the design of polymer-protein conjugates formed by disulfide exchange. For this purpose, polymers are developed that bear pending pyridyldisulfide moieties on their backbone. The latter allows for reversible disulfide bonds with either cysteine moieties or synthetically introduced thiols on the protein backbone. Finally, the effect of polymer conjugation on the *in vitro* interaction with dendritic cells is investigated.

Chapter 4 elaborates on the encapsulation of cancer cell lysate into porous calcium carbonate ($CaCO_3$) microparticles by a one-step co-precipitation reaction. To enhance the immunogenicity of the microparticles polymer-conjugated small molecule TLR7/8-agonists are adsorbed onto the microparticle surface. Additionally, the ability of these microparticles to activate dendritic cells is investigated *in vitro*.

Chapter 5 and 6 focus on the formulation of whole cancer cells into microparticles. Whole tumor cells comprise cell membrane components, and when translated to whole tumor tissue, also offer the possibility to co-encapsulate stromal proteins.

In Chapter 5 living cancer cells are used as templates for layer-by-layer assembly of hydrogen bonding species followed by hypo-osmotic treatment to obtain bio-hybrid capsules loaded with cancer cell lysate. Immunogenic properties are engineered into the capsules by pre-treatment of the cancer cells with heat shock to induce expression of damage-associated molecular patterns.

As the layer-by-layer approach is however labor-intensive and time-consuming, an alternative strategy is developed in Chapter 6, which presents a single-step method to encapsulate whole cancer cells in matrix microparticles composed of oppositely charged polyelectrolytes. In analogy to Chapter 4, the immunogenicity of the microparticles is enhanced by incorporation of a polymer-conjugated small molecule TLR7/8-agonist. Finally, the interaction of these microparticles with dendritic cells is investigated *in vitro*.

To conclude, an overview of the broader international context and relevance of this thesis alongside future perspectives of anti-cancer immune-therapy is provided in Chapter 7.

PART I

GENERAL INTRODUCTION AND BACKGROUND

CHAPTER 1

THE IMMUNE SYSTEM

INNATE AND ADAPTIVE PROTECTION AGAINST INFECTION

ABSTRACT

The immune system is the defense mechanism of the body against damage and infiltrating pathogens such as viruses and bacteria. In addition, it is also involved in avoiding auto-immunity, i.e. recognition of self-antigens, by induction of self-tolerance. In this regard, the immune system consists out of soldiers that are specifically trained to recognize damage and foreign patterns and can distinguish between non-self and self. It comprises different cell populations and molecules that are part of the innate immune system – i.e. the initial and non-specific defense mechanism – or the adaptive immune system – i.e. the slower but specific and stronger immune response.

THE INNATE IMMUNE SYSTEM

Innate immunity is the first line defense of the body against infectious agents. It acts immediately upon recognition of damage or pathogen signals in order to confine any threat to the host as fast and efficiently as possible. It is non-specific as it can only recognize patterns that are common to many pathogens. Nevertheless, it is a very important first line defense mechanism to protect the body against any invading pathogen. This ideally leads to elimination of the invader but can also lead to merely dampening of the infection as the innate immune system is often not strong enough to immediately eradicate the pathogen. This dampening effect is however of high importance as it delays the infection and allows the adaptive system to gain specificity and strength to ultimately eradicate the respective pathogen (*vide infra – the adaptive immune system*). The major effector mechanisms involved in innate immunity rely on neutrophils, monocytes, natural killer (NK)-cells, the complement system and macrophages. These innate components all have the ability to bind or take up and subsequently eliminate undesirable material through different mechanisms. In addition, they induce inflammation and recruit more immune cells to the infected or damaged site to increase the probability of pathogen elimination or repair respectively[1]. Furthermore, this chapter will devote focus to NK-cells, the complement system and macrophages due to their importance in the following chapters.

Natural killer cells are specialized in clearance of cells that are infected with pathogens, mainly viruses, and rely on the balance between activation and inhibition. NK-cells express two different type of receptors, i.e. inhibiting receptors and activating receptors. First, NK-cells express killer-cell immunoglobulin-like receptors (KIRs) and regulate the killer function upon interaction with major histocompatibility complex class I (MHC-I) molecules[2-4]. MHC class I molecules are expressed in nucleated cells and interaction of MHC-I with KIRs on the cell surface of NK-cells leads to inhibition of the killer function. In contrast, infected cells or cancer cells downregulate MHC-I expression to evade immune recognition by the adaptive immune system (*vide infra – Chapter 2: Tumor immune-escape*). Therefore, they do not provide the inhibitory signal necessary to suppress NK-cell mediated elimination resulting in lysis of the target cells via secretion of perforin and granzymes[5,6]. Second, activating receptors on the surface of NK-cells activate NK-cell reactivity upon interaction with their ligands. These include

receptors that interact with cytokines – i.e. interleukin (IL)-1, IL-2, IL-15 and IL-18 – or cell surface molecules such as natural killer group 2 member D (NKG2D) and immuno-receptor tyrosine-based activating motifs (ITAMs)[4,7,8]. Therefore, NK-cells can be activated by two cell types: [1] cells that lack MHC-I expression such as cancer cells or infected cells; and [2] cells that express MHC-I but also exhibit expression of activating cytokines or molecules due to stress or damage. In addition, activated NK-cells also produce pro-inflammatory mediators to further enhance inflammation and recruitment of other immune cells.

Another important innate protection strategy of the immune system is associated with the complement system. The complement system comprises around 35 different soluble and membrane-bound proteins and activation leads to a cascade of proteolytic reactions[9]. Initiation of the complement pathway occurs when foreign material is recognized or upon recognition of a foreign antigen-antibody complex (*vide infra – B-lymphocytes*). This results in opsonization of the material followed by cell lysis or removal by phagocytes[10-12].

Macrophages on the other hand are very important phagocytes, are resident in many tissues and are primarily designed to ingest and eliminate dead or dying cells, cell debris and pathogens. Due to the presence, although limited in number, of germline-encoded pathogen recognition receptors (PRRs) on the cell surface of macrophages, they recognize conserved damage- and pathogen-associated molecular patterns, DAMPs and PAMPs, very efficiently[13]. Macrophages ingest pathogens upon recognition of PAMPs resulting in entrapment of the respective pathogen in the phagosome followed by fusion with a lysosome leading to enzymatic destruction of the infectious agent. In addition, macrophages are also programmed to avoid toxic accumulation of cell debris, dead or dying cells and prevent further cell damage upon recognition of endogenous DAMPs[14]. Besides induction of uptake of foreign and dead cell material, activation of these PRRs additionally evokes production of pro-inflammatory cytokines, type I interferons (IFN) and chemokines leading to local and systemic inflammation which in turn results in recruitment and activation of other immune cells[1,15,16].

Altogether, the innate immune system is a very important part of immunity as it protects the body against infiltrating pathogens and tissue damage while inducing recruitment of other innate immune cells and adaptive immune cells. Protection is based on recognition of highly conserved patterns, whether or not of microbial origin, and is non-specific which enables

immediate interaction and thus fast elimination of the originator. It is however often not sufficient to completely eradicate the cause and relies on the adaptive immune system to fully eradicate the infection.

DENDRITIC CELLS: THE LINK BETWEEN INNATE AND ADAPTIVE IMMUNITY

Dendritic cells (DCs) are necessary to direct the adaptive immune system specifically against invading pathogens and are therefore the critical factor in the interplay between the innate and the adaptive immune response. They are the most potent class of antigen presenting cells (APCs) due to their high antigen presentation capacity and high PRR expression number compared to other APCs such as macrophages or B-cells of which the primary function is phagocytosis of foreign and dead cell material (*vide supra – The innate immune system*) or antibody secretion respectively (*vide infra – B-lymphocytes*), rather than antigen presentation. DCs are highly efficient in pathogen recognition, uptake, processing and antigen presentation alongside providing the necessary signals for optimal activation of lymphocytes.

1. ANTIGEN PRESENTATION AND CROSS-PRESENTATION

Two main types of DCs exist, i.e. migratory DCs or resident lymphoid DCs. Migratory DCs migrate to the site of infection followed by transport to the draining lymph nodes whereas resident lymphoid DCs do not migrate and are stimulated in the lymph nodes upon interaction with migratory DCs[17]. In addition a more accurate classification of DCs is currently accepted, involving plasmacytoid DCs, CD11b+ DCs and XCR1 DCs of which the latter can be subdivided into CD103+ DCs and CD8+ DCs. Detailed description of these subtypes will, however, not be adressed in this chapter as it is out of scope and not relevant for the following chapters.

Naive or immature DCs recognize pathogens immediately due to the presence of a high number of PRRs on their surface and are therefore experts in capturing antigens via phagocytosis yet are inefficient in antigen processing and presentation. However, stimulation of these highly expressed PRRs by PAMPs or DAMPs results in strong maturation of the dendritic cells which exhibit much lower phagocytic properties in contrast to naive DCs and redistribute MHC molecules to the cell surface accompanied with antigen presentation[18-20]. Prior to antigen presentation, the phagocytosed foreign material is processed in the phagosome of the

dendritic cell. This occurs through fusion of the phagosome with lysosomes and subsequent acidification (pH 4.5-5) resulting in degradation of the material in the presence of lysosomal hydrolases. Depending on the origin of material, i.e. exogenous or endogenous, the obtained peptides are loaded onto MHC class II or MHC class I molecules respectively which are assembled and provided by the endoplasmic reticulum (ER). They differ in tissue distribution and type of peptides that are presented due to different processing pathways. Class I molecules are expressed by nucleated cells and are therefore widely abundant, whereas MHC-II is primarily expressed by antigen presenting cells of which DCs are the most efficient.

MHC class II loading of exogenous peptides is triggered upon release from class II molecules of the invariant chain (Ii), a transmembrane chaperone protein, in response to proteases from the MHC-II peptide binding site only leaving a short MHC class II-associated invariant-chain peptide (CLIP) behind. Subsequently, exchange of CLIP with exogenous peptides can occur in the presence of a catalyst chaperone protein HLA-DM which facilitates CLIP release from the MHC class II binding cavity and enhances antigen presentation. Following MHC-II loading of these peptides, the MHC-II-antigen complex is transported to the cell membrane and presented to the environment[21-24]. In contrast, MHC class I loading occurs to present cytosolic and nuclear derived protein fragments at the cell surface. MHC-I presentation is important for the establishment of self-tolerance and eradication of intracellular microorganisms and tumors. The proteins are degraded in the cytosol of DCs by the proteasome followed by translocation of the resulting peptides to the ER via TAP, a transporter associated with antigen presentation. Like MHC class II molecules, the MHC-I molecules are stabilized by chaperone proteins in the ER lumen and ensure efficient peptide loading. These chaperone proteins include calreticulin, ERp57, tapasin and TAP, also called the peptide-loading complex (PLC). In addition, the exchange of endogenous peptides with the PLC is facilitated by tapasin similar to HLA-DM that catalyzes the exchange on MHC class II molecules[25]. Finally, the MHC-I-antigen complex is transferred to the cell membrane via the Golgi apparatus for antigen presentation[22,26,27].

Because exogenously derived proteins are presented onto MHC class II molecules, this implicates pathogens should be primarily presented on MHC-II by APCs. However, this is often not the case due to the induction of a process called cross-presentation. This process only occurs efficiently in dendritic cells which once again points out the unique ability of DCs in

priming the immune system. Cross-presentation is favored upon interaction of APCs with particulate antigens such as pathogens and is a critical process for the eradication of infectious agents via the adaptive immune system (*vide infra – T-lymphocytes*). It involves two main pathways, i.e. the TAP-independent vacuolar pathway and the TAP-dependent cytosolic pathway. The latter pathway is similar to the standard MHC-I antigen presentation pathway for endogenous peptides and is therefore the most abundant. It comprises transfer to the cytoplasm of the engulfed proteins mediated by the ER-derived Sec61 translocation complex followed by degradation through the proteasome prior to MHC-I loading in the phagosome or the endoplasmic reticulum via TAP. In contrast, MHC-I loading in TAP-independent cross-presentation occurs in the phagosome vesicles and is also proteasome-independent. After uptake, the material is degraded by proteases present in the phagosome followed by immediate loading of the peptides onto MHC class I molecules provided by the ER and transport to the cell membrane[28-33]. An overview of the different MHC antigen presentation pathways is illustrated in Figure 1.

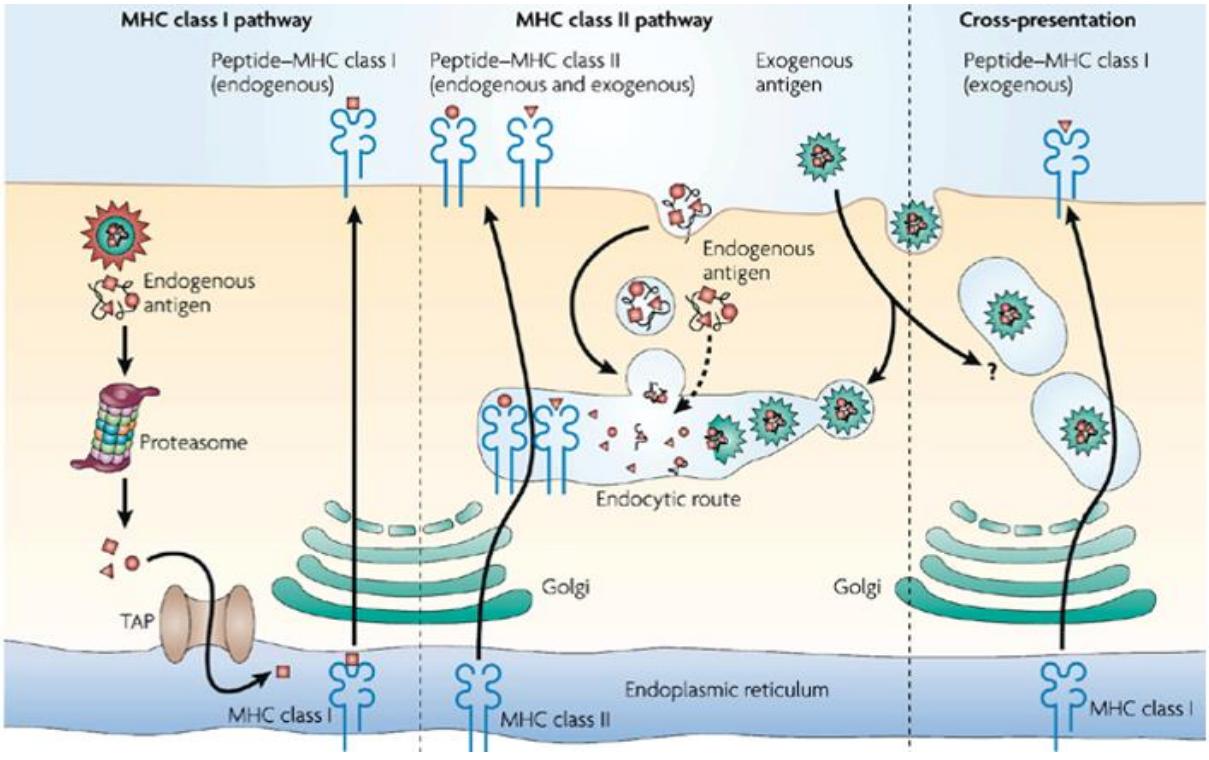

Figure 1. MHC antigen presentation pathways in dendritic cells - (*Figure adjusted from reference 33*).

2. DC MATURATION

Upon recognition of a pathogen by naive DCs, redistribution of MHC molecules and increased antigen presentation ability occurs alongside maturation of the DCs and upregulation of costimulatory molecules and cytokine secretion, providing the second and third signal for activation of T-cells (*vide infra – T-lymphocytes*). Co-stimulatory molecules involve the cluster of differentiation (CD) molecules, CD80 and CD86, which provide the second signal for optimal T-lymphocyte activation via interaction with the CD28 receptor on the T-cell surface[34,35]. In addition, the third signal for T-cell activation induces, depending on the type of cytokines, different T-lymphocyte populations. Taken together, this shows the high potential of dendritic cells as they not only very efficiently present pathogenic peptides to the adaptive immune system but are also able to optimally prime the immune cells against the respective pathogen[36-38].

Depending on the type of pathogen, different PRRs are triggered. Several PRRs exist, involving toll-like receptors (TLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and C-type lectin receptors (CLRs) of which the TLRs are the most widely explored and well characterized[15,39,40]. Different types of TLRs exist and so far ten toll-like receptors have been identified in humans referred from TLR1 up to TLR10. Some TLRs are cell membrane bound involving the TLRs 1, 2, 4, 5 and 6, all of which primarily recognize bacterial products whereas the TLRs 3, 7, 8 and 9 are confined in intracellular compartments and focus on viral material[41-44]. Cell surface TLRs mainly interact with microbial cell membrane components such as lipids, lipoproteins and protein. TLR1 recognizes lipoproteins and oligosaccharides originating from the cell wall of bacteria and involves hydrophobic interactions whereas TLR6 interacts with lipoteichoic acids and is characterized by hydrophilic ligand binding. Both TLR1 and TLR6 form heterodimers with TLR2 and by consequence lead to hydrophobic and hydrophilic interactions respectively[45,46]. TLR4 is the most studied receptor and is targeted by lipopolysaccharide (LPS) and its derivatives such as monophosphoryl lipid A (MPLA)[47]. LPS or endotoxins are a part of the cell membrane of Gram-negative bacteria. Note, that TLR2 and TLR4 are also present in intracellular compartments of antigen presenting cells[48,49]. TLR5 recognizes the motility filament of bacteria flagellin and flagellin-related peptides[50,51]. Despite the fact that TLR10 is an orphan receptor without defined ligands, it has been shown to recognize specific pathogens such as Listeria, in

collaboration with TLR2, and influenza A virus[43,52,53]. Intracellular TLRs are exclusively found in the phagosomal compartments of dendritic cells and macrophages. TLR3 binds specifically to double-stranded viral ribonucleic acid (dsRNA)[54] whereas TLR7 and TLR8 recognize single-stranded RNA (ssRNA) [55]. The TLR9 receptor on the other hand interacts with foreign deoxyribonucleic acid (DNA) and is activated by oligonucleotides such as CpG (unmethylated cytosine guanine) sequences[41,43,56,57].

ADAPTIVE IMMUNITY

In contrast to the innate immune system, the adaptive immune system requires more time to gain reactivity against invading pathogens. Adaptive immunity is specifically primed against every new infectious agent that enters the body by antigen presenting cells, in particular by dendritic cells (*vide supra – Dendritic cells*) and is therefore slower but stronger and highly specific. It involves B- and T-lymphocytes that, upon optimal activation by dendritic cells, differentiate into plasma cells or memory B-cells, CD4+ T-helper (TH) cells and CD8+ effector T-cells or memory T-cells respectively which all have different functions in combatting infections. In general, B-lymphocytes give rise to humoral responses whereas T-lymphocytes induce cellular immunity.

1. T-LYMPHOCYTES AND CELLULAR IMMUNITY

Activation of T-lymphocytes results in cell-mediated immune responses as it gives rise to effector T-cells such as CD8+ cytotoxic T-lymphocytes (CTLs), CD4+ T-helper cells and memory T-cells which are primed upon recognition of antigen by naive T-cells followed by activation and clonal expansion upon optimal stimulation. T-cells require three signals provided by antigen presenting cells to be fully activated: [1] recognition of peptide-MHC complex by the T-cell receptor (TCR); [2] co-stimulation of the CD28 receptor on the T-cell receptor; and [3] cytokines that influence the differentiation into the different types of effector cells, i.e. CTLs or T-helper cells (*vide supra – T-lymphocytes*). Inappropriate stimulation of naive T-cells however results in functional inactive or anergic T-cells and does not result in an immune response[58].

T-helper cells do not directly eradicate infected cells but aid the immune reaction via various mechanisms. The major subtypes of TH cells are TH1-, TH2-, TH17-cells and T follicular helper

cells or T_{FH} cells and are defined by their different cytokine profile production[59,60]. TH2-cells are involved in allergic responses and immunity against parasites through secretion of IL4 and IL-5 whereas T_{FH} cells provide direct help to B-cells via CD40 ligand expression (*vide infra – B-lymphocytes*). TH17 cells are involved in early adaptive responses against extracellular pathogens and fungi and produce IL-17, IL-21 and IL-22. TH1-cells facilitate CTL- and macrophage-mediated killing of microorganisms by producing TH1-cytokines such as IFN-γ and IL-2 but also enhance the CTL-response by expression of the CD40 ligand[58,61-63]. IL-2 is a cytokine that is also secreted by the T-cell itself to induce rapid proliferation by interaction of the interleukin with the IL-2-receptor that is highly expressed on the surface of T-cells. Therefore, secretion of IL-2 by TH1-cells acts as a growth factor which further amplifies the CD8+ T-lymphocyte proliferation. Expression of the CD40 ligand on the other hand has an indirect effect on T-cells by increasing the expression of co-stimulatory molecules on antigen presenting cells. Upon efficient T-cell priming and clonal expansion, the resulting CTLs very efficiently recognize

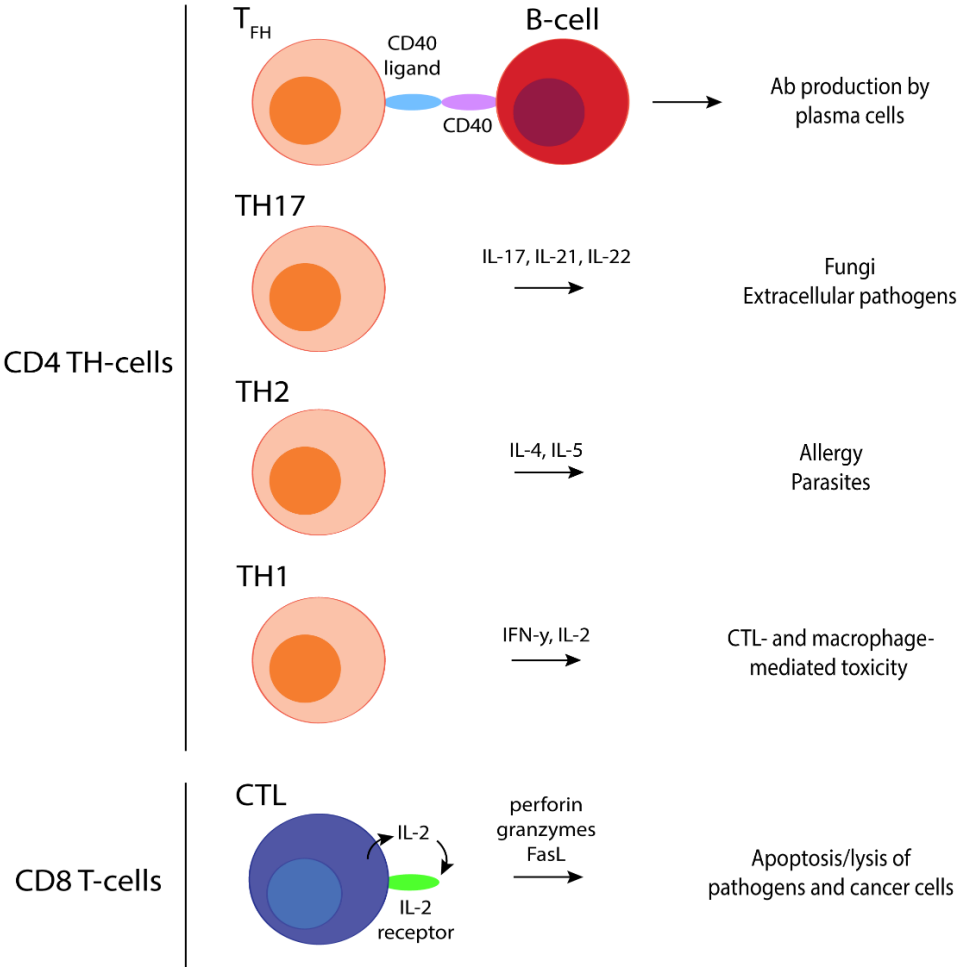

Figure 2. Cellular immunity against infection.

and kill their target cells such as intracellular microorganisms or cancer cells upon recognition of the foreign antigen presented on the cell surface via MHC class I molecules. The target cells are lyzed or killed by apoptosis induction upon binding of the TCR with the MHC-antigen complex on the target cell through secretion of cytotoxins such as perforin, granzymes and Fas ligand (FasL)[64]. **Figure 2** illustrates cellular immunity to combat infection.

2. B-LYMPHOCYTES AND HUMORAL IMMUNITY

In contrast to T-cell mediated immunity, B-lymphocytes evoke a humoral immune response via secretion of antibodies. B-lymphocytes are triggered by antigens and T-helper cells and differentiate into antibody-producing plasma cells and memory B-cells. Following uptake of an antigen by B-cells, the antigen is processed and presented via MHC class II on their surface[65-67]. Recognition and binding of the peptide-MHC-II complex to the TCR of CD4+ T_{FH} cells, stimulated by the same antigen, offers additional stimulation of B-cells via interaction of the CD40 receptor with the CD40 ligand and cytokines leading to proliferation and differentiation into plasma cells and B memory cells[68-71]. Plasma cells secrete antibodies directed to the respective antigen and practice immunity through three mechanisms. The first pathway involves neutralization prior to the invasion of the target in healthy cells. Second, antibody binding gives rise to opsonization as it facilitates uptake by phagocytes such as macrophages and leads to antibody-dependent cellular cytotoxicity (ADCC). Macrophages express the Fc-receptor on their surface which recognizes the constant and invariable region of antibodies, i.e. the Fc-region, very efficiently.

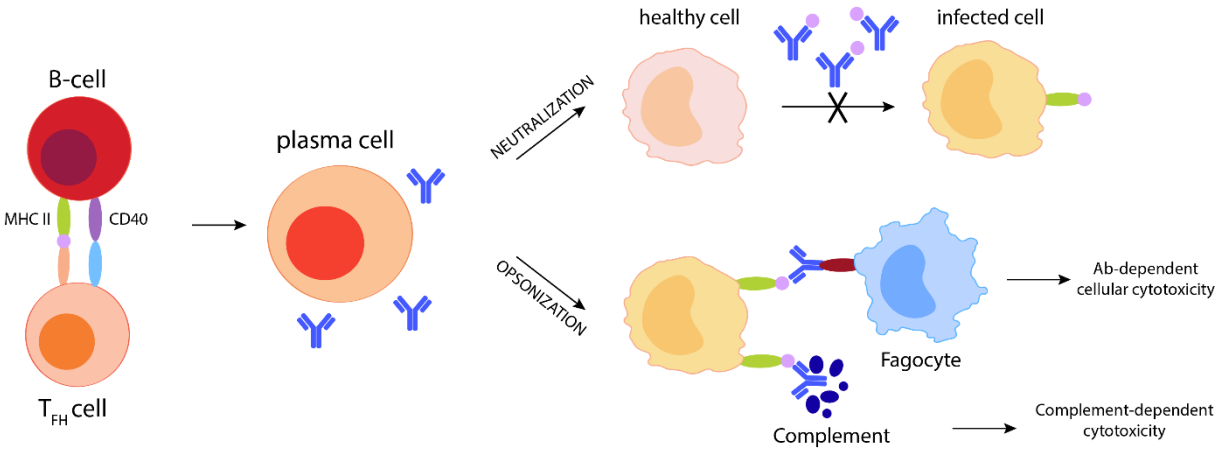

Figure 3. Humoral immunity against infection.

Thirdly, the antigen-antibody complex can also trigger removal by the complement system through Fc-region recognition followed by complement-dependent cytotoxicity (CDC)[58]. Activation of B-cells and humoral immune mediated fight against infection is illustrated in Figure 3.

CONCLUSION

To conclude, the immune system is a complex network of innate and adaptive immune cells that work together to eradicate invading pathogens through unspecific and specific pathways respectively. There is an important interplay between the innate and the adaptive immune response which allows the adaptive immune system to gain specificity and power against the infection that is initially attacked and restrained by the innate immune system. The essential immune cells linking the innate and adaptive immunity are the dendritic cells that prime the adaptive immune system specifically against every new pathogen leading to effector T-lymphocytes and antibodies that target and kill the invader directly or indirectly while establishing immunological memory that protects the host upon encounter of the same infection in the future.

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CHAPTER 2

ONCOLOGY MEETS IMMUNOLOGY

SHIFTING THE BALANCE OF IMMUNE-SUPPRESSION TOWARDS IMMUNE-ACTIVATION VIA ANTI-TUMOR IMMUNE-THERAPY

INTRODUCTION

Cancer remains one of the leading causes of death worldwide and it is stated by the WHO that, in the next 20 years, the number of new cancer patient cases will almost double. It is predicted that 40 % of men and women will be diagnosed with cancer in their life. Despite this increase in incidence, the mortality rate is decreasing which can be attributed to the extensive research that has been performed the past years resulting in new strategies to tackle malignancies more efficiently. Conventional therapies for cancer treatment involve hormone therapy, radiotherapy and chemotherapy. Hormone therapy is only applicable to malignancies that rely on hormones to grow such as breast and prostate cancer, therefore the range of application is narrow. The two most conventional treatments are radiotherapy and chemotherapy, which aim to destroy rapidly dividing cancer cells by respectively X-ray radiation – to evoke DNA-damage – or by drugs – to affect cancer cells at different stages of cell division. Both techniques however are prone to serious side effects due to lack of specificity. Moreover, these therapies do not seem to evoke a prolonged protection of the patient against relapse and metastasis. One of the most exciting developments in this regard lies in the interface of oncology and immunology and prompted the emergence of the immuno-oncology field. The rationale is based on a term named 'cancer immunoediting'[1]. This states that the immune system protects the host against tumor development (immunosurveillance) but interestingly also can promote tumor growth (tumor-immune-escape)[2-4]. Intensive research has been done to elucidate this dual role and the complex relationship between the immune system and cancer in order to find similarities in cancer pathogenesis to potentially enable targeting of the foundation of malignancies in general and induce a prolonged protective effect against cancer.

Cancer immunoediting consists out of three E's: elimination, equilibrium and escape and explains the link between cancer pathogenesis and immunology as a dynamic bidirectional cross-talk[3,5]. The elimination phase comprises the immunosurveillance stage where malignant cells are successfully eradicated by a competent immune system. Innate cells are alarmed by inflammation or by malignant cellular transformation and lead to immune cell recruitment (NK-cells, macrophages, dendritic cells) via local production of IFN-γ and chemokines (*vide supra – Chapter I: The innate*

immune system). Immature dendritic cells are activated after which they mature and migrate to the draining lymph node where CD4+ TH1 T-cell activation and CD8+ CTL proliferation occurs followed by T-cell homing to the tumor site and tumor-specific eradication of the cancer cells[3,4,6]. Unfortunately, some tumor cells are able to avoid immune-destruction by entering a dynamic equilibrium also called the tumor dormant state, the longest of the three phases, which can persist for several years in the host. This equilibrium encompasses a balance between promotion of elimination versus persistence and relies mainly on CTL-mediated immunity[7,8]. In this phase cancer immunoediting occurs, yielding potential resistance to an immune attack which arises from the enormous plasticity of the cancer cell genome as a result of the heterogeneity and multiple types of genetic instability[9-11]. The resulting resistant variants enter the escape phase via a combination of three mechanisms involving lower immunogenicity, increased survival and an immune-suppressive tumor microenvironment (TME) allowing cell expansion.

TUMOR IMMUNE-ESCAPE

The first escape mechanism leads to lower immunogenicity of the tumor evoking a decreased recognition of the cancer cells by the adaptive immune system. One of the major effectors of this escape strategy implies the modification of the antigen presentation machinery[12]. This process is altered through loss of MHC class I molecules or downregulation in more than 50 % of all tumors but can also include antigenic drift, lack of co-stimulation, TAP-defects and low-molecular-weight protein (LMP)-2 and LMP-7 deficiencies[13-15]. Another important cause of obtained lower immunogenicity and growth facilitation involves the absence or abnormal function of the IFN-γ receptor pathway. As described above, IFN-γ plays a role in increasing tumor immunogenicity via promotion of tumor cell recognition and elimination. Thus, by lowering their IFN-γ sensitivity tumors can escape these events[6,16,17].

Second, tumor cells can avoid immune-destruction via survival strategies and thus CTL-induced apoptosis resistance. This is obtained through over-expression of anti-apoptotic molecules (such as B-cell lymphoma-2 (Bcl-2), B-cell lymphoma-extra large (Bcl-x_L), Fas-associated death domain (FADD)-like interleukin (IL)-1β-converting enzyme (FLICE)-inhibitory protein (FLIP) and

survivin), signal transducer and activator of transcription (STAT)-3 activation and/or through lysis resistance acquired by expression of mutated death receptors[2,18-21].

And finally, the third tumor-escape mechanism comprises the immune-suppressive microenvironment enclosing and protecting the tumor cells by counterattacking the immune response. The tumor microenvironment ensures that cancer cells cannot be reached or eliminated by anti-tumor effector cells via different routes. Several cell populations play a vital role in tumor cell development, survival, growth and metastasis, all giving rise to multiple immune-escape or immune-suppressive actions forming a complex network that very efficiently evades or influences the immune system. One of the most critical cell types involved in immune-suppression are myeloid-derived suppressor cells (MDSCs). They accumulate preferably in peripheral lymphoid organs or in tumor tissue, depending on their main function. Recruitment of MDSCs is regulated by a variety of chemokines produced by the tumor such as CC chemokine ligand (CCL)-2, CCL5, CCL7, CXC chemokine ligand (CXCL)-1, CXCL5 and CXCL8[22]. Following chemoattraction, production of chronic inflammation factors by the malignant tissue finally drives the MDSCs into their immune-suppressive function. It is known that the tumor environment is chronically inflamed which maintains tumorigenesis[23] and evokes MDSC recruitment via production of pro-inflammatory growth factors such as vascular endothelial growth factor (VEGF), granulocyte(-macrophage) colony-stimulating factor (G(M)-CSF) and pro-inflammatory mediators such as prostaglandin E2 (PGE-2), IL-1β and IL-17[22,24].

MDSCs can influence the immune system via multiple mechanisms and suppress the cytotoxic activity of CD8+ T-cells in various ways involving expression arginase (Arg1) and indoleamine 2,3-dioxygenase (IDO) giving rise to depletion of L-arginine (L-Arg) and L-tryptophan (L-Trp) respectively[22,25-27]. Arginase is an enzyme that degrades L-Arg and leads to anergic T-cells unable to proliferate as L-Arg is essential for expression of CD3, which is necessary for signal transduction of the T-cell receptor (TCR). IDO, on the other hand, degrades the essential amino acid L-Trp for T-cell survival and expansion[28-30]. In addition to depletion of L-Arg and L-Trp, the availability of L-cysteine (L-Cys) is also lowered by MDSCs. Naive T-cells rely on APCs to acquire L-Cys as they are unable to take up or to *de novo* synthesize this amino acid. MDSCs are also unable to synthesize

L-Cys independently and consequently take up high amounts of L-Cys thereby quickly limiting the availability of L-Cys for T-cells in their proximity[22,27,31,32]. Summarized, by limiting several amino acids, T-cell activation is halted by MDSCs as metabolic changes are essential for T-cell proliferation[22,24].

Next to deprivation of nutrients, MDSCs develop several other immune-suppressive strategies to evade the anti-tumor immune attack force. On one hand, MDSCs produce NAPDH oxidase (Nox-2) and nitric oxide synthase 2 (Nos-2) yielding hyper-production of reactive oxygen species (ROS) and nitric oxide (NO)[22,33]. ROS evoke cellular damage, thereby enhancing inflammation and apoptosis of T-cells whereas NO can react with different compounds yielding toxicity. The latter prevents IL-2 signaling which negatively regulates effector and memory T-cell proliferation and also synergistically enhances the activity of Arg1. On the other hand, MDSCs also inhibit T-cell migration and induce T-cell exhaustion by downregulation of selectins and expression of the programmed death-ligand 1 (PDL1) respectively[22,31,34]. T-cell migration is impaired as selectins are required for naive T-cells to adhere to and enter the tumor draining lymph nodes or the tumor microenvironment[35,36]. T-cell exhaustion achieved by PDL1-expression on the surface of MDSCs will be discussed in detail further on in this section.

MDSCs alongside tumor cells also recruit regulatory T-cells (Treg cells) to the TME which are, just as MDSCs, crucial cells involved in immune-escape. Treg cells are a subpopulation of CD4+ T-cells that, under normal conditions, suppress the activation of self-antigen effector immune cells to limit autoimmunity and inflammation. However, in cancer tissue, natural residing Treg cells are recruited alongside induction of Treg cells arising from naive CD4+ T-cells in order to avoid cancer cell elimination by anti-tumor specific T-cells and to escape immune-destruction. Treg cells suppress the immune system via various ways one of which is secretion of immune-suppressive cytokines such as IL-10 and TGF-β to evoke inhibition of expansion and function of CD8+ effector T-cells and dendritic cell maturation[26,37]. In addition, Treg cells secrete VEGF which positively influences angiogenesis, the formation of new blood vessels, and granzymes for cytolysis of effector immune cells[37,38]. Treg can also cause local IL-2 depletion and starve effector cells leading to apoptosis[24,39-41]. Next to cytokine production and IL-2 deprivation, Treg cells can very efficiently

promote tolerogenic dendritic cells (tol-DCs) through inhibition of costimulatory molecules[37]. In healthy individuals tolerogenic dendritic cells maintain peripheral tolerance against self-antigens, however, in a tumor setting tol-DCs induce T-cell anergy, T-cell deletion and/or T-cell suppression. T-cell anergy is induced when one of the three crucial signals are not provided to the T-cells by the dendritic cells (*vide supra – Chapter 1*). Tolerogenic DCs often lack co-stimulatory molecule stimulation by CD80 or CD86 and downregulate pro-inflammatory cytokines while upregulating anti-inflammatory cytokines such as TGF-β and IL-10[26,42-44]. In addition, Treg cells directly impair the cytotoxic activity and proliferation of CD8+ T-cells via immune checkpoint molecules programmed death ligand-1 (PDL1) and cytotoxic T-lymphocyte-associated antigen (CTLA-4) through interaction with the programmed death 1 (PD-1) and CD28 receptors respectively[45-48]. Expression of CTLA-4 on the surface of the Treg cells results in downregulation of costimulatory molecules and thus T-cell anergy alongside increased induction of IDO secretion by DCs. CTLA-4 is a CD28 homolog and has a much higher affinity for CD80 and CD86 compared to the CD28-receptor present on the surface of T-cells. Unlike CD80/CD86, co-stimulation of naive T-cells upon CD28-receptor interaction with CTLA-4 does not evoke a stimulatory signal which in turn inhibits optimal T-cell activation[48-50]. Whereas CTLA-4 is confined to T-cells, PD-1 is more broadly expressed on activated T-cells, B-cells and myeloid cells. Expression of the programmed death ligand 1 (PDL1) by Treg cells induces a co-inhibitory signal that is correlated with activation-induced apoptosis and anergy[47,48,51]. Note that PDL1 can also be expressed by the tumor cells themselves as well as MDSCs, macrophages and dendritic cells.

Finally, in addition to T-lymphocyte reprogramming, MDSCs also account for reprogramming of other immune cells such as macrophages, natural killer cells and dendritic cells, further fortifying the unfavorable atmosphere for anti-tumor immune cells near the TME[22,52]. These three immune cell populations are all attracted by the tumor tissue itself through production of pro-inflammatory mediators such as chemokines, cytokines and eicosanoids evoked by chronic inflammation of the tumor site. First, macrophages are part of the initial barrier of the innate immune system against intracellular pathogens. They differentiate under influence of IFN-γ and/or LPS into M1-macrophages and promote TH1-polarization of CD4+ lymphocytes by IL-12 production. In turn, MDSCs can subvert macrophages to the M2-phenotype via production of IL-10. This phenotype is

also described as tumor-associated macrophages (TAMs) which are tumor-promoting instead of tumoricidal[53-58]. TAMs promote tumor growth and angiogenesis and suppress anti-tumor immune responses through production of activation factors such as VEGF and epidermal growth factor (EGF) or through expression of type-2 inflammatory cytokines like IL-10 and TGF-β respectively. The second population involves NK-cells which are the cytotoxic innate equivalent of CTLs in the adaptive system. They can very efficiently kill tumor cells that lack or downregulate MHC-I expression and therefore can't be recognized anymore by cytotoxic CD8+ T-cells[59,60]. In proximity to the TME and MDSCs however, the cell-mediated cytotoxicity of NK-cells is damaged as MDSCs inhibit the production of perforins by the cells which is essential for apoptosis induction via cell lysis[22,61]. Thirdly, DCs can also be reprogrammed by MDSCs, just as Treg cells. Subversion towards the type-2 tolerogenic phenotype favoring tumor outgrowth and immune tolerance by locally releasing factors as IDO, TGF-β and IL-10[22,24,43,62]. On the other hand, T-cell deletion is achieved by the tol-DCs through production of the Fas ligand which interacts with the Fas receptor present on the T-cell membrane triggering a cascade of intracellular signaling for induction of programmed cell death or apoptosis[63,64]. Additionally, suppression of T-cell mediated anti-tumor immunity is obtained by a defective antigen presentation function of the DCs. Finally, tol-DCs can also influence T-cell proliferation via expression of PDL1 like MDSCs and Treg cells[42,65,66].

Altogether, the tumor microenvironment, whether directly or indirectly, recruits immune cells via secretion of chemokines and reprograms immune cells in pro-inflammatory and pro-tumoral phenotypes via release of cytokines and co-inhibitory signals. In turn, these immune-suppressive immune cells recruit more immune cells enabling a vicious circle of a strong unfavorable atmosphere for anti-tumor immune cells. The recruitment and activation of this complex network of immune-suppressive cells and the applied strategies to lower the immunogenicity and increase apoptosis resistance by cancer cells, are summarized and illustrated in **Figure 1**.

Importantly, immune-suppressive cells are part of a strong network surrounding the tumor, called the tumor stroma, involving stromal cells, blood vessels, cancer-associated fibroblasts (CAFs) and the extracellular matrix (ECM). The tumor stroma provides three essential pro-tumoral strategies: [1] an immune-suppressive environment for anti-tumor immune cells near the TME via various

mechanisms that are described above in detail by immune-suppressive cells; [2] a physical barrier for immune cells to enter the microenvironment; and [3] the supply of the necessary high amount of nutrients[67]. The invasion of the tumor tissue with anti-tumor immune cells is physically blocked due to stiffening of the ECM caused by crosslinking of structural proteins such as collagen and elastin catalyzed by lysyl oxidase (LOX) produced by CAFs[68,69]. Due to rapid proliferation, cancer cells quickly exhaust nutrients and oxygen which limits tumor progression and triggers the release of pro-angiogenic factors such as VEGF, EGF and fibroblast growth factor (FGF) by tumor cells, immune cells, stromal cells as well as CAFs to promote angiogenesis[69-72]. However, the synthesis of new blood vessels results in immature vessels leading to irregular blood flow, vascular leakiness, chaotic architecture and thus fails to provide the required amount of oxygen to the cancer cells. The tumor microenvironment stays therefore always hypoxic which was initially thought to be a limiting factor for tumor growth. However, it has been elucidated that the TME can adapt itself to prolonged hypoxic states and the latter promotes malignant progression (invasion, metastasis) and increases resistance to conventional therapies[73-75]. In addition, cancer cells upregulate glycolysis upon nutrient shortage to support cell viability and proliferation resulting in increased conversion of glucose into lactate[73,76-79]. The latter evokes acidosis, acts as metabolic fuel for aerobic tumor regions, induces angiogenesis and suppresses immune cells. This further contributes to tumor growth and immune evasion.

In conclusion: tumor immune-escape is a very complex strategy of cancer cells to evade eradication by the immune system comprising lower immunogenicity of the tumor cells, apoptosis resistance, recruitment of immune-suppressive MDSCs and Treg cells, damaged CTL and NK-cell activity, impaired functionality of dendritic cells, shift from TH1 to TH2 immune responses, physical barrier formation preventing tumor infiltration of immune cells, angiogenesis and hypoxia. The combined immune-suppressive functions of the different cell populations along with the pro-tumoral influence of the chronically inflamed tumor tissue clearly show the complexity of the paradoxical role of the immune system in cancer progression and points out the evident need for a multistep approach in the battle against cancer.

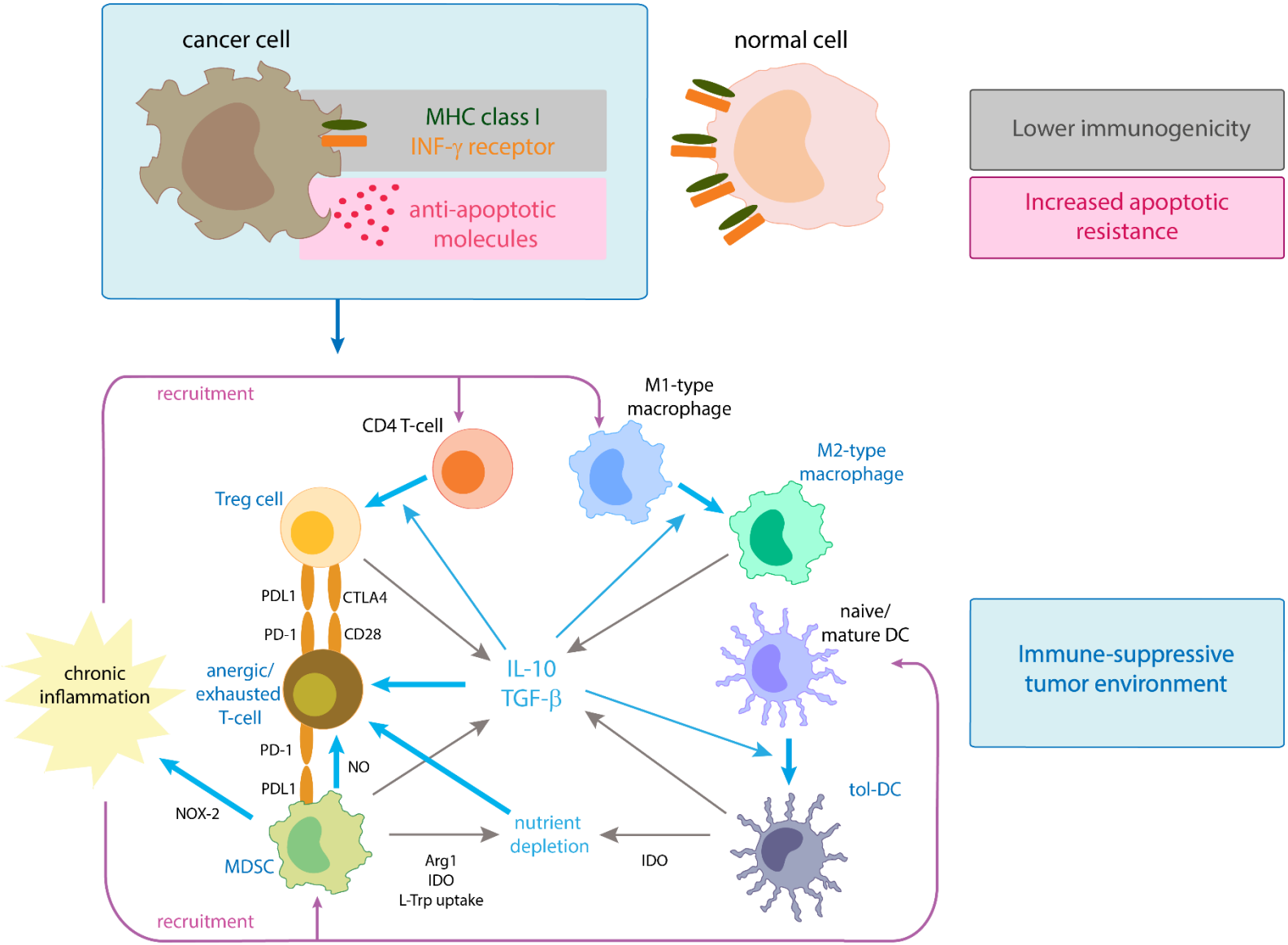

Figure 1. Tumor immune-escape mechanisms.

CANCER IMMUNE-THERAPY

Extensive research over the past two decades yielded better insight in how the immune system can positively or negatively influence malignant transformation and it is clear that subtle differences in the immune cell population can drastically change the impact of the immune system. The interplay of oncology with immunology is therefore a very important key, if not the most important, in the fight against cancer. Taking this into account, it is evident that trying to alter the immune response in order to shift the balance of a pro-tumoral environment towards an unfavorable setting for cancer cells is one of the most promising strategies to battle cancer. This approach is recognized as cancer immune-therapy and aims to manipulate the immune-suppressive immune cells and tumor microenvironment via different routes. In comparison to conventional therapies that are unspecific and evoke severe adverse events (alopecia, gastrointestinal symptoms, myelosuppression, immune-suppression), cancer immune-therapy has great potential as the side effects of immune-therapy are less severe which improves patient outcome, adherence and compliance. In general, cancer immune-therapy involves both passive or active therapies and can be specific or non-specific.

Passive immune-therapy does not rely on the immune system of the patient to attack cancer cells but employs immune cells or other components that are, prior to administration into the patient, synthesized outside the body (*ex vivo*). This aids and strengthens the immune system of the patient to fight tumor growth by providing an immediate and stronger immune cell force. Passive immune-therapy bypasses the necessity to activate endogenous immunity and can be advantageous when the immune system of the patient is strongly weakened[80,81].

In contrast to passive immune-therapy, active immune-therapy re-activates the suppressed and/or weakened immune system of the patient and shifts the balance from immune-suppression towards immune-activation by increasing the amount of anti-tumoral specific immune cells. It relies on the ability of the patient's immune system to recover from prolonged immune-suppression and induces an endogenous immune reaction against the malignancy[80,82]. Because of the patient´s own immune system is activated and induces proliferation of lymphocytes, active immune-therapy does not have an immediate positive effect opposed to passive immune-therapy but it does produce a strong memory response which is an advantage for long-term survival of the patient. Conventional therapies for cancer treatment often result

in significant reduction of the tumor or complete remission, however, the prognosis for advanced tumors is not optimistic. In addition, the majority of cancer-related deaths is not caused by the primary tumor but by metastasis and relapse. The induction of immunological memory provides the patient with prolonged protection contrary to conventional applied therapies, even after treatment, whilst decreasing the possibility of tumor relapse that leads to more resistant and more aggressive malignancies, which are often harder to treat and more prone to metastasis[83].

An overview of the different cancer immune-therapies will be discussed in detail below thereby highlighting the most promising strategies regarding personalized medicine for cancer treatment.

1. MONOCLONAL ANTIBODY THERAPY

Monoclonal antibody (mAb) therapy involves selective targeting of a specific protein that is overexpressed, mutated or selectively expressed on tumor cells involved in cancer initiation and/or progression. It is a passive immune-therapy that, upon binding of the mAb with the respective target, leads to blocking of receptor binding sites such as grow factor receptors or to elimination of the cancer cell mediated by antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) [80,84,85]. Elimination of the malignant cells can additionally evoke release of cancer antigens within the tumor proximity which can in turn result in uptake by DCs and possibly an anti-tumor directed adaptive response.

Many antibodies have received approval from the FDA for the treatment of various solid tumors and hematological malignancies[85,86]. Although mAb therapy has shown promise, resistance due to immune-escape via downregulation or loss of the expression of the target antigen is one of the main reasons responsible for low response rates.

2. ADOPTIVE CELL TRANSFER

Adoptive cell transfer (ACT) involves isolation of lymphocytes from the patient's peripheral blood, draining lymph nodes or tumor tissue followed by *ex vivo* priming into tumor-specific CD8+ CTLs, prior to administration back into the patient and is illustrated in Figure 2.

Priming of the CTLs is achieved by co-incubation with dendritic cells that are pulsed with tumor-associated antigen(s) or by cytokine (Il-2, IFN-γ) activation. After generating large numbers of analogous tumor-reactive CTLs, the T-cells are reinfused back into the patient often in combination with IL-2 administration to boost the T-cell proliferation[87,88]. In this way, the balance is shifted from T-cell anergy and tolerance to a superior amount of high avidity effector T-cells that exert cytotoxic effects against malignant cells.

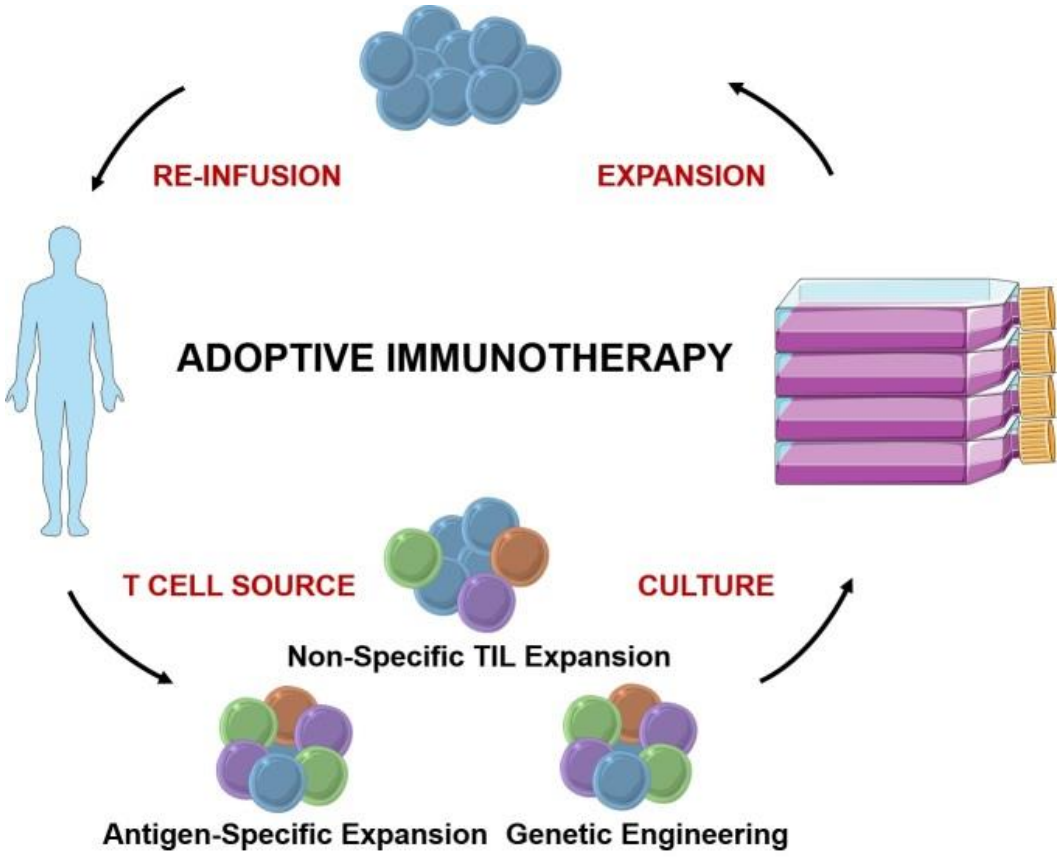

Figure 2. Adoptive autologuous T-cell transfer of either tumor-infiltrating T-cells (TIL) or peripheral blood cells requiring respectively non-specific or specific expansion via antigen-specific expansion (TCR T-cells) or genetic engineering (CAR-T-cells) - (*Figure adjusted from reference 88*).

An increased amount of effector T-cells is however not sufficient for tumor eradication as the T-cells need to be able to infiltrate the tumor tissue in order to perform their function. The dense tumor stroma serves as a physical barrier withholding tumoricidal immune cells entrance to the tumor alongside the immune-suppressive environment created by MDSCs and Treg cells, thereby limiting the efficacy of adoptive transfer of cytokine-induced T-lymphocytes. To overcome functional impairment of the T-cells adoptive transfer with tumor-infiltrating

lymphocytes (TILs) directly isolated from tumor mass and boosted with IL-2, is a promising alternative provided that administration of the non-specifically expanded TILs is preceded by lymphodepletion. Lymphodepletion encloses deletion of immune-suppressive Treg cells and MDSCs through chemotherapy alone or in combination with radiation and alleviates the immune-suppressive pressure near the tumor microenvironment leading to a more durable response[89-92]. This approach faces unfortunately several disadvantages: [1] lymphodepletion can be very dangerous and is life-threatening for immune-comprised patients or patients with an impaired immune system; [2] the expansion of a large amount of TILs is costly, time-consuming and requires highly specialized personnel; [3] administration in conjunction with IL-2 can stimulate expansion of Treg cells; and [4] isolation of reactive TILs from tumor tissue is only possible for melanoma (in 50 % of the cases) while other cancer types rarely contain sufficient tumor-reactive lymphocytes[90,93].

An exciting new approach that aims to improve and broaden TIL adoptive transfer therapy involves adoptive transfer of genetically engineered autologous normal peripheral blood cells with receptors capable of recognizing cancer-specific antigens in combination with preconditioning of the patient with lymphodepletion. There is no need for surgical resection of tumor tissue and other cancer types besides melanoma are also feasible for T-cell engineered recognition of tumor antigens, in contrast to ACT with TILs, providing tumor-associated antigens are identifiable. Genetically modified T-cells involve expression of naturally occurring T-cell receptors (TCRs) or chimeric antigen receptors (CARs). TCRs, on one hand, recognize antigens presented via MHC molecules whereas CARs can identify antigens independent from MHC-presentation. Modification of the T-cells is obtained via transduction with viral vectors containing TCR genes that specifically recognize tumor-associated antigens. TCR T-cell recognition of antigens is however restricted to MHC-presentation and downregulation of MHC by the tumor to evade cytolysis is a major obstacle[88,90,94]. CAR T-cells, on the other hand, are T-cells transfected with a viral construct encoding an extracellular single-chain antibody variable fragment against tumor surface antigens fused to the T-cell signaling domains. The antibody targets the respective target antigens in a MHC-independent manner and thereby avoids immune-escape by downregulation of MHC expression. Clinical studies showed that part of the adoptively transferred CAR T-cells develop into memory T-cells who can survive for years and offer prolonged and possibly lifelong lasting protection against relapse[95-98].

A great challenge for both TCR and CAR engineered T-cells is the selection of appropriate antigens to refine the affinity and specificity in order to avoid toxicity that results in immune-mediated destruction of normal tissues and treatment failure due to mutation of the target antigen or absence of its expression. In addition, CAR T-cells also face another disadvantage called the cytokine release syndrome (CRS). Toxic quantities of cytokines can be produced by large numbers of activated CAR T-cells and evoke fever, hypotension and neurologic symptoms which can sometimes be life-threatening[87,96,97,99]. Therefore, the off-target side effects due to immune-destruction of healthy cells, the lymphodepletion related adverse effects and limitations alongside the high cost and time consuming synthesis indicate that additional research is required to optimize the adoptive transfer therapy as a broadly applicable anti-cancer immune-therapy.

3. CYTOKINE THERAPY

Cytokines are the messengers of the immune system and regulate both the innate and the adaptive immune cells mediated by receptors. They play a crucial part in homeostasis and function of lymphocytes. Since cytokines function in cascade, the administration of a single cytokine is unlikely to be sufficient and is the reason for the modest therapeutic effect in the clinic[80]. In addition, systemic administration of an effective dosage of cytokines is often associated with severe toxicities. In this regard, fusion with targeting antibodies appears to be an attractive option that is currently explored to overcome these limitations. Moreover, combining several cytokines or combination with other immunotherapies may potentially lead to a more optimal response that better covers the cytokine cascade while reducing adverse events as lower concentrations can be used opposed to monotherapy.

Three cytokines have been already approved by the FDA involving IL-2 for lymphoma, leukemia, metastatic renal cell carcinoma and melanoma, IFN-α which is additionally used in various types of leukemia and Kaposi's sarcoma and G(M)-CSF to stimulate hematopoiesis following chemotherapy[84,100]. IL-2 is a T-cell growth factor that is involved in the regulation of tolerance and proliferation of Treg cells and effector lymphocytes respectively. In detail, IL-2 influences the ratio of effector T-cells to Treg cells depending on its concentration. At low levels (during homeostasis or steady-state), Treg cells are superior as they express high affinity receptors whereas in higher concentrations the lower affinity receptors present on effector T-cells and

other immune cells are also activated. Due to this contradictory function, IL-2 therapy suffers from the disadvantage that Treg cells are simultaneously stimulated which dampens the anti-tumor immunity. In addition, severe dose-dependent adverse events are reported involving vascular leak syndrome (VLS) – related to septic shock – and activation-induced cell death (AICD) of T-lymphocytes[101-103].

IFN-α acts directly on tumor cells via: [1] growth inhibition through down-regulation of oncogene expression and induction of tumor-suppressor genes; and [2] immune recognition promotion through induction of MHC class I molecules expression. It can promote differentiation of monocytes to highly active dendritic cells and proliferation of NK-cells and T-cells. Systemic infusion of IFN-α however evokes severe hypotension[103-105]. Although IL-2 and IFN-α both are potent cytokines and have shown significant positive results in cancer patients, additional research is needed in order to obtain formulations that are less toxic and more specific to ameliorate the efficacy of other anti-tumor immunotherapies.

At last, G-CSF and GM-CSF are involved in differentiation of hematopoietic stem cells and are used to help patients to recover from leucopenia or, in combination with adoptive immune cell transfer, to aid stimulation of a strong immune response[106,107].

4. ONCOLYTIC VIROTHERAPY

Oncolytic viruses are self-replicating and can be naturally occurring or genetically modified to provide tumor selectivity. Naturally occurring viruses enter both normal and cancer cells but, due to the immediate recognition and rapid clearance, the virus is eliminated in normal cells whereas in cancer cells the virus is not cleared[108]. Therefore, many viruses preferentially infect cancer cells as they have a selective advantage for viral replication because they suppress normal immune recognition/destruction and resist apoptosis. On the other hand, oncolytic viruses can be genetically modified to exert more tumor selectivity through e.g. targeting a specific protein that is overexpressed on the cancer cell surface. Once the oncolytic virus has infected the cancer cell, it destroys the cancer cell through oncolytic lysis, thereby enhancing the immunogenicity of the tumor microenvironment due to production of endogenous danger signals (DAMPs) and release of tumor-derived cytokines (Type I IFNs), viral PAMPs and cancer antigens within the vicinity of the tumor[109,110]. Due to this change in the tumor

microenvironment, systemic immunity is activated specifically against the malignancy of the patient. In addition, the induced cancer cell lysis also gives rise to release of the virus, enabling spreading of the latter in neighboring cancer cells. Figure 3 provides an overview of the anti-tumor mechanisms induced by oncolytic virotherapy.

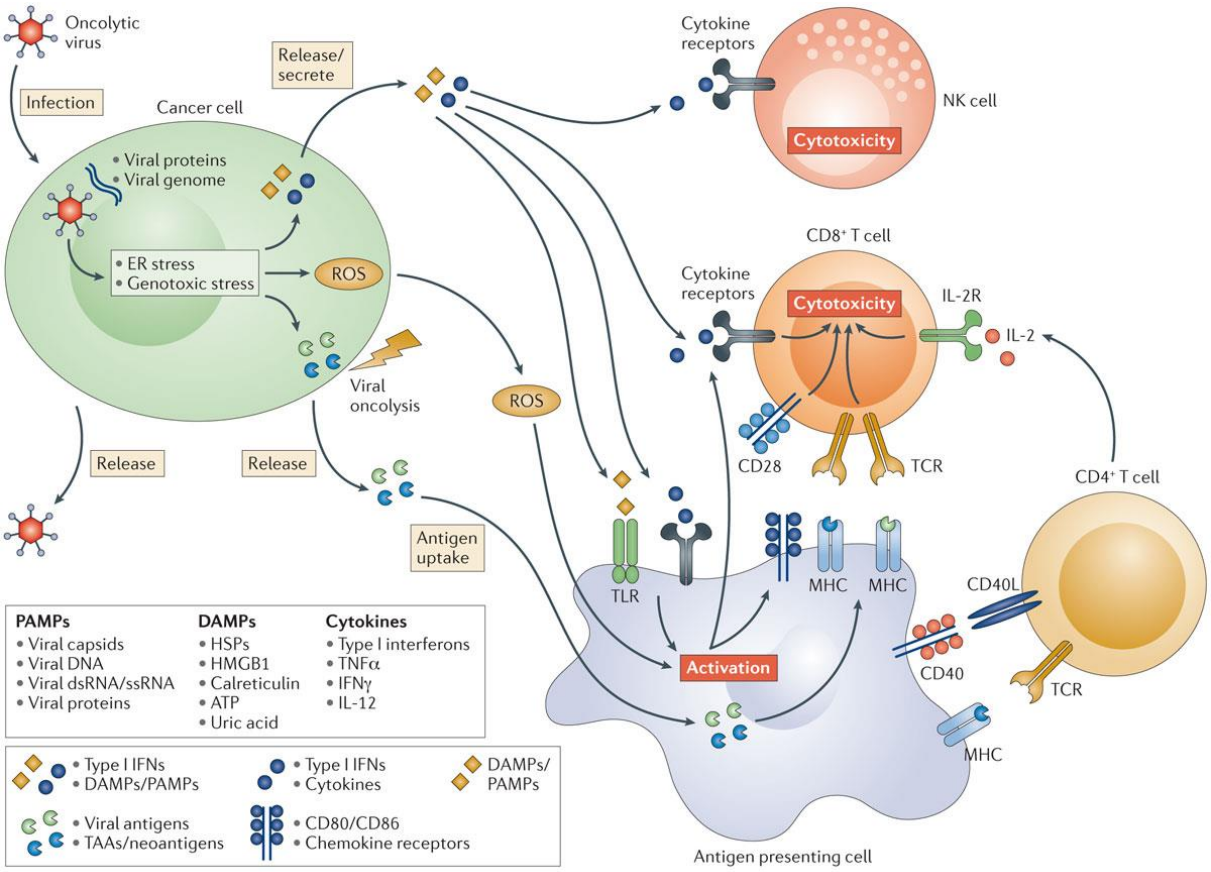

Figure 3. Oncolytic virotherapy exerts its function through a combination of direct cancer cell lysis (viral oncolysis) and indirect activation of anti-tumor immune responses – (*Figure adjusted from reference 110*).

In most cases oncolytic virotherapy is injected intra-tumoral to avoid low efficacy due to the presence of the physical, dense ECM barrier[109,110]. This however limits its applicability to malignancies that are physically accessible through palpation or imaging and is consequently unlikely to infect and eliminate distant metastases. If oncolytic viruses could be delivered intravenously rather than directly into the tumor, the range of targetable cancer types would dramatically increase and oncolytic virotherapy would also potentially be an option to treat metastasis. Another challenge using oncolytic viruses is the risk of being cleared by the patient's immune system through neutralizing viral antibodies or cytotoxic CD8+ T-cells[110]. In this regard,

it is important to consider the pre-existence of antibodies or memory T-cells prior to therapy decisions. Strategies circumventing this initial response of the immune system involve PEGylation and polymer coating which prevents antibody binding and neutralization or modification of the viral genome to express products that inhibit antigen presentation and thus avoids recognition by the patient's immune system[111]. Additionally, careful patient selection is necessary to avoid immunocompromised patients due to the risk of infection and biosafety issues need to be taken into account during production, handling and administration. Another risk of oncolytic virotherapy is that the virus can mutate to regain its pathogenic potential[110].

Recently oncolytic virotherapy has gained a lot of interest due Talimogene laherparepvec (T-VEC) or Imlygic® for treatment of unresectable and recurrent melanoma, the first FDA approved virotherapy. T-VEC consists out of a herpes simplex virus type I that is genetically modified to attenuate the virus, to increase tumor selectivity and to secrete granulocyte macrophage-colony stimulating factor (GM-CSF). Through deletion of two non-essential viral genes, i.e. a neuro-virulence gene and an inhibiting gene for antigen presentation, the pathogenesis of the HSV is reduced and the tumor selectivity is increased respectively. Secretion of GM-CSF additionally recruits and activates antigen presenting cells to evoke a more potent anti-tumor response[110,113]. Phase III clinical trials indicate that T-VEC virotherapy improves durable response rates, defined as partial or complete response lasting continuously for a minimum of six months, in patients with advanced melanoma. However, no significant difference in overall survival was reported and no effect was seen for the metastatic lesions of the melanoma spread in internal organs. Common side effects that have been reported involve flu-like symptoms (fever, fatigue, chills), pain at the injection site and/or herpetic infections[114]. Overall clinical tolerability of oncolytic viruses and safety is very good, even at the highest feasible doses and clinical results are encouraging and promising for the future. The recent FDA approval of the first oncolytic virotherapy will further boost research aiming to increase the clinical efficacy, feasibility and applicability[115,116].

5. IMMUNE CHECKPOINT INHIBITORS

One of the most important tumor escape mechanisms involves immune-suppression by cancer cells and immune cells such as MDSCs, tolerogenic DCs, TAMs and regulatory T-cells through immune checkpoint activation of tumor-infiltrated T-cells resulting in loss of CTL function (*vide*

infra – Tumor immune escape). Inhibition of these pathways has been extensively studied, in particular the PD-1 and the CTLA-4 pathway, which has led to the development the most promising immune-therapy strategy so far comprising immune checkpoint inhibitors. Recent FDA-approval in 2011 of the CTLA-4 inhibitor Ipilimumab (Yervoy®)[117,118] and two PD1-inhibitors Nivolumab (Opdivo®)[119] and Pembrolizumab (Keytruda®)[120,121] in 2014 for the treatment of renal cell carcinoma and for the treatment of melanoma and non-small cell lung cancer respectively has dramatically boosted the field of immuno-oncology. Opposed to the rapid response obtained with chemotherapy and other more traditional therapeutic strategies within a few weeks after initiation, the responses to immune-checkpoint blockers is a lot slower and can take up to six months. In some cases this is even preceded by size increase of metastatic lesions before regression occurs.

6. INTRATUMORAL INJECTION OF PRR-AGONISTS

Intratumoral injection of pathogen recognition receptor (PRR)-agonists aims to re-activate tolerogenic DCs through induction of maturation. Tolerogenic DCs are unable to boost the immune system against the malignancy and attribute to tumor immune escape (*vide supra – Tumor immune escape*). Mature DCs, on the other hand, can prime T-cells via co-stimulation and cytokine signaling to proliferate and expand into effector or helper T-cells depending on the cytokine spectrum rather than induction of T-cell anergy. DCs highly express pathogen-recognition receptors on their cellular membranes and cytoplasm. Stimulation of the latter leads to maturation of DCs and by consequence efficient priming of an anti-tumor immune response. In this regard, triggering of PRRs is explored to redirect the immune system against the malignancy. Toll-like receptor (TLR) agonists in particular have shown promise as potent activators of tolerogenic DCs following intratumoral injection leading to tumor regression[122-124].

7. CANCER VACCINATION

Cancer vaccination fights cancer by re-activating the suppressed and/or weakened immune system. It aims at the generation of a tumor-specific immune response by the host's immune system and evokes relatively mild side effects (local erythema, flu-like symptoms) compared to the harsh side effects seen with chemotherapy[125]. Different from preventive vaccination, that aims to provide protection against a possible future infection or disease, cancer vaccination

induces a therapeutic effect in patients that have already developed a malignancy and aims at boosting the malfunctioning immune system of the patient to recognize and kill off the cancer cells specifically[126,127]. Immunization for prevention of diseases has dramatically changed the burden of infectious diseases worldwide as many of them are dramatically reduced or eliminated. Therapeutic cancer vaccination has however not yet met the high expectations which can be attributed to the immune-suppressive microenvironment that was not well understood up to a few years ago. The recent gain in knowledge about how the TME influences the immune balance in the patient and how tumor-escape occurs, has revolutionized the cancer immune-therapy field and will aid in the design of more potent vaccines with increased efficacy in the near future.

Cancer vaccination involves dendritic cells, the most potent class of antigen presenting cells in recognition, uptake, processing and presentation of foreign material and are a critical factor in the interplay between the innate and the adaptive immune response. Dendritic cells can very efficiently process exogenous and endogenous antigens followed by presentation onto MHC-I or MHC-II respectively. Recognition of the MHC-I or MHC-II epitope complex by CD8+ T-cells or CD4+ T-cells respectively subsequently evokes activation and expansion of the lymphocytes[128-131]. In order to obtain tumor eradication, cancer vaccines need to very efficiently induce a strong CD8+ cytotoxic T-cell response as CTLs can specifically recognize the target cells for which they are primed and eliminate them via cell lysis or apoptosis induction. In addition, CD4+ TH1 helper T-cells are required for optimal priming of the CTLs and for expansion of memory cells. Thus, cancer vaccination aims to target and activate dendritic cells to induce CD8+ cytotoxic and CD4+ TH1-cells that specifically eliminate the malignant cells and induce expansion of memory T-cells[127,132-134].

An important factor in vaccine design is the choice of an immunogenic antigen. The idea of ‘one size fits all’ is a false premise due to inter-tumoral heterogeneity, diversity between tumor in different patients, or intra-tumoral heterogeneity which involves the complexity of individual tumors[11,135,136]. Further, immune-escape through mutation as well as downregulation or lack of expression of the vaccine antigen also need to be taken into account. Two main approaches for antigen selection are currently implemented either comprising vaccines containing defined synthetic antigens or patient-derived cancer tissue.

7.1.1 Defined synthetic antigen vaccines

Antigen vaccines involve tumor-associated non-mutated antigens (TAAs) that are over-expressed by cancer cells but can also be present on normal cells. The requirements that the antigens need to meet in order to be attractive targets are: [1] tumor-specificity: no or highly restricted expression in normal tissue; [2] immunogenicity: constitutive expression during oncogenesis; and [3] oncogenicity: expression is essential for cell survival[137]. If those conditions are not met, the T-cells that recognize the respective antigen will have poor reactivity which will not lead to tumor regression. Vaccines containing TAAs are not universal because they are unlikely to be relevant for every single patient, they can be ineffective due to immune-escape (mutation, downregulation) and they can also potentially encounter lower efficiency due to inhibition of the immune response caused by thymic tolerance. This can partly be circumvented by using multiple cancer antigens which will give rise to a broader immune response directed to a variety of antigens and loss of activity due to mutation or the lack of expression of one specific antigen is a less detrimental factor. However, relevance for every single patient, thymic tolerance and the fact that specific antigens still need to be identified for numerous cancer types are still issues encountered by TAA-vaccines[138-140].

In contrast, antigen vaccines that include tumor-specific mutated antigens (TSAs), also known as neo-antigens, do not face these challenges. Neo-antigen vaccines involve formulation of proteins that are absent from the normal human genome, recognized as 'foreign antigens', and are created by tumor-specific mutations yielding tumor antigens, different from shared non-specific TAAs[141,142]. Identification of neo-antigens is performed by parallel genomic analysis of patient-derived tumor tissue compared to normal tissue of the patient followed by filtering for gene expression and prediction of proteasomal processing and human leucocyte antigen (HLA) class I binding affinity. The peptides with the highest HLA-affinity are selected and subsequently synthesized to allow analysis of TIL-reactivity against the epitopes via *in vitro* T-cell assays [132,143,144]. Unfortunately this analysis procedure, illustrated in **Figure 4**, can be costly, labor intensive and complex. In addition, neo-antigen identification requires a solid tumor that can be surgically resected.

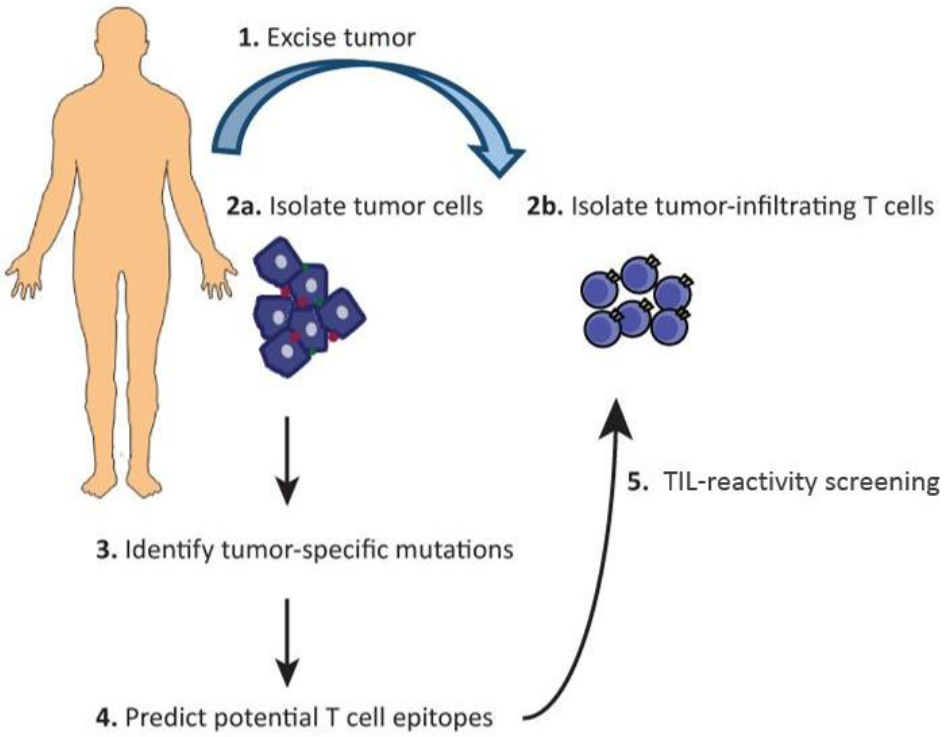

Figure 4. Overview of the genome screening procedure of patient-derived tumor tissue to identify immunogenic neo-antigens – (Figure a*djusted from reference 144*).

Interestingly, the immunogenicity of neo-antigens is not hampered due to thymic tolerance, contrary to non-mutated self-proteins, because they are patient- and tumor-specific and potentially evoke a more powerful T-cell response with affinity that is only restricted to tumor cells of the patient[137,142,145]. Therefore, this is a very attractive approach in the search for more personalized immunotherapies and is currently widely explored for its potency as cancer vaccine. The ongoing clinical studies suggest neo-antigen vaccination is feasible and holds promise as a personalized cancer immune-therapy.

In general, antigen vaccines can involve vaccination with synthetic peptides, recombinant proteins or RNA/DNA-encoding proteins derived from TAAs or neo-antigens. Peptide-based cancer vaccines rely on the ability of T-lymphocytes to recognize antigen-derived epitope peptides. The short amino acid sequences of single MHC-bound peptides highly expressed on cancer cells or DCs loaded with cancer cell lysate are identified using proteomic technologies and subsequently synthesized for vaccination[137]. Although short peptides have shown to elicit anti-tumor immune responses, they rarely had effect on the tumor growth. This can likely be attributed to induction of T-cell tolerance or anergy due to the absence of co-stimulation as

short peptides can directly bind to MHC-molecules on every MHC-expressing cell without the need of be processed. As consequence no DC-targeting and no memory induction occurs[141].

Long peptide vaccines, on the other hand, consist out of one epitope with flanking amino acids or out of several epitopes. Both require processing prior to MHC-presentation and are more likely to be recognized by both CD8+ and CD4+ T-cells, of which the latter are required for memory T-cell induction. Normally only endogenous proteins can be presented onto MHC-I molecules, however longer peptide vaccines can also be presented via MHC-I after processing through a process called cross-presentation. This makes long-peptide vaccines superior for anti-tumor immune-therapy opposed to short-peptide vaccines as both CTLs as CD4+ TH1-cells are induced[137,146]. Despite this, complete recombinant protein vaccination is often favored as peptide vaccines may not contain all important epitopes and experience higher elimination rates[99].

Another approach implements RNA or DNA-encoding proteins which allows easy delivery of multiple antigens and is not restricted to the patient's HLA-type unlike peptide- and protein based proteins. The main advantage of nucleic acid vaccines is the possibility of large-scale production and storage as they can be amplified yielding unlimited supply of antigen. DNA-based vaccines require the DNA plasmids to cross both the cellular and the nuclear membrane of the dendritic cells and subsequent transcription and translation into the respective cancer antigen followed by presentation of the processed peptides onto MHC[147]. In contrast, RNA-based vaccines only require cell membrane crossing to enter the cytosol for translation and has no oncogenic potential as it cannot integrate into the host genome. This way, RNA-based vaccines are considered to be superior to DNA-based vaccines. However, RNA degradation due to extracellular exonucleases remains a significant stability concern for RNA-based vaccines[148,149].

Summarized, antigen vaccines are gaining more and more potency due to more personalized approaches that are emerging, involving the formulation of multiple TAAs or identification of neo-antigens. In particular, the neo-antigen screening to develop vaccines specifically tailored for every individual patient has shown great promise and strengthens the importance of the concept of personalized cancer immune-therapy.

7.1.2 Patient-derived cancer tissue vaccines

Another attractive approach to obtain a personalized vaccine comprises the incorporation of patient-derived antigens isolated from the patient´s own tumor tissue. This method includes all potentially relevant antigens, both TAAs and TSAs and contains the entire spectrum of antigenic targets[149,150] unlike other techniques (*vide supra – Defined synthetic antigen vaccines*). In addition, vaccines derived from patient-derived cancer tissue circumvent MHC-restriction[141] and the need for epitope identification of which the latter is a major advantage in comparison to neo-antigen vaccines in terms of cost, labor burden and complexity.

Autologous cancer tissue vaccines contain cancer cell lysate or whole tumor cells including cell membrane and possibly stroma[151]. The latter can potentially be interesting to evoke a broad, all-embracing cytotoxic T-cell response that attacks not only the tumor cells but also the immune-suppressive surrounding stroma. The induction of a potent anti-tumor immune response following cancer vaccination involving autologous tumor cell material has already been reported[152,153]. One should consider that autologous cancer tissue vaccines require sufficient amount of tumor and thus are only applicable for solid tumors that can be surgically resected which is also true for neo-antigen vaccines. To overcome this, allogenic cancer tissue vaccines based on two or three human tumor cell lines are a potential alternative that enables large-scale production and standardization of quality and composition[141,149,154]. This can however evoke an anti-MHC immune response which can interfere with the anti-tumor response[155].

7.1.3 *Ex vivo* vs *in vivo* targeting

Dendritic cell cancer vaccination can be achieved by two strategies: [1] adoptive transfer of autologous dendritic cells stimulated with cancer antigens and adjuvants *ex vivo*; and [2] *in vivo* targeting of dendritic cells. *Ex vivo* dendritic cell-based vaccines involve electroporation of autologous DCs derived from the cancer patient's own blood monocytes *ex vivo* with cancer antigens and an adjuvant prior to reinfusion in the patient[156]. The culturing of autologous DCs with antigens will provide immunity against the respective antigens and the immune-stimulating adjuvant is crucial to obtain mature DCs that are able to induce activation and expansion of T-lymphocytes. In 2010, the FDA approved the first DC-based vaccine Sipuleucel-T (Provenge®) for men with metastatic castration-resistant prostate cancer.

Sipuleucel-T involves autologous APCs and blood monocytes exposed *ex vivo* to PA2402, a recombinant fusion protein composed of prostatic acid phosphatase (PAP) and GM-CSF[157-159]. Despite the proven survival benefit, many other clinical studies of *ex vivo* DC-based vaccines have failed to demonstrate clinical benefits. In addition, *ex vivo* DC vaccination is a highly costly, labor-intensive procedure and does not take advantage of the physiological stimuli that occur in direct *in vivo* DC targeting[149,156].

Optimal targeting and activation of DCs *in vivo* to induce a strong anti-tumor immune response however requires the design of a vaccine to resemble pathogens. This involves encapsulation of cancer antigens into particulate carriers co-formulated with pathogen-recognition receptor agonists to mimic a pathogenic infection[160-164]. First, it is known that particulate-based antigens have a dramatic advantage over soluble antigens in terms of recognition and uptake efficiency by dendritic cells[160]. Particle-based vaccines, in the size range of 50 nm to 10 µm, resemble viruses and bacteria and are therefore immediately recognized by dendritic cells and processed. Peripheral migratory DCs recognize and take up particles at the injection site followed by transportation via the lymphatic system to the draining lymph nodes and antigen presentation to lymphocytes. In addition, only small particles (sub 200 nm range) can target lymph-node resident DCs through drainage to the lymph nodes via passive diffusion into the lymphatics from the injection site. Therefore, nanoparticles are preferred over microparticles due to their higher tissue mobility and their ability to target different DC-subsets[160,162,166,167].

Second, a process called cross-presentation is favored in dendritic cells when particulate material is taken up comprising presentation of the processed peptides onto MHC-I instead MHC-II, which is normally the case for exogenously derived proteins. In this way, dendritic cell can prime a cytotoxic T-cell response against exogenously acquired proteins, which is essential to elicit an anti-tumor effect[165,167,168].

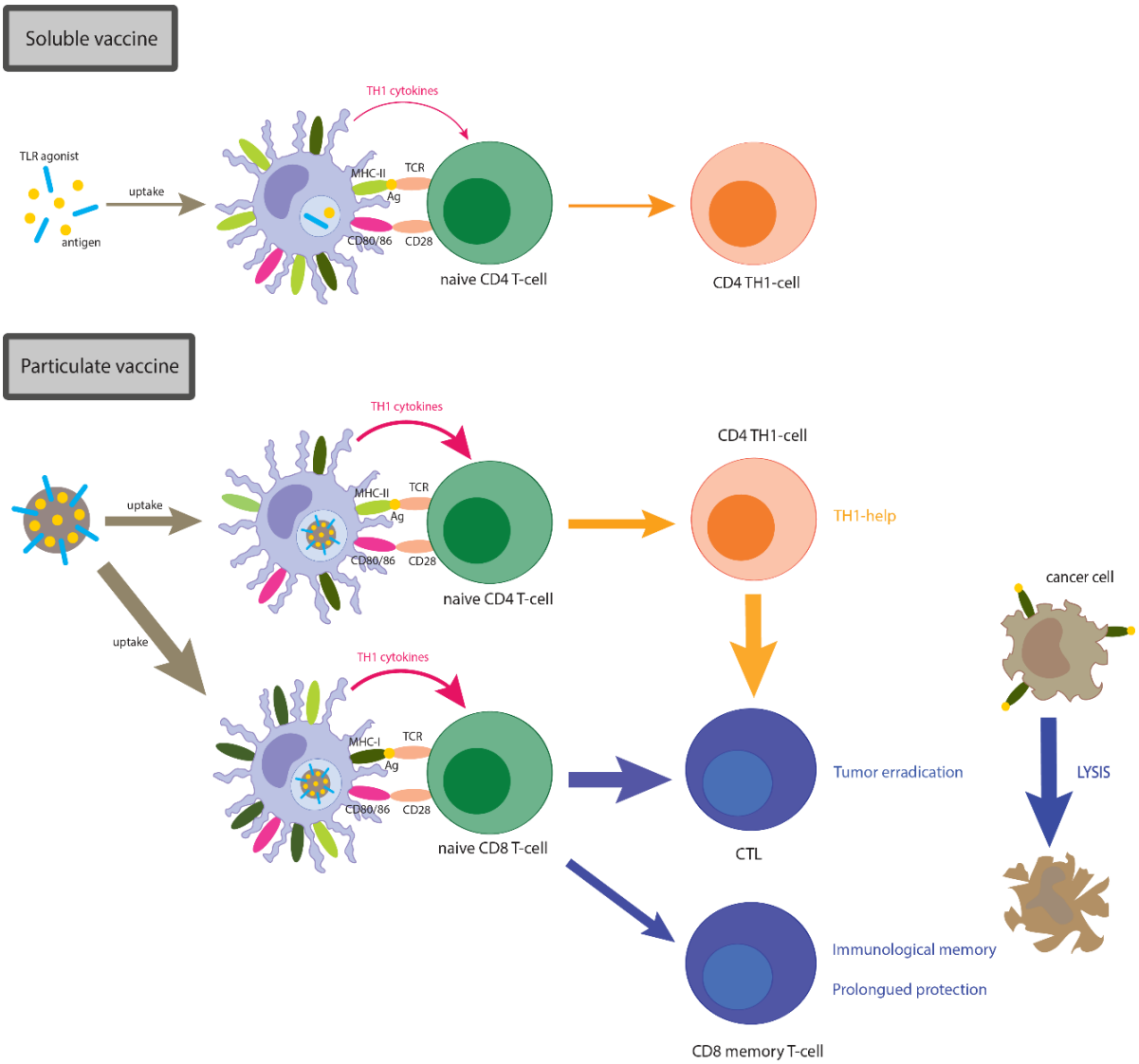

Figure 5. Optimal targeting of dendritic cells for inducing a strong CTL-induced anti-tumor response requires the formulation of immunogenic antigens in pathogen-like vaccine particles.

Third, whereas formulation of cancer antigens into particulate carriers yields in efficient targeting of dendritic cells and favors cross-presentation, it is not sufficient for optimal activation of dendritic cells. Triggering of DC maturation is essential for optimal priming of the T-cells via co-formulation of the cancer vaccine with agonists that trigger pathogen recognition receptors that skew TH1 and CTL immune responses[163,169,170]. Upon triggering of PRRs, the dendritic cell undergoes maturation and is able to deliver the three signals necessary for optimal priming of T-cells. In this regard, it is essential to co-formulate cancer antigens vaccine particles with pathogen-recognition receptor agonists to mimic a pathogenic infection[42,171-174]. **Figure 5** illustrates the anti-tumor immune response mechanism that is generated by dendritic

cells upon recognition of a particulate vaccine containing both cancer antigens and TH1-inducing adjuvants.

VACCINE-RELATED IMMUNOGENICITY: COUNTERING T-CELL ANERGY

Optimal priming of T-cells is essential to induce a strong anti-tumor immune response. Lack of vaccine immunogenicity is however a major limitation of DC-based vaccines due to suboptimal adjuvants. The induction of peripheral tolerance due to T-cell anergy (unresponsive T-cells) is one of the main reasons why therapeutic vaccines have failed to elicit a strong anti-tumor immune response with clinical benefit for the patient. Dendritic cells require certain activation stimuli to transform from naive DCs into mature DCs in order to prime T-cells rather than tolerogenic DCs that induce T-cell anergy.

Currently, adjuvants licensed for use in human vaccines only involve a few possibilities including aluminum salts, oil-in-water emulsions (MF59, AS03, AF03), virosomes and monophosphoryl lipid A (MPLA). Alum is the most widely explored adjuvant used in numerous vaccines, however it can only induce a strong TH2-type immune response which is unfavorable for cancer vaccination as it does not promote CTL-activation[175]. Oil-in-water emulsions are licensed for flu vaccines and induce both TH1- and TH2-type immunity and are thus also less suited for cancer vaccine purposes[176-179]. In contrast, virosomes – spherical lipid vesicles that contain functional viral influenza glycoproteins[180,181] – and MPLA – derived from the natural adjuvant lipopolysaccharide (LPS)[182,183] – both induce strong TH1-type immune responses. They differ from the other licensed adjuvantia in their mechanism of action. Both virosomes and MPLA are adjuvants derived from pathogens, in other words PAMPs, and trigger PPRs on dendritic cells very efficiently. PAMP-related adjuvants are therefore interesting to increase the immunogenicity of cancer vaccines.

1. PAMP-BASED ADJUVANTS

One of the most effective vaccines is a live attenuated vaccine, the yellow fever vaccine, that induces DC-maturation via signaling through TLR2, TLR7, TLR8 and TLR9 and appears to evoke lifelong immunity after only one vaccination. Despite the high efficacy of the yellow fever vaccine, the vaccine is contraindicated in immune-suppressed patients, infants (younger than

6 months), elderly and pregnant women due to infection risk[184-186]. For adjuvants derived from pathogens in general, in addition to infection risk, these natural products experience variability and sometimes difficulties to be extracted in sufficient quantities. Due to the discovery of TLRs as key sensors in pathogen recognition, TLR-agonists hold promise as potent adjuvants for cancer vaccines as the PAMP motifs are immediately recognized by the immune system and elicit a strong and broad immune response. In this regard, the design of synthetic potent TLR-agonist analogues that exhibit maximal immunogenicity without compromising on tolerability and safety is extensively studied and has shown promise. Surprisingly, despite the wide use of TLR-agonists as adjuvants, the complex mechanisms of action are not well characterized and therefore there is still room for improvement regarding optimizing the immunogenicity of therapeutic cancer vaccines.

In order to determine the optimal conditions for immune stimulation, some fundamental questions still need to be answered about the immune system. Current cancer vaccine research mainly aims at co-formulation of synthetic TLR-agonists with cancer antigens without detailed knowledge about how the adjuvant structure and combined signals interact with their receptors and how this results in different immune responses. Therefore, there is a need for a multidisciplinary approach in order to define the parameters that need to be met by a cancer vaccine and to maximize the immunogenicity without compromising on tolerability, which will require close collaboration of chemists, biologists, immunologists and pharmacists.

Four factors can potentially fine-tune the immunogenicity of cancer vaccines involving more detailed knowledge about: [1] structure requirements; [2] concentration range; [3] spacing; and [4] synergism of adjuvants. Better understanding of the chemical structure holds promise for designing more immunogenic and more effective vaccines and requires specific knowledge about how and which chemical structures interact with immune cell receptors and are recognized as non-self[187-190]. Aside from chemical structure of adjuvants, the concentration can be another important factor in optimizing vaccine immunogenicity. Hyper- or hypo-activation of immune cell receptors results in incomplete activation of dendritic cells causing T-cell anergy[188,191]. It is therefore of high interest to pay attention to the amount of adjuvant that is co-delivered with the cancer antigens upon vaccination. Third, influencing immunogenicity involves spacing of the TLR-agonist, i.e. the distance between the TLR-agonist molecules. It is

hypothesized that understanding of the spacing of PRRs on antigen presenting cells can potentially increase activation of DCs via simultaneous association of the agonist molecules with the receptors[192-194]. Lastly, this hypothesis also holds true for the combination of different adjuvants because inter-agonist proximity, taking into account the spacing of the different PRRs, will evoke confined and optimal interaction of the different adjuvants with their respective receptors. The reasoning for combining two or more TLR-agonists is based on the fact that one TLR-agonist stimulates only one receptor leading to a partial response whereas two or more simultaneously interact with multiple receptors which amplifies the immune response and is also called synergism[195-199]. Exploring different spacing lengths and combinations of agonists will further ameliorate the design of immunogenic vaccines. Altogether, fine-tuning of the immunogenicity of vaccines can significantly enhance DC activation efficiency leading to a stronger and broader immune response lowering the risk of induction of T-cell anergy.

Importantly, optimized adjuvant structure properties, concentration, spacing and synergism combinations will not only lead to optimal activation of the immune system but will also result in reduction of the side effects related to adjuvants. Indeed, optimal efficacy of adjuvants could be obtained if the inflammation induced immune response is more tightly controlled. However, this will not be sufficient to avoid side effects as systemic dissemination of adjuvants is often the cause of the severe adverse events. In this regard, it is of high interest to fine-tune the physicochemical properties of synthetic TLR-agonists in order to localize the inflammation at the site of injection and limit systemic exposure and generalized inflammation.

A new and promising generation of TLR-agonists involves small molecule immune potentiators (SMIPs). Low molecular weight molecules allow easy and inexpensive synthesis which can be optimized to increase specificity and potency while avoiding toxic effects. In addition, they can be relatively easily modified and combined for efficient formulation in vaccine particulates and delivery[200-204]. The latter is an appealing feature of SMIPs as this can reduce systemic exposure and by consequence systemic adverse events. This requires research collaborations combining formulation chemistry with immunology and pharmacology and is currently investigated by several groups showing that the potency *in vivo* of several TLR-agonists can be enhanced while reducing the systemic side effects through adaption of the physicochemical properties of the

adjuvantia. Several studies have shown lipid-, polymer-, polysaccharide- and nanoparticle-conjugation of small molecule ligands strongly reduce systemic inflammation and yield potent lymph node localized responses that enhance the adaptive immune response against co-delivered antigens[205-207].

2. DAMP-BASED ADJUVANTS

Aside from PAMPs, DC maturation can also be achieved by damage-associated molecular patterns (DAMPs). DAMPs are, unlike PAMPs, endogenous factors of non-microbial origin that are produced by the cells of the host. DAMPs are secreted or exposed on the cell membrane of cells and can be a variety of molecules such as adenosine triphosphate (ATP), high mobility group box 1 (HMGB1), calreticulin (CRT) and heat shock proteins (HSPs)[208]. Dead or dying cells release or expose DAMPs on their surface which are recognized by the immune system that is programmed to remove the respective cells through phagocytosis to avoid toxic accumulation[209]. In contrast, when DAMPs are secreted as consequence of severe tissue damage by stressed, injured or dying cells, they act as danger signals and adjuvants to mobilize an immune defense mechanism to protect the host from further damage. DAMPs can bind PRRs on dendritic cells and evoke subsequent maturation and activation of an immune response against the originator of the tissue damage[210-212].

A process related to this phenomenon is called immunogenic cell death (ICD) and has gained a lot of interest recently for its potential adjuvant properties in anti-cancer immune-therapy due strong induction of a broad range of DAMPs. This leads to release of these DAMPs alongside cancer antigens upon cell death. The process of ICD underlines again the role of the immune system in the efficacy of cancer therapy and suggests that the type of tumor cell death mechanism can be a key factor in initiating an anti-tumor response. This knowledge widens the potency of patient-derived cancer tissue vaccines as the immunogenicity can be strongly augmented through induction of ICD prior to vaccine design. In this way, the released cancer antigens and DAMPs will simultaneously interact with the PRRs resulting in a stronger anti-tumor immune response[213-215]. Interestingly, ICD can be induced by existing anti-cancer therapies, i.e. low dose chemotherapeutic drugs and radiotherapy (*vide infra – Chapter 7*), broadening the application field of these conventional therapies.

Other strategies to increase the immunogenicity of patient-derived cancer tissue vaccines, which are not endorsed by the immunogenic cell death theory but merely increase the immunogenicity of cells, can involve heat shock treatment, oxidative treatment or UV irradiation of the cells prior to vaccine design[216-221]. Subjection of cells to these immunogenic treatments induces production of specific DAMPs and release of cancer antigens which will, upon DC targeting, support and strengthen the immunogenicity of the vaccine in eliciting a stronger anti-tumor immune response. The mechanisms of ICD-based vaccines and immunogenic DC vaccines are illustrated in Figure 6.

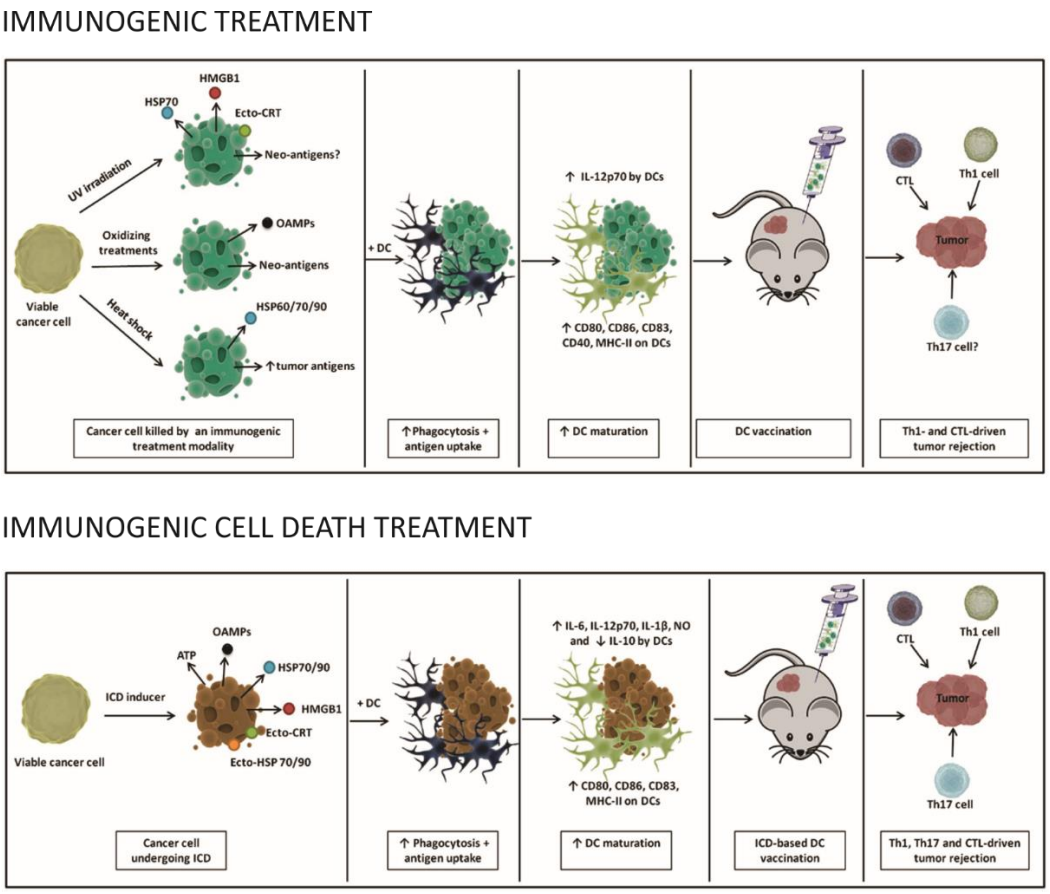

Figure 6. Illustration of the induction of anti-tumor immune responses evoked by cell-based vaccines killed by immunogenic treatment compared to ICD – (*Figure adjusted from reference 221*).

Although much progress has been made in the development of potent vaccines, there is still a lot of room for improvement in determining the optimal conditions for TLR immune stimulation, i.e. taking structure characteristics, concentration and spacing into account without compromising on tolerability and safety. Moreover, evidence of synergistic effects of

combining adjuvants alongside the potential of synthetic small molecule TLR-agonists can also contribute to optimization of cancer vaccine design. This will hopefully lead to licensed use of small molecule TLR-agonists and to more potent formulations with limited systemic toxicity profiles. Of note, the other innate immune receptors such as NOD-like receptors (NLRs), RIG-I-like receptors (RLRs) and C-type lectin receptors (CLRs) can also potentially broaden the adjuvant synergistic possibilities. However, further research is required to assess their potency. Altogether, fine-tuning and combining these strategies holds promise to significantly increase vaccine immunogenicity and decrease the risk of T-cell anergy through the induction of a more potent and focused immune response against malignancies.

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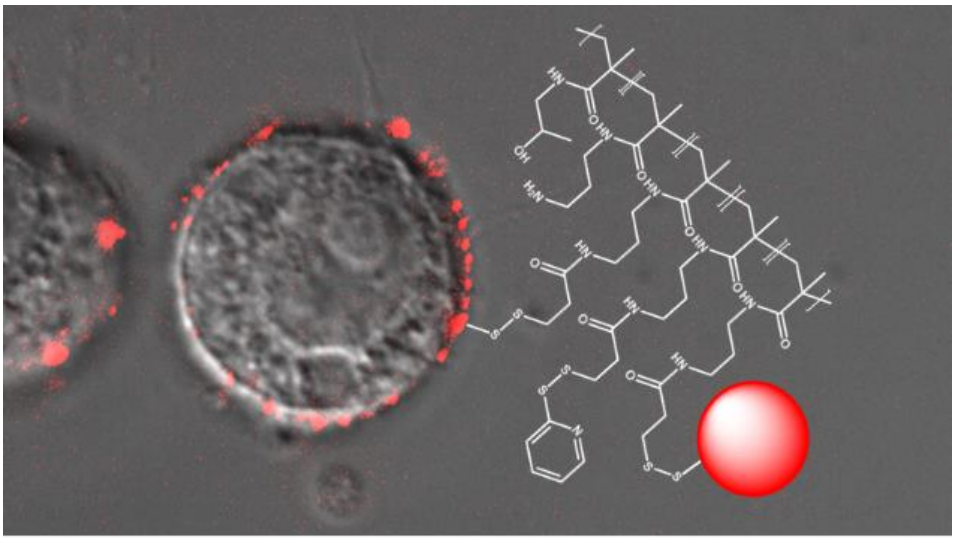
PART II

DESIGN OF IMMUNE-MODULATING POLYMERIC MICROPARTICLES



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A generic polymer-protein ligation strategy for vaccine delivery

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CHAPTER 3

POLYMER-PROTEIN LIGATED NANO-CONJUGATES

ABSTRACT

Although the field of cancer immune-therapy is intensively investigated, there is still a need for generic strategies that allow easy, mild and efficient formulation of vaccine antigens. Here we report on a polymer-protein ligation strategy to formulate protein antigens into reversible polymeric conjugates for enhanced uptake by dendritic cells and presentation to CD8+ T-cells. A N-hydroxypropyl methacrylamide (HPMA) based co-polymer was synthesized via RAFT polymerization followed by introduction of pyridyldisulfide moieties. To enhance ligation efficiency to ovalbumin, which is used as model protein antigen, protected thiols were introduced onto lysine residues and deprotected *in situ* in presence of the polymer. The ligation efficiency was compared for both the thiol-modified versus unmodified ovalbumin and the reversibility was confirmed. Furthermore, the obtained nano-conjugates were tested *in vitro* for their interaction and association with dendritic cells, showing enhanced cellular uptake and antigen cross-presentation to CD8+ T-cells.

INTRODUCTION

Dendritic cells (DCs) were first described by Ralph Steinman and co-workers as heterogeneous, large stellate cells that are potent stimulators of the immune system with a much higher antigen presentation capacity compared to other cell subclasses such as macrophages and B-lymphocytes[1,2]. This discovery has led to major insights in how the immune system interacts with foreign material, revealing dendritic cells as the critical factor in the interplay between the innate and the adaptive immune response[3-14]. Therefore cancer immune-therapy, focusing on stimulating DCs with cancer antigens[15-18], is a promising strategy that aims for harnessing the patient´s immune system without facing the ubiquitous side effects of chemo- and radiotherapy[3,12].

In this regard, formulating protein antigens into particulate carriers is highly attractive as this dramatically promotes cross-presentation relative to soluble antigens[15,19-22]. Sub-micron particles are preferred over bigger ones due to their higher tissue mobility and by consequence ability to target different DC subsets. On the one hand, peripheral migratory DCs can recognize and take up particles at their injection site followed by lymphatic transport to the lymph nodes and antigen-presentation to T-cells. On the other hand, only small nanoparticles, in the sub 200 nm range, will additionally drain to the lymph nodes via passive diffusion into the lymphatics and also target lymph-node resident DCs[23-26].

This chapter reports on a generic strategy for the formulation of antigens into nano-scaled polymeric conjugates based on co-polymers of N-hydroxypropyl methacrylamide (HPMA) with 3-aminopropyl methacrylamide (APMA). The neutral hydrophilic HPMA moieties provide water solubility and biocompatibility[27-29]. Additionally, the APMA units will be used to introduce pyridyldisulfide moieties for reversible protein conjugation via disulfide formation and enhancement of cell uptake by interaction with exofacial thiols[30,31]. Figure 1 shows a schematic representation of the concept.

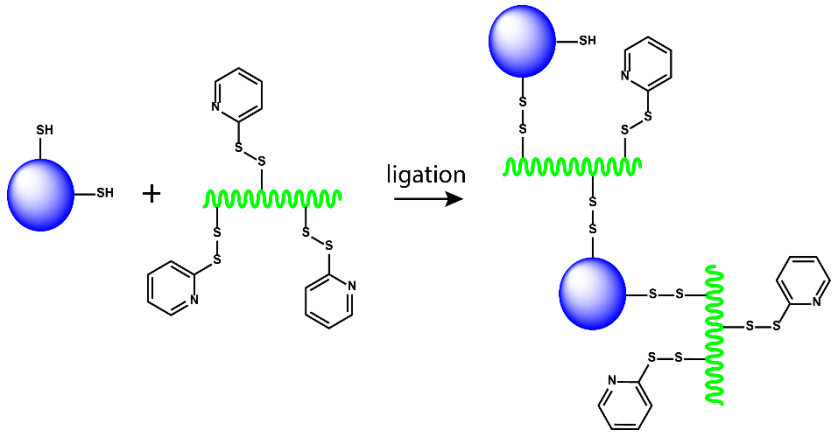

Figure 1. Schematic illustration of a polymer-protein ligation strategy based on reversible disulfide formation between free thiols on a protein and pyridyldisulfide moieties on a polymer backbone. A mixture is obtained composed of multiple polymers per protein and/or multiple proteins per polymer.

RESULTS AND DISCUSSION

1. Synthesis and modification of poly(HPMA-co-APMA)

Co-polymerization of HPMA and APMA was performed by reversible addition-fragmentation transfer (RAFT) polymerization according to Zhu Qin *et al*[32] for a targeted degree of polymerization (DP) of 100 repeating units (corresponding to a target molecular weight of 14 kDa) composed of 80 HPMA repeating units and 20 APMA repeating units. Being a controlled radical polymerization technique, RAFT yields access to polymers with a well-defined chain length and a low dispersity[36,37], which is of interest to obtain polymers with a molecular weight below the renal clearance threshold to allow elimination from the body.

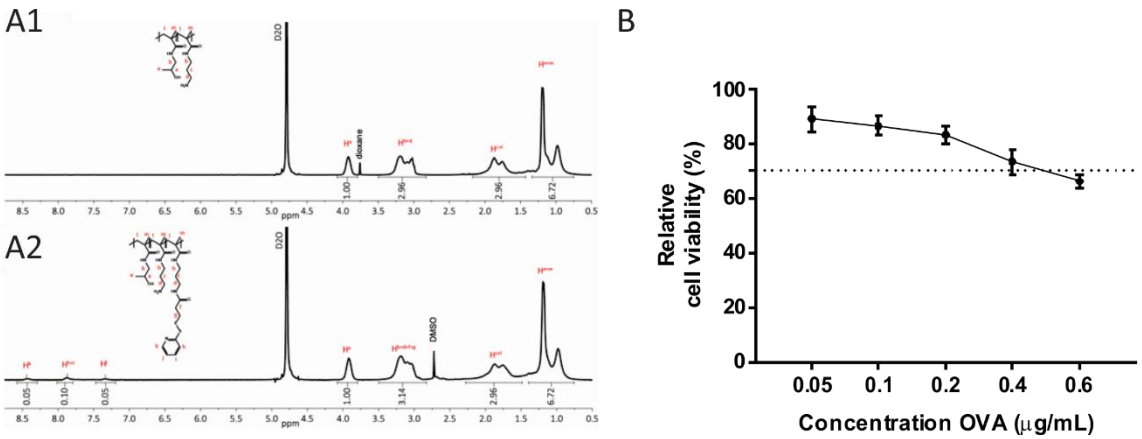

Figure 2. (A1) ^{1}H-NMR spectrum of poly(HPMA-co-APMA) and (A2) ^{1}H-NMR spectrum of poly(HPMA-PDS). (B) *In vitro* MTT cytotoxicity assay of poly(HPMA-co-APMA) on DC2.4 cells.

^{1}H NMR analysis indicated a conversion of 76 % and a composition of 80 % HPMA and 20 % APMA, which is in good accordance to the monomer composition (**Figure 2A**). A low dispersity of 1.08 and a M_n of 16 kDa was obtained via size exclusion chromatography (SEC) in dimethylacetamide.

Subsequently we engineered antigen-binding properties onto the polymers (**Figure 3A**). First, we synthesized 3-(2-pyridyldithio)-propanoic acid (PDPA) by thiol-disulfide exchange of 2,2-dipyridyldisulfide and 3-mercapto propionic acid in the presence of acetic acid, based on Hugh *et al.*[33]. Secondly, the APMA moieties were substituted with PDPA using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) as amidation reagent to introduce pending pyridyldisulfide moieties onto the poly(HPMA) backbone. ^{1}H-NMR spectroscopy revealed a degree of substitution of 20 % of the primary amine moieties. This polymer will be further denoted as poly(HPMA-PDS) (**Figure 2B**). Higher substitution percentages were also obtained (results not shown) but were not of interest as sufficiently high conjugation efficiencies were achieved by 20 % substitution of the APMA moieties with PDS (v*ide infra – Antigen conjugation*). The latter can undergo disulfide exchange with free thiols present on cysteine residues of proteins and pyridyldisulfide based protein conjugation strategies have been widely explored in combination with RAFT polymerization[38-42]. Indeed, disulfides are attractive moieties for designing drug delivery systems. Firstly, they are readily formed with free thiols of cysteine residues. Secondly, disulfides are stable under (oxidative) extracellular conditions but can be reduced to free thiols in the cytoplasm of cells. Thirdly, disulfide exchange with cell surface thiols can enhance cellular uptake and can also trigger dissociation of disulfides[30,31]. The potential cytotoxicity of the residual amine groups on the poly(HPMA-PDS) was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assay on the immortalized DC2.4 cell line for increasing concentrations of poly(HPMA-co-APMA). As shown in **Figure 2B**, up to a polymer concentration of 0.2 mg/mL no cytotoxicity is observed, evinced by a cell viability above 70%. Note, that the MTT assay measures the activity of mitochondrial enzymes (dehydrogenases) rather then cell death. Therefore it can not be excluded that poly(HPMA-co-APMA) partially inhibits these enzymes rather then induces cell death above 0.2 mg/mL. Nevertheless, polymer concentrations well below this threshold were used in further experiments (concentration

range between 0.0011 mg/mL-0.017 mg/mL) to ensure optimal cell viability during these experiments.

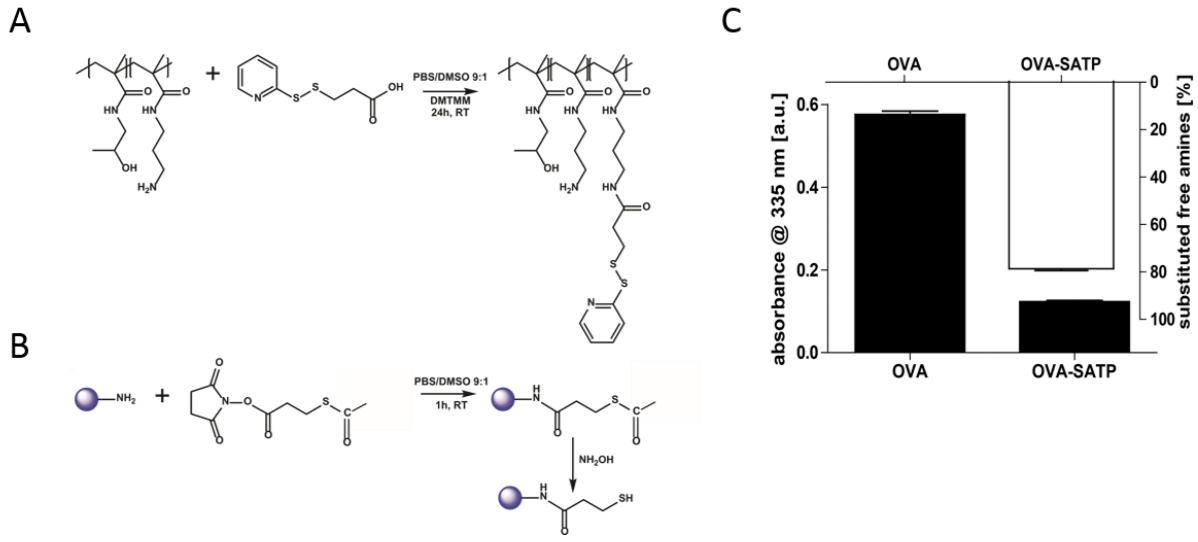

Figure 3. (A) Modification of poly(HPMA-co-APMA) with PDPA. (B) Substitution of lysine residues with SATP and subsequent deprotection to free thiols. (C) Determination of the extent of OVA modification with SATP determined by TNBSA assay.

2. Antigen modification with protected thiols

Antigen conjugation to poly(HPMA-PDS) requires the presence of free thiols on the protein backbone for thiol-disulfide exchange with the PDS moieties. As the composition of every protein differs, variable conjugation efficiency is likely for every other antigen. In addition, it is possible that the amount of thiols per protein will not be sufficient to afford efficient formulation of the respective antigen into nano-conjugates. Therefore, we opted to generalize the conjugation strategy. In this regard, we introduced protected thiols onto lysine residues that are more abundantly present on proteins than cysteines. As a model protein antigen we used ovalbumin (OVA) because it contains peptide sequences that are recognized by the murine immune system as CD4+ and CD8+ epitopes. Moreover, a wide variety of *in vitro* and *in vivo* tools are available for immunological assessment of OVA based vaccine formulations. OVA was incubated with a molar ratio of OVA to S-acetylthiopropionate N-succinimidyl ester (SATP) of 1 to 15. This compound substitutes primary amines on lysine residues with acetylated thiols that can be deprotected *in situ* in presence of hydroxylamine (**Figure 3B**), and subsequently undergo disulfide exchange with poly(HPMA-PDS). The decrease in free amines was quantified

by spectrophotometry using the (2,4,6-trinitrobenzene sulfonic acid) assay (TNBSA), thereby indicating 78 % of all available lysines to be substituted (Figure 3C).

3. Antigen conjugation

In a first series of experiments, the polymer-conjugation efficiency of OVA-SATP was compared versus unsubstituted OVA. SDS-PAGE was used to assess protein conjugation of poly(HPMA-PDS) to OVA and OVA-SATP. For unsubstituted OVA, no difference could be observed upon incubation of the protein with the polymer. In contrast, as shown in Figure 4, the free OVA band disappears almost completely when OVA-SATP is used. This confirms that SATP substitution strongly increases the polymer-protein conjugation efficiency. Interestingly, three distinct bands appear that correspond either to OVA that is ligated to several polymer strands or *vice versa*, along with a broad spread of higher molecular weight bands. These data suggest that besides polymers being grafted onto the protein, also polymeric crosslinking among different proteins occur.

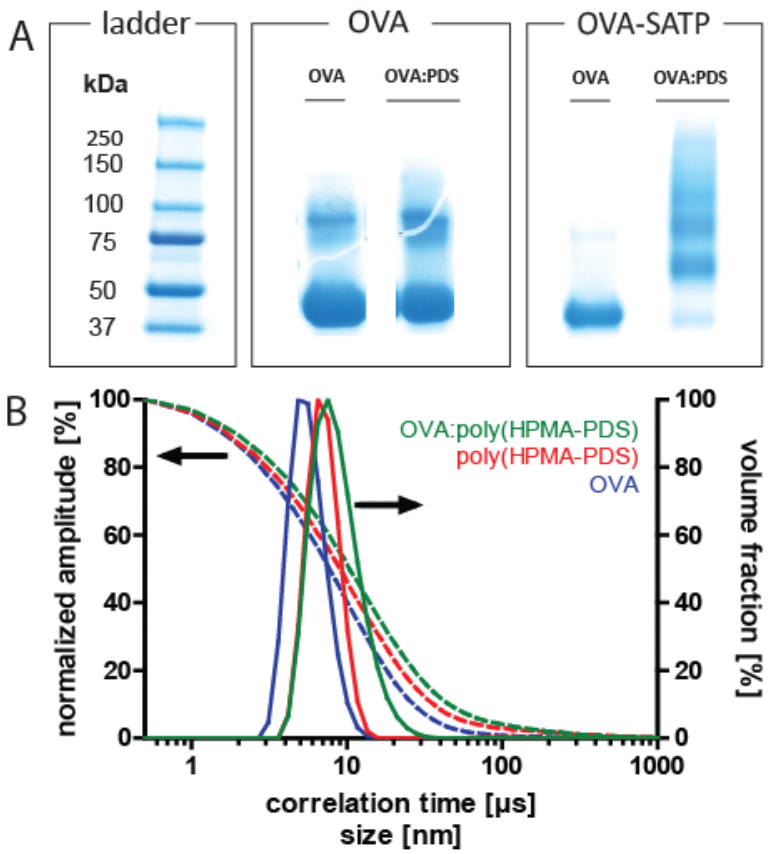

Figure 4. (A) SDS-PAGE analysis of OVA and OVA-SATP ligated to poly(HPMA-PDS) in a 1:1 molar ratio. (B) DLS data of soluble OVA, poly(HPMA-PDS) and the OVA:poly(HPMA-PDS).

Dynamic light scattering (DLS; **Figure 4B**) revealed the presence of small conjugates without extensive inter-particle crosslinking, as evidenced by both the size distribution and the correlation curves. This property is of importance with regard to tissue mobility *in vivo*.

The required ratio of polymer to OVA-SATP to yield optimal conjugation was determined by visualization of the conjugation efficiency of different molar ratios of OVA-SATP to poly(HPMA-PDS), by SDS-PAGE. **Figure 5** indicates that approximately 100 % conjugation can be already obtained at an equimolar ratio of OVA-SATP to poly(HPMA-PDS). Therefore, we decided to use a ratio of OVA to polymer of 1:1 in further experiments. To assess the reversible nature of the disulfide bonds formed between OVA-SATP and the poly(HPMA-PDS), SDS-PAGE was performed in presence and absence of 2-mercaptoethanol as a reducing agent. As shown in **Figure 5**, in presence of 2-mercaptoethanol, the band corresponding to OVA-SATP reappears again as a single protein band confirming the reversibility of the conjugation.

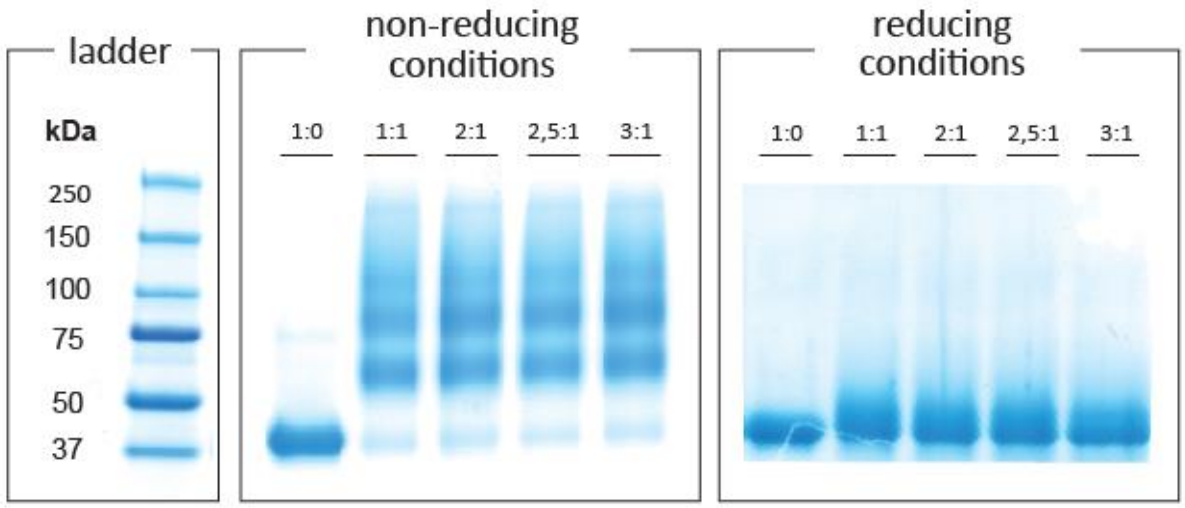

Figure 5. SDS-PAGE analysis of OVA-SATP to poly(HPMA-PDS) conjugation for varying protein to polymer ratios. Gel electrophoresis was performed under non-reducing and reducing (presence of 2-mercaptoethanol) conditions to investigate the reversibility of the conjugation.

4. In vitro characterization

Next we aimed at investigating the effect of polymer conjugation on the uptake of OVA by DCs. First, poly(HPMA-PDS) was fluorescently labeled with Atto647 NHS ester and conjugated to AlexaFluor488-labeled OVA (OVA-AF488) to allow analysis of the uptake of both OVA and the respective polymer. The resulting conjugates were incubated in different concentrations overnight at 37 °C with DC2.4 cells (an immortalized murine dendritic cell line)[36]. Flow

cytometry analysis of the mean AF488 fluorescence per cell (Figure 6A1), showed a dose-dependent cellular association of the antigen and indicates that poly(HPMA-PDS) conjugation leads, relative to soluble OVA, to higher uptake efficiency of OVA. A similar dose-dependent trend was observed for the mean Atto647 fluorescence per cell for poly(HPMA-PDS) (Figure 6A2). This data indicates that both OVA and the polymer associate with the dendritic cells.

To investigate whether the conjugates are internalized by the DCs or merely bound to the cell surface, confocal microscopy was performed. Figure 6B reveals that the conjugates are indeed internalized by the DCs. The co-localizing signals of OVA-AF488 and of the polymer-Atto647 inside the cells also confirms that, upon uptake of the conjugates, the polymers are trafficked within the same intracellular vesicles as OVA and the conjugates are not cleaved at the cell surface.

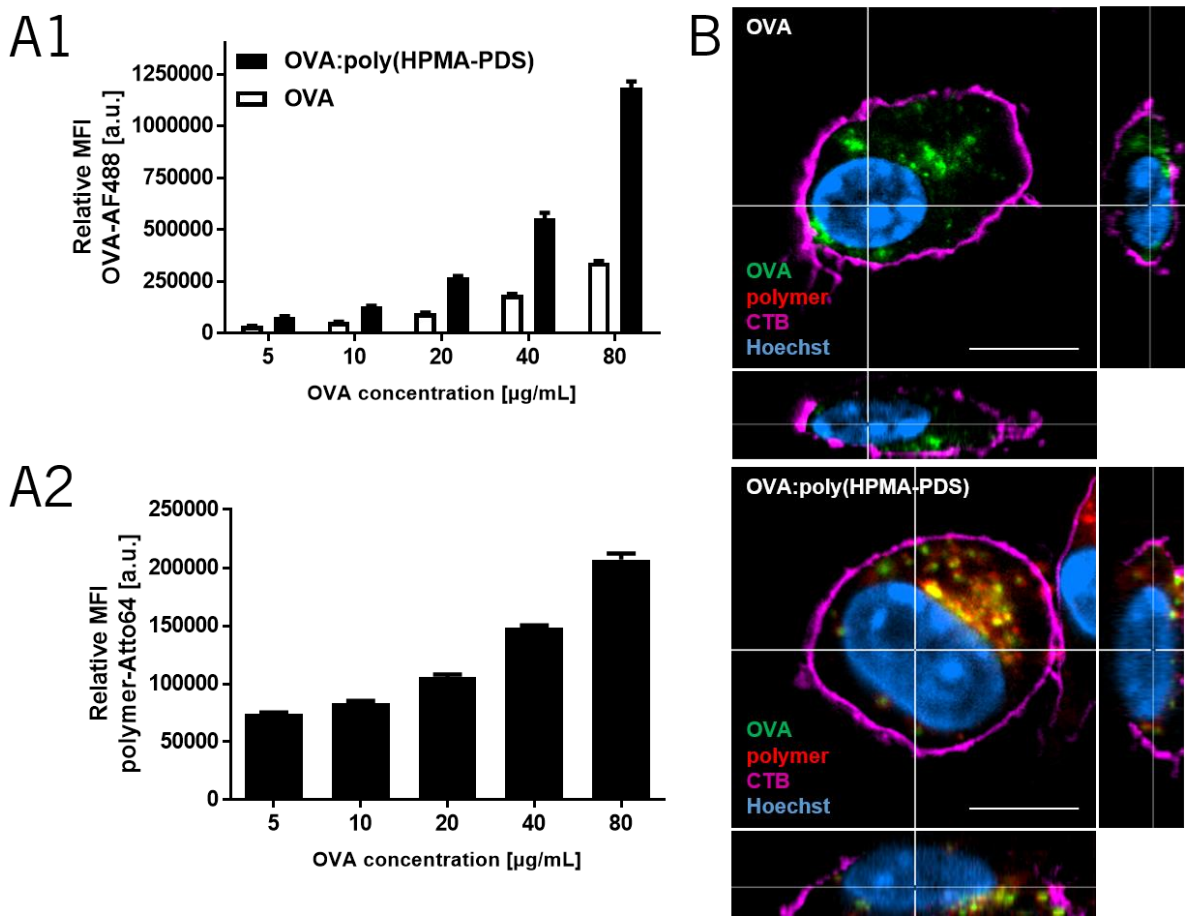

Figure 6. *In vitro* interaction of dendritic cells with OVA and OVA:poly(HPMA-PDS): (A) Flow cytometry analysis of the interaction of DCs with OVA (A1) and with poly(HPMA-PDS) (A2), as function of OVA concentration. (B) Confocal microscopy images. Cell membrane is stained with AF555-labeled cholera toxin B (CTB-AF555). Cell nuclei are stained with Hoechst. Scale bar represents 15 µm.

To identify the reason because of which polymer-conjugated OVA shows higher uptake than soluble OVA, we pulsed DCs with soluble OVA and poly(HPMA-PDS) separately at 4 °C. Under these conditions, energy dependent uptake mechanisms are blocked. As shown in Figure 7A, soluble OVA is not associated with the DCs at 4 °C, opposed to poly(HPMA-PDS) which does show dose-dependent cellular association. Confocal microscopy (Figure 7B; overlaying the DIC and the Atto647 channel) confirmed that at 4 °C, poly(HPMA-PDS) is indeed bound to the cell membrane.

Taken together, we hypothesize that remaining pyridyldisulfide moieties on the conjugates promote interaction with cysteine residues of cell surface proteins and thereby bind to the cell membrane[29, 30].

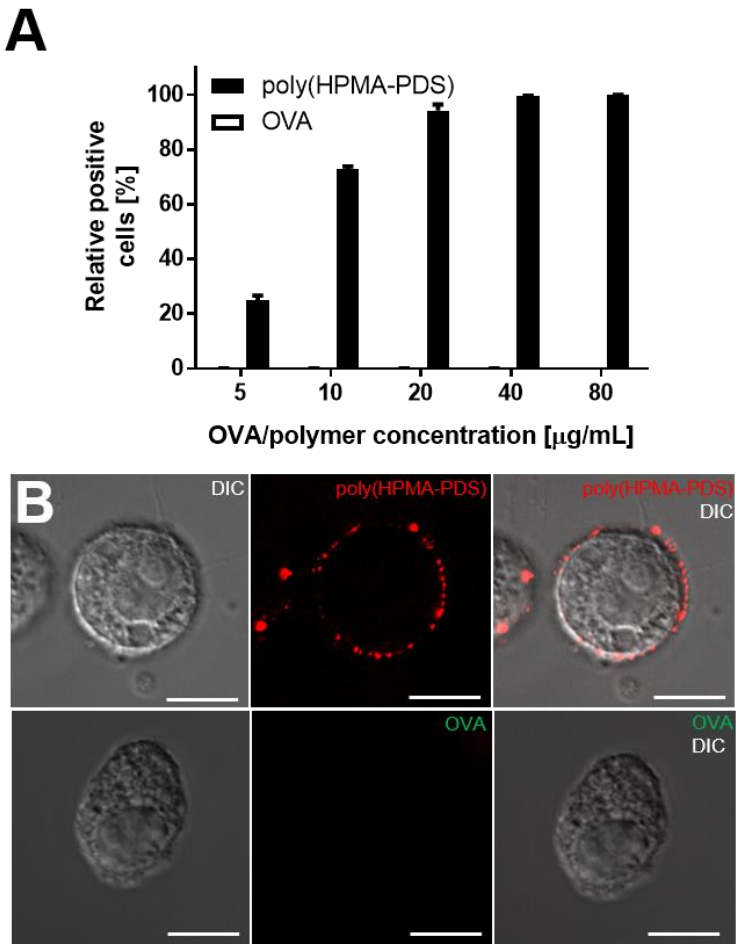

Figure 7. Investigation of energy independent cellular association of DCs pulsed with soluble OVA and poly(HPMA-PDS) at 4 °C: (A) Flow cytometry analysis. (B) Confocal microscopy images. Scale bar is 15 µm.

5. Immuno-biological evaluation.

Taking into account the high efficiency of OVA conjugation to poly(HPMA-PDS) together with the high uptake efficiency by DCs and the reversibility of the conjugation, we found the poly(HPMA-PDS) based system to have potential for the formulation of protein vaccine antigens.

To assess the immunological potential of the conjugates, we determined whether or not poly(HPMA-PDS) conjugation enhances cross-presentation by DCs to CD8+ T-cells via an *in vitro* CD8+ T-cell proliferation assay. Mouse bone marrow derived dendritic cells (bmDCs) were isolated, pulsed with soluble OVA and OVA:poly(HPMA-PDS) in three different OVA concentrations (0.2, 2 and 5 mg/mL) followed by co-culture with OT-I cells. OT-I cells are CD8+ T-cells that express the transgenic T-cell receptor that specifically recognizes the CD8+ epitope of OVA (i.e. SIINFEKL) presented via MHC-I. These OT-I cells were labeled with the fluorescent label CFSE in order to allow monitoring of the CD8+ T-cell proliferation via flow cytometry analysis. Upon division of the OT-I cells, the fluorescent CFSE marker will be equally divided into the daughter cells leading to a decrease of the fluorescent signal.

Figure 8A demonstrates that the OVA:poly(HPMA-PDS) conjugates enhance CD8+ T-cell proliferation over soluble OVA as evinced by a more pronounced decrease of the CFSE signal. Additionally, ELISA was performed to determine the amount of IFN-γ that is produced by the CD8+ T-cells. IFN-γ is a pro-inflammatory cytokine that is highly upregulated upon activation of the OT-I cells and drives the effector T-cell response. As depicted in Figure 8B the IFN-γ production is indeed higher when CD8+ T-cells are co-cultured with bmDCs that are pulsed with the OVA:poly(HPMA-PDS) conjugates compared to soluble OVA confirming the data obtained by the T-cell proliferation assay.

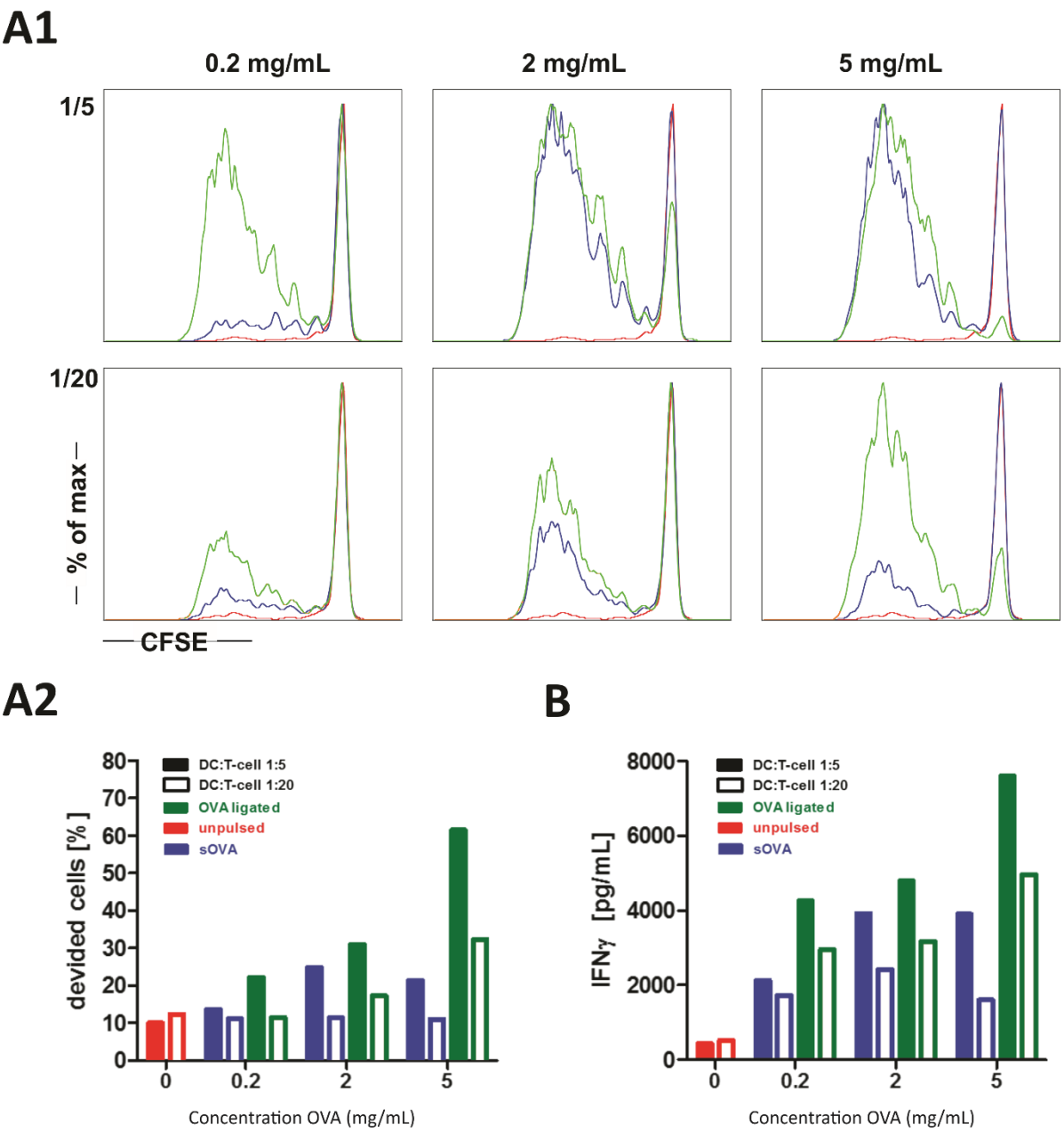

Figure 8. *In vitro* immuno-biological evaluation: (A1) Flow cytometry histograms of CFSE labeled transgenic OT-I OVA-specific CD8+ T-cells co-cultured with bmDCs pulsed with different concentrations of OVA and OVA:poly(HPMA-PDS) respectively. BmDC to T-cell ratios of 1/5 and 1/20 were used (A2) Corresponding quantification of T-cell proliferation expressed as the percentage of divided labeled transgenic OT-I OVA-specific CD8+ T-cells. (B) IFN-γ secretion in the supernatant of the bmDC/T-cell co-cultures measured by ELISA.

CONCLUSION

To summarize, we show in this chapter that water-soluble HPMA-based RAFT polymers with multiple pending pyridyldisulfide moieties are well suited for protein conjugation, on the condition that these proteins are modified with protected thiols that can be deprotected *in situ*. The obtained conjugates are stable in aqueous medium and can be disassembled in response to reducing conditions. *In vitro* experiments on dendritic cells show that the polymer conjugation of a model antigen resulted in an increased cellular uptake, relative to unconjugated protein, which we attribute to thiol-disulfide exchange between remaining pyridyldisulfide moieties and exofacial thiols present on the cell surface. Furthermore, we demonstrate that polymer conjugation increases antigen presentation by bmDCs to CD8+ T-cells *in vitro*. In future research, the effect of polymer conjugation on lymphatic antigen transportation and *in vivo* immune-activation will be studied as well as conjugation of molecular adjuvants to these polymers.

EXPERIMENTAL SECTION

Materials. Hydroxypropyl methacrylamide (HPMA) and aminopropyl methacrylamide (APMA) were obtained from Polysciences. Anhydrous acetic acid was purchased from Biosolve chemicals. Dimethylsulfoxide (DMSO), mercapto-propanoic acid, 4-cyanovaleric acid dithiobenzoate (CTP), 4,4'-azobis(4-cyanovaleric acid) (ACVA), basic alumina, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent, sodium dodecyl sulfate (SDS), ethanol, dichloromethane, Na_2EDTA, hydroxylamine Atto 647N NHS ester, $NaHCO_3$, paraformaldehyde and PD10 desalting columns were obtained from Sigma Aldrich. Phosphate buffered saline pH 7,2 (PBS), RPMI-glutamax 1640 medium, fetal bovine serum EU qualified (FBS), penicillin/streptomycin (5000 U/mL), sodium pyruvate (100mM), cell dissociation buffer (PBS based), Hoechst and cholera toxine B conjugates to AlexaFluor555 (CTB-AF555) were purchased from Invitrogen. Dipyridyldisulfide (DPDS) and 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium Chloride (DMTMM) were obtained from TCI America. 2-Mercaptoethanol, Laemli sample buffer (4x), Coomassie blue stain (G-250) and the 4-20 % mini-protean TGX gels were purchased from Bio-rad. S-acetylthiopropionate N-succinimidyl ester (SATP), TNBSA solution and hydrochloric acid (HCl) 37% v/v were obtained from Thermo Scientific whereas the pre-treated Spectra/Por 7 dialysis membranes were purchased from Spectrumlabs.

Instrumentation.

^{1}H-Nuclear magnetic resonance (NMR). NMR spectra were recorded on a Bruker 300 MHz FT NMR in D_2O and d_6-DMSO. Chemical shifts (δ) are provided in ppm relative to TMS.

Size exclusion chromatography (SEC). SEC elugrams were recorded on a Shimadzu 20A system in dimethylacetaminde (DMAc) as solvent containing 50 mM LiBr. The system was equipped with a 20A ISO-pump and a 20A refractive index detector (RID). Measurements were recorded at 50 °C with a flow rate of 0.7 mL/min. Calibration of the 2 PL 5 µm Mixed-D columns was done with polymethylmethacrylate (PMMA) standards obtained from PSS (Mainz, Germany).

Electron spray ionization-mass spectroscopy (ESI-MS). ESI-MS was carried out on a Waters LCT Premier XETM TOF mass spectrometer with a ZsprayTM source, ESI and modular LocksprayTM interface, coupled to a Waters alliance HPLC system.

UV-VIS spectrophotometry. UV-VIS spectra were recorded on a Shimadzu UV-1650PC spectrophotometer in 1 cm x 1 cm quartz cells.

Dynamic light scattering (DLS). DLS analyses were performed on a Zetasizer Nano S (Malvern Instruments Ltd., Malvern, U.K.) with a HeNe laser (λ = 633 nm) at a scattering angle of 173 °.

Synthesis of poly(HPMA-co-APMA). Based on Qin *et al.*[32] a copolymer composed of hydroxypropyl methacrylamide and aminopropyl methacrylamide (HPMA-co-APMA) was synthesized via aqueous reversible addition-fragmentation chain transfer (RAFT) polymerization using 4,4'-azobis(4-cyanovaleric acid) as the initiator and 4-cyanovaleric acid dithiobenzoate as the RAFT agent. The RAFT polymerization was set for a HPMA/APMA ratio of 80/20 and a target degree of polymerization (DP) of 100. After dissolution of HPMA (34.91 M), APMA (8.73 M) and ACVA (0.218 M) in deionized water, CTP was dissolved in 1,4-dioxane and added in a final concentration of 0.436 M. Subsequently, oxygen was removed via four cycles of freeze-pump-thaw. The polymerization was initiated by heating the mixture to 70 °C in an oil bath for 24 h. Purification of the co-polymer was achieved by precipitation in acetone followed by removal of residual solvent via vacuum pump (24 h). Confirmation of the co-polymerization was assessed by ^{1}H NMR analysis and the DP was determined by GPC analysis. Conversion rate of the RAFT polymerization was achieved by ^{1}H NMR analysis of the reaction mixture at different time points. ^{1}H NMR peak assignment (300 MHz, D_2O): δ (ppm) 0.99-1.20 ($CH_2CH(OH)C\mathit{H_3}$, $CH_2C(C\mathit{H_3})CO$), 1.80-1.92 ($C\mathit{H_2}C(CH_3)$-CO, $CH_2C\mathit{H_2}CH_2NH_2$), 3.08 ($CH_2CH_2C\mathit{H_2}NH_2$), 3.24 ($CONHC\mathit{H_2}CH(OH)CH_3$, $CONHC\mathit{H_2}CH_2CH_2NH_2$), 3.94 ($CH_2C\mathit{H}$-$(OH)CH_3$).

Synthesis of PDPA. Based on Hugh *et al.* 3-(2-pyridyldithio)-propanoic acid (PDPA) was synthesized[33]. In brief, 3-mercapto-propanoic acid (0.0249 mmol) was added to a 0.623 M solution of 2,2'-dipyridyldisulfide (DPDS) in anhydrous ethanol in the presence of acetic acid. The solution was subsequently stirred for 2 hours at room temperature. Purification of PDPA was assessed by basic alumina column chromatography using a 3:2 mixture of dichloromethane and ethanol followed by elution of PDPA via addition of 4 % of acetic acid to the eluent mixture. Traces of acetic acid were removed under high vacuum for 48 h. The synthesis and purity of the product was confirmed by ^{1}H NMR analysis and LC-MS (ESI^+) analysis. ^{1}H NMR peak assignment (300 MHz; d_6-DMSO): δ (ppm) 2.59 (2H, t, J = 7.0), 2.98 (2H, t, J = 7.0), 7.23 (1H, ddd, J = 7.1, 4.8, 1.2), 7.75 (1H, d, J = 8.0), 7.8 (1H, td, J = 7.9, 1.4), 8.4 (1H, d, J = 4.9), 12.43

(1H, broad s). Theoretical mass of PDPA: 215.01 and experimental mass: ESI+ m/z in MeOH – 216.0 (MH+).

Synthesis of poly(HPMA-PDS). To a 5 mg/mL solution of poly(HPMA-co-APMA) in a phosphate buffered saline (PBS) 1.5 M excess of DMTMM was added followed by an equimolar amount of PDPA dissolved in DMSO. The reaction was stirred overnight at room temperature followed by purification via dialysis against deionized water (MWCO 3.5 kDa) for 24 h and lyophilization. The synthesis was confirmed via ^{1}H NMR analysis.

Cell lines. The DC2.4 cell line was a kind gift from Dr. Kenneth Rock (University of Massachusetts, Boston, US).[34] DC2.4 cells were cultured in RPMI-glutamax medium supplemented with 10 % fetal bovine serum, 1 % penicillin/streptomycin and 1 mM sodium pyruvate and incubated at 37 °C with 5 % CO_2 saturation. Isolation of the cells for experiments was performed by incubation of the cells in a PBS-based dissociation buffer for 10 minutes.

MTT assay. Cell toxicity was measured by seeding DC2.4 in 96 well plates at a density of 50000 cells/mL in complete RPMI medium (total volume 100 µL) 1 day prior to addition of increasing concentrations of poly(HPMA-co-APMA) in PBS. Subsequently the cells were cultured for 24 h followed by addition of 40 µL of the MTT reagent (1 mg/mL). After an incubation period of 2-3 h the formed formazan crystals were dissolved in 100 µL of a 10 % m/v SDS/0.01 M HCl solution overnight protected from light. The absorbance was measured by a microplate reader at 570 nm. As a negative and positive control PBS buffer and DMSO respectively were added to the wells.

Synthesis of OVA-SATP. Protected thiol groups were introduced via interaction with SATP. First a 120 µM solution of ovalbumin (OVA) was prepared in PBS pH 7,2. Second, SATP dissolved in dry DMSO was added in different molar ratios of SATP to OVA, i.e. 1:5, 1:10, 1:20, 1:50. After incubation of 45 min at room temperature the unreacted fraction was eliminated via a disposable PD10 column. The pure fractions of protein were distinguished from the waste fractions by UV spectrophotometry followed by lyophilization. Quantification of the introduced thiol percentage was assessed by the trinitrobenzene sulfonic acid assay (TNBSA) through measurement of the residual amine content.

TNBSA assay. The residual amine content of OVA after SATP substitution was determined by the (2,4,6-trinitrobenzene sulfonic acid assay (TNBSA) according to the manufacturer's instructions. First OVA and OVA-SATP were dissolved in a 0.1 M sodium bicarbonate buffer pH 8.5 at 50 µg/mL followed by addition of 0.25 mL of a 0.01 % TNBSA solution to 0.5 mL of each sample. After incubation of 2 hours at 37 °C 0.25 mL 10 % SDS and 0.125 mL 1 N HCl were added. Subsequently, absorbance was measured at 335 nm.

Polymer-protein conjugation. To a 0.465 M OVA solution in deacetylation buffer different molar polymer:OVA ratios were added to a final volume of 1 mL in PBS. The molar ratios used were 1:1, 2:1, 2.5:1, 3:1 and 4:1. The reaction was incubated for 2 hours at room temperature followed by visualization of the conjugation efficiency via SDS-PAGE and semi-quantitative analysis of the encapsulation via Image J.

Gel electrophoresis (SDS-PAGE). To analyze protein conjugation or to determine the reversibility of the synthesized particles, gel electrophoresis was performed. The samples were diluted with respectively Laemli sample buffer solution (4x) or with a 1:9 2-mercaptoethanol:Laemli sample buffer solution (4x), incubated for 5 minutes at 95 °C and loaded on 4-20 % precast gels. After the run (150 kV), visualization of the protein bands was achieved by incubation of the gels into Coomassie blue stain.

Fluorescent labeling. The remaining unsubstituted APMA units of poly(HPMA-PDS) were labeled with the Atto647-NHS ester according to the manufacturers' instructions. In brief, the fluorescent dye was dissolved in dry DMSO (2.3 mM) and added to a 5 mg/mL polymer solution in 0.1 M $NaHCO_3$ buffer (pH 8.3) aiming a target degree of 2.5 %. After incubation of 30 to 60 minutes protected from light at room temperature the excess was removed via PD10 column purification followed by lyophilization. Second, the fluorescent conjugates were synthesized as described above using the fluorescently labeled poly(HPMA-PDS) and a molar ratio of OVA:OVA-AF488 49:1. After 2 h incubation the particles were dialyzed against deionized water for 48 h (MWCO 100 kDa).

In vitro DC2.4 uptake assay. DC 2.4 cells were seeded at a density of 0.4 x 10^6 cells/mL in a 24 well plate one day prior to the addition of the fluorescent particles at different concentrations. After 24 h incubation the cells were dissociated using cell dissociation buffer followed by

centrifugation for 5 min at 200 G at 0 °C. After resuspension, the samples were stored on ice and measured by the BD Accuri C6 flow cytometer. The data was analyzed using FlowJo.

Confocal microscopy imaging. DC2.4 cells were seeded at a density of 0.2 x 10^6 cells/mL in a glass bottom Will-co dish and incubated overnight. Next, the fluorescent particles were added, incubated for 24 h and fixated in a 2 % paraformaldehyde solution for 10-15 minutes. The cells were subsequently washed and simultaneously stained by CTB-AF555 and Hoechst for 1 h at room temperature. Finally, the samples were washed with PBS and visualized by confocal microscopy. This was carried out on a Leica DMI6000 B inverted microscope equipped with an oil immersion objective (Zeiss, 63×, NA 1.40) and attached to an Andor DSD2 confocal scanner. Images were processed with Image J.

In vitro OT-I proliferation assay and ELISA. Mouse bone marrow derived DCs (bmDCs) were isolated by flushing femurs of C57BL/6 mice with complete RPMI with a 26 G needle. The cell suspension was filtered through a 100 µm cell strainer and incubated for 3-5 minutes in red blood cell lysis buffer on ice. The cells were subsequently seeded into a 24 well plate at a density of 3 x 10^5 cells/mL in complete RPMI containing 20 ng/mL of GM-CSF and incubated at 37 °C/5 % CO_2 for 7 days. To ensure optimal bmDC growth, fresh medium containing 20 ng/mL GM-CSF was added on day 3 and on day 6 the medium was refreshed. On day 7 the bmDCs were isolated and pulsed with the test compounds containing 0.2, 2 and 5 mg/mL OVA followed by co-culture with CFSE-labeled OVA specific transgenic CD8+ T-cells, according to previously described protocols.[35]

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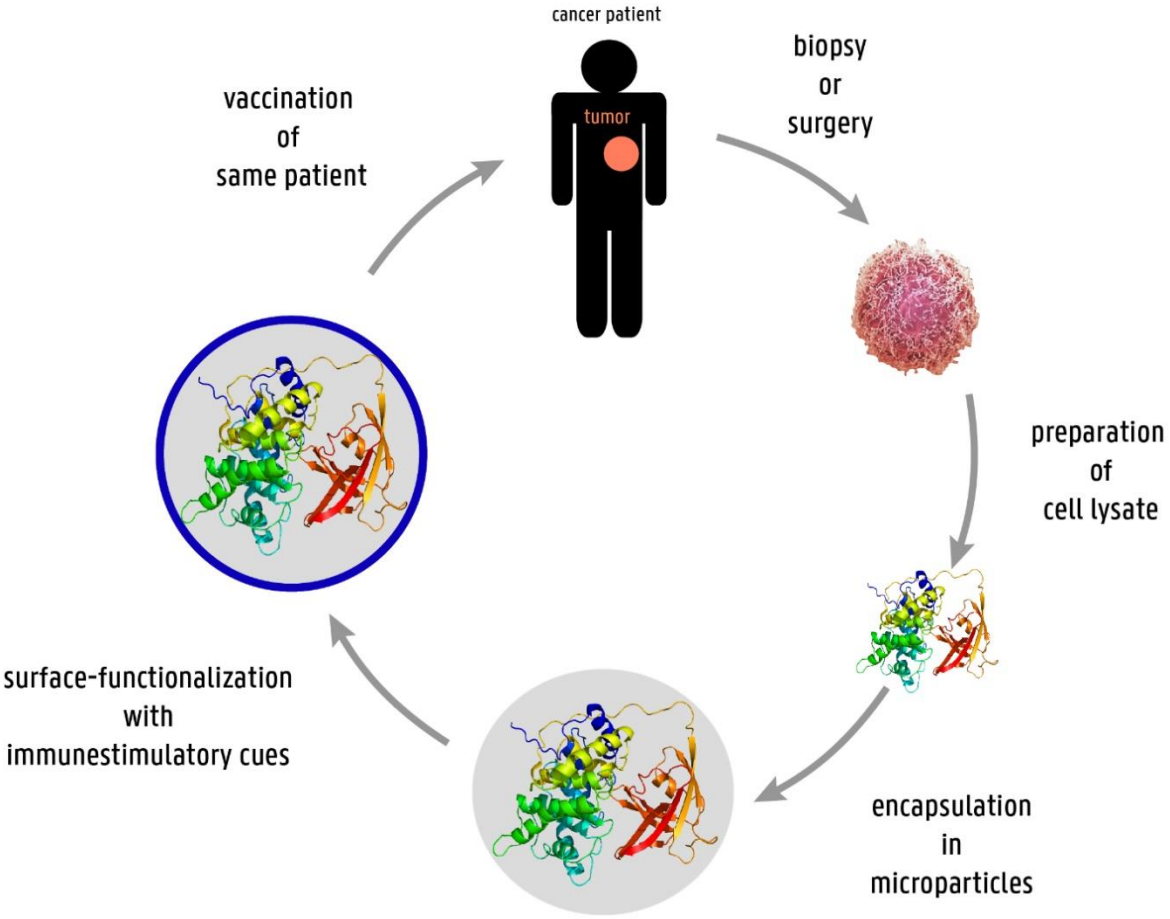

Parts of this chapter were submitted in:

Cancer cell lysate entrapment in $CaCO_3$ engineered with polymeric TLR-agonists – immune-modulating microparticles in view of personalized anti-tumor vaccination.

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CHAPTER 4

IMMUNE-MODULATING POLYMERIC $CaCO_3$ PARTICLES

ABSTRACT

Personalized anti-tumor immune-therapy has recently gained major interest in the fight against metastatic cancer. Here we report on a two-step strategy to encapsulate patient-derived cell lysate into immune-modulating microparticles as a general personalized vaccine formulation approach. In a first step, cell lysate produced from cancer cells was entrapped into stable porous calcium carbonate ($CaCO_3$) microparticles by a co-precipitation reaction. Second, the surface of these microparticles was adsorbed with a polymer substituted with a small molecule TLR7-agonist to enhance the immunogenicity. Relative to soluble cell lysate, microparticle-encapsulation yielded higher uptake of cell lysate by dendritic cells *in vitro*. Also, microparticle-adsorbed polymeric TLR7-agonists retained their TLR-triggering capacity resulting in immunogenic vaccine particles. We anticipate this strategy might hold potential to turn patient-derived tumor tissue – comprising the patient's own mutanome – into potent personalized anti-cancer vaccines.

INTRODUCTION

One of the main reasons why DC targeting and anti-tumor immune-therapy in general has great potential[1-5], is the lack of unfavorable and unspecific side effects caused by conventional therapies[6-8]. In addition, and perhaps even more important, is the induction of immunological memory which provides the patient with prolonged protection, even after treatment, whilst decreasing the possibility of tumor relapse that leads to more resistant and more aggressive malignancies, often harder to treat and more prone to metastasis.

Recently, personalized medicine has gained major interest in the field of anti-cancer immune-therapy and involves a patient-specific approach that can potentially give rise to a more efficient, more specific and more potent immune-activation[9]. Personalized medicine encompasses different approaches such as the use of more than one tumor-associated antigen to increase the potency of the vaccine, the implementation of neo-antigens or the incorporation of patient-derived cancer tissue.

In the case of using multiple tumor-associated antigens, a broader immune response is directed against a variety of known non-mutated antigens. In this way, loss of activity due to mutation or the lack of expression of one specific antigen[10,11] is a less detrimental factor as multiple antigens are formulated together. Despite these advantages, this approach is not universal because tumor-associated non-mutated antigens are unlikely to be relevant for every single patient and can also potentially encounter lower efficiency due to inhibition of the immune response caused by thymic tolerance.

Implementation of neo-antigens, on the other hand, involves formulation of proteins that are absent from the normal human genome and are created by tumor-specific mutations yielding patient-specific tumor antigens, different from shared non-specific cancer antigens[12, 13]. Neo-antigens are not subjected to thymic tolerance, in contrast to non-mutated self-proteins, because they are patient- and tumor specific and potentially evoke a more powerful T-cell response that is only restricted to tumor cells of the patient. Identification of neo-antigens however requires genomic analysis of patient derived tumor cells which is nowadays still costly, labor intensive and complex.

In contrast, incorporation of patient-derived cancer tissue is more straightforward, less complicated and preferentially induces a broad immune response against non-mutated cancer antigens as well as neo-antigens leading to a more robust, a more powerful and all-embracing cytotoxic T-cell response compared to the other personalized treatment strategies[14-16].

To induce a strong anti-tumor response, cross-presentation of cancer antigens by DCs to T-cells is required. Second, DCs need to be activated by the correct stimuli to further engage with CD4+ and CD8+ T-cells to skew TH1-driven and CTL-responses[2, 3, 17]. With regard to DC activation, this is achieved when pathogen-recognition receptors (PRRs) – present on cellular membranes and cytoplasm – are triggered by pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). The latter are endogenous factors of non-microbial origin, including heat shock proteins (HSPs), which originate from necrotic or stressed cells[18-20]. Therefore, strategies that can co-deliver tumor-associated antigens and activation stimuli to DCs *in vivo* are attractive in view of anti-cancer vaccination.

This chapter reports on a generic approach to encapsulate cancer cell lysates into calcium carbonate ($CaCO_3$) microparticles that are subsequently engineered with immune-stimulating cues to improve their immunogenicity as depicted in **Figure 1**. Calcium carbonate microparticles are known in a wide field of different applications and are used as templates for encapsulating of protein and peptide antigens[21-27]. $CaCO_3$ particles have a high loading capacity for macromolecules, are inexpensive, and easy to produce and exhibit high porosity, biocompatibility alongside with biodegradability[28-30]. Therefore, they are an appealing platform for vaccine formulation. Moreover, the synthesis is very mild and can fully be performed in aqueous medium without the need of any organic solvents, reactive chemistry or high energy input and only requires $CaCl_2$ and Na_2CO_3 which spontaneously form $CaCO_3$ microparticles. Performing the synthesis in the presence of macromolecules allows for the encapsulation of biomacromolecules into the porous interior of the microparticles while the surface of the microparticles can also be functionalized through adsorption of cationic polymers equipped e.g. with immune-activating cues.

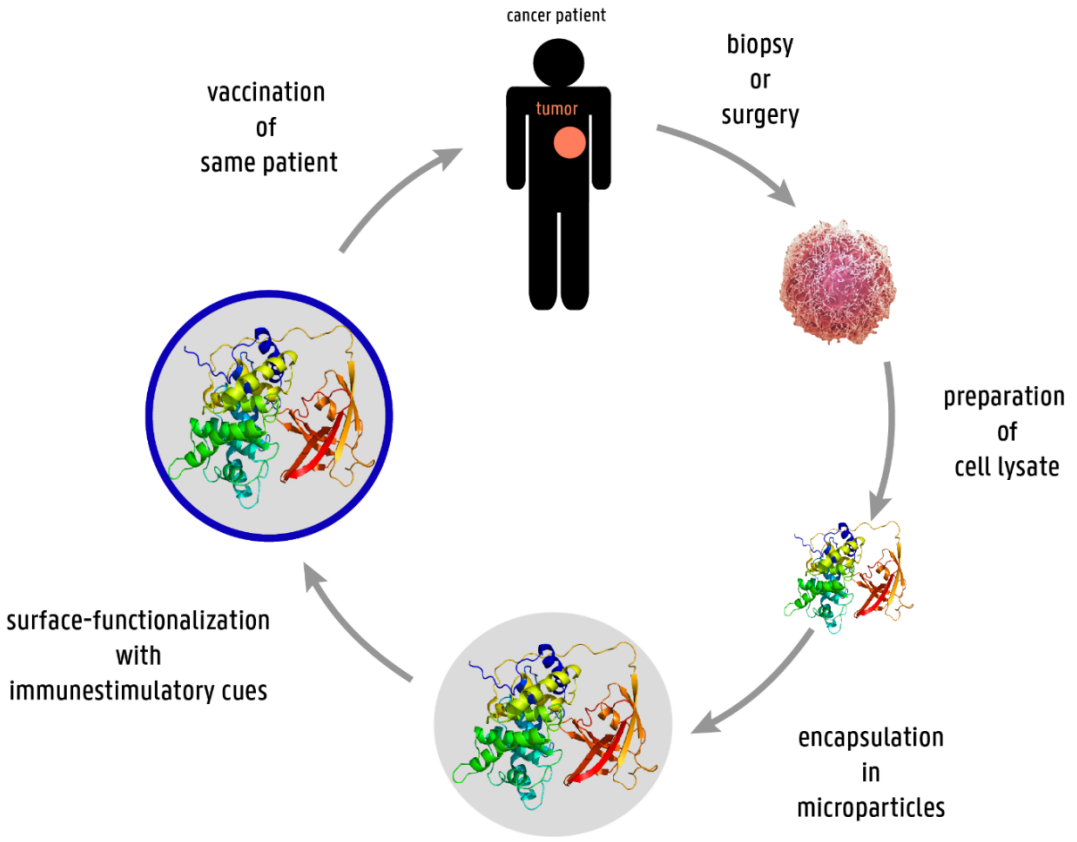

Figure 1. Schematic illustration of the vaccine approach for patient-specific antigen formulation into immune-modulating microparticles.

RESULTS AND DISCUSSION

1. Synthesis of antigen-loaded $CaCO_3$ microparticles

$CaCO_3$ microparticles were synthesized based on a procedure reported by Volodkin *et al.*[28] Initially we used ovalbumin (OVA) as model antigen. The latter was encapsulated by co-precipitation of Na_2CO_3 and $NaCl_2$ as reported earlier by De Koker *et al.*[21-23, 31]. Interestingly, we found that non-aggregated spherically shaped vaterite $CaCO_3$ microparticles (**Figure 2B-D**) could be reproducibly obtained when the mixing of the respective reaction components was performed on ice whereas at room temperature aggregate formation was commonly observed (**Figure 2A**). An OVA concentration of up to 1 mg/mL could be used while avoiding aggregation. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) gave further proof of successful microparticle formation. Interestingly, SEM imaging showed a clearly visible porous structure of the obtained microparticles. The loading efficiency of OVA into the $CaCO_3$

microparticles was determined by measuring the free (i.e. non-encapsulated) protein concentration in the supernatant by UV-VIS spectrophotometry to be 85 % (+/- 4.14).

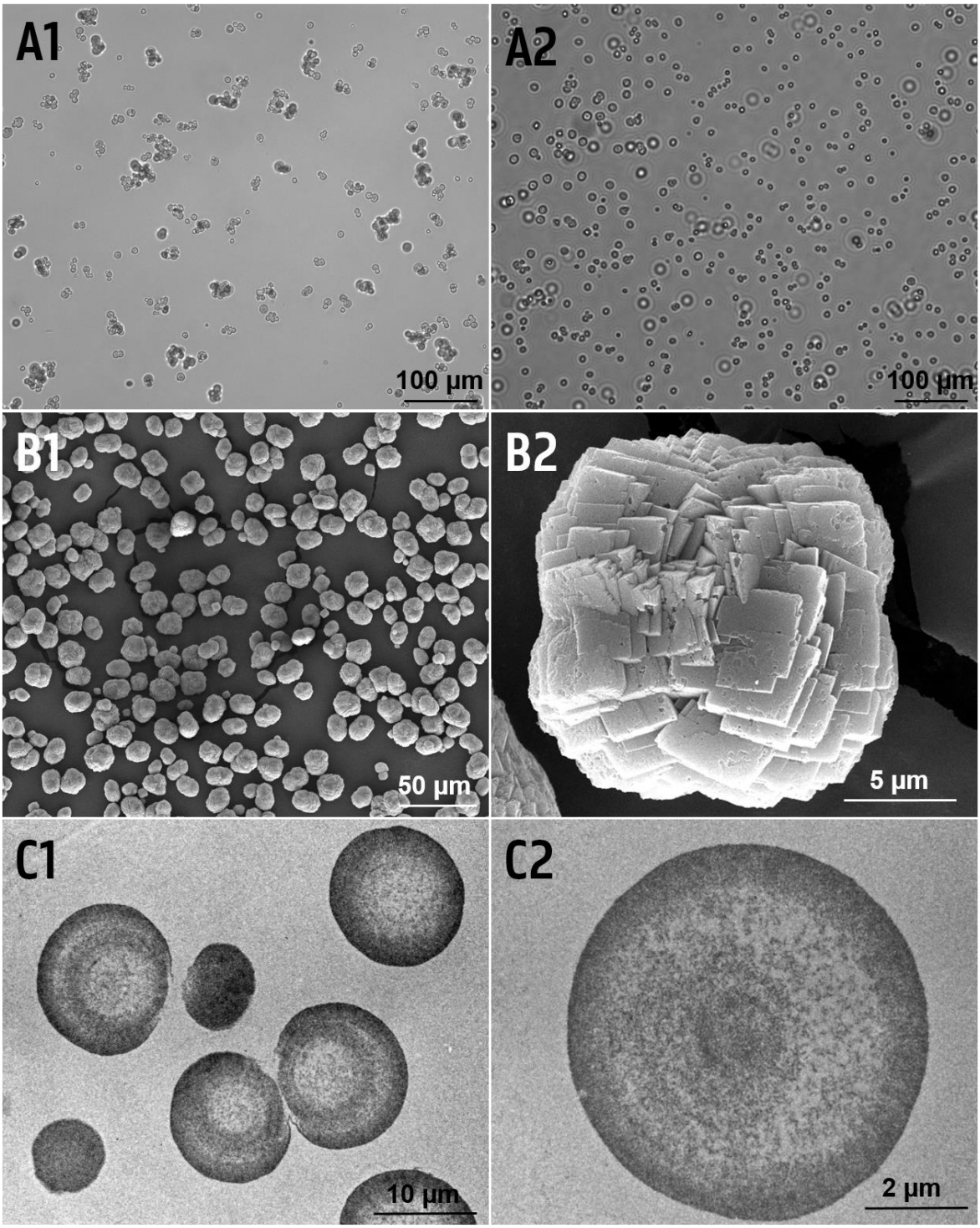

Figure 2. Optical microscopy imaging of $CaCO_3$ particles: (A1) aggregated particles (A2) non-aggregated particles – Electron microscopy (EM) imaging of the $CaCO_3$ microparticles: (B) Scanning EM images and (C) Tranmission EM images.

2. Polymer adsorption on antigen-loaded $CaCO_3$ microparticles

To engineer the $CaCO_3$ microparticle surface with molecular adjuvants – and thus allow for co-delivery of antigen and immune-stimulatory cues – we used a copolymer of N-(hydroxypropyl) methacrylamide (HPMA) and N-(3-aminopropyl) methacrylamide (APMA) composed of 80 HPMA and 20 APMA repeating units, synthesized by RAFT polymerization as earlier reported[32]. Further on in this chapter (*vide infra – Synthesis of a polymeric TLR7/8-agonist*), this polymer will be substituted with a small molecule TLR-agonist.

$CaCO_3$ microparticles were dispersed in deionized water at a concentration of 13 mg/mL and incubated with different concentrations of polymer (i.e. 0.5 – 50 mg/mL). Subsequently the microparticles were centrifuged and the amount of adsorbed polymer was determined by measuring the concentration of free polymer in the supernatant. As shown in Figure 3A, which expresses the percentage of polymer that is adsorbed onto the $CaCO_3$ microparticles, an increasing amount of polymer can be deposited up to 0.285 mg polymer (+/- 0.605) per 1 mg $CaCO_3$. Based on these results a 10 mg/mL polymer concentration was used for further experiments.

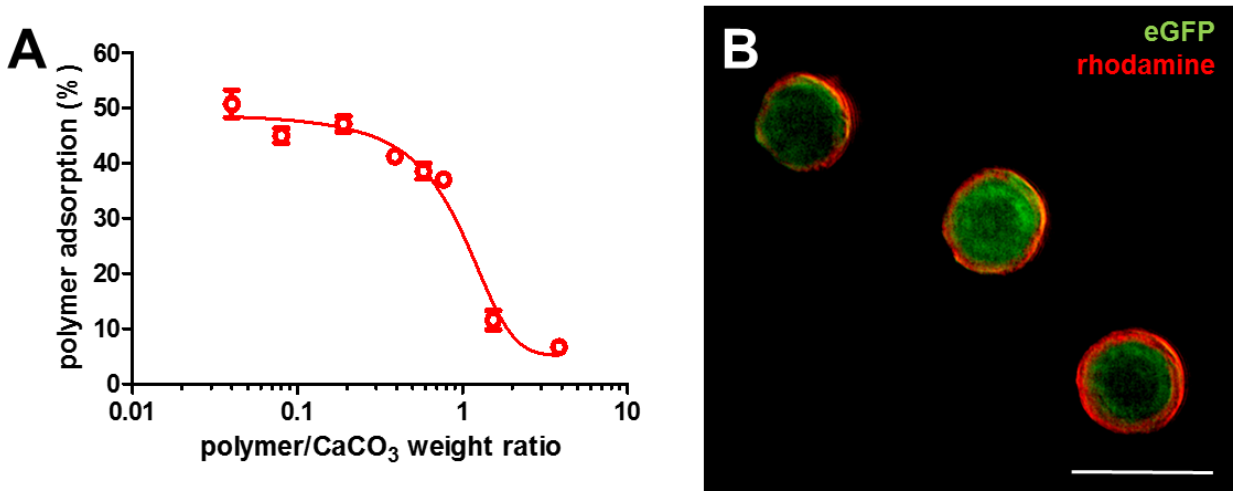

Figure 3. Adsorption of poly(HPMA-APMA) on antigen-loaded $CaCO_3$ particles: (A) Efficiency of deposition determined by UV-VIS. (B) Confocal image of the absorbed polymer layer (in red) on antigen-loaded (in green) particles. Scale bar represents 10 µm.

Zeta-potential measurements before and after polymer deposition indicated a charge reversal from -23.73 mV (+/-0.416) to 3.52 mV (+/- 0.586). The negative value of the OVA-loaded $CaCO_3$ microparticles can likely be attributed to the isoelectric point of 4.3 of OVA, meaning that it will

bear a net negative charge at neutral pH. Protonation of the primary amine groups of poly(HPMA-APMA) will confer a cationic charge and thus accounts for charge reversal upon polymer adsorption based on a combination of electrostatic interaction and physisorption. Polymer localization was visualized by confocal microscopy using poly(HPMA-APMA) that was labeled with rhodamine (**Figure 3B**). This clearly shows the presence of the adsorbed polymer layer on the surface of the $CaCO_3$ microparticles.

3. *In vitro* screening

Next, in a series of experiments, we investigated the interaction between the microparticles and DCs *in vitro*. For this purpose, we used bone marrow derived murine DCs and pulsed with microparticles loaded with AlexaFluor488-labeled OVA (1:50 ratio to unlabeled OVA) to allow for detection by flow cytometry and confocal microscopy. In these experiments we compared OVA-loaded microparticles with and without polymer coating with free soluble OVA. A dose-dependent increase in mean cellular fluorescence is observed (**Figure 4**), with a dramatic difference between soluble OVA and $CaCO_3$-encapsulated OVA. Furthermore, no significant influence of the polymer coating on mean cellular fluorescence was observed.

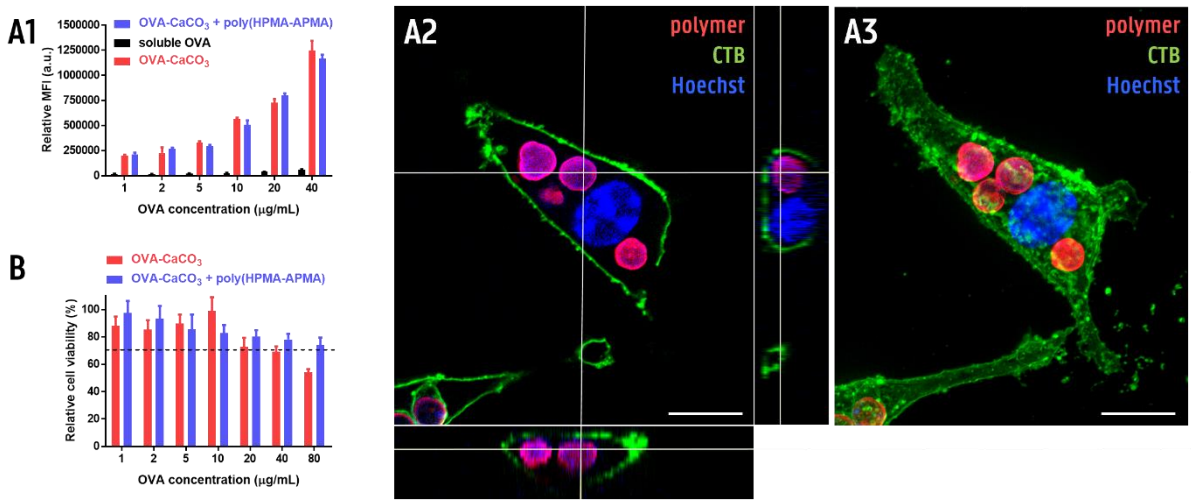

Figure 4. (A) *In vitro* uptake characterization of OVA-AF488 loaded $CaCO_3$ particles: (A1) Flow cytometry analysis and (A2-A3) confocal imaging: the cell membrane was stained with AF555-labeled cholera toxin B (CTB-AF555) and the cell nuclei were stained with Hoechst. Scale bar represents 10 µm (A2) shows one z-plane plus corresponding cross-sections (A3) shows the maximum intensity projection of the full z-stack. (B) Cell viability measured by MTT assay of DC2.4 cells pulsed with different concentrations of $CaCO_3$ microparticles, with and without polymer coating.

Both findings indicate that the particulate nature of the $CaCO_3$-encapsulated OVA and likely the fact that microparticles tend to sediment account for a better association of encapsulated relative to soluble OVA by DCs. Confocal microscopy gave further proof that the microparticles were internalized by DCs and were not merely bound to the cell membrane. MTT cytotoxicity assay showed only a moderate decrease in cell viability at higher particle concentrations up to 40 µg/mL OVA (corresponding to 0.3 mg/mL of $CaCO_3$), again with no influence of the polymer coating.

4. Synthesis of a polymeric TLR7/8-agonist

Microparticulate formulation of antigens is known to enhance CD8+ T-cell presentation but is insufficient to promote the induction of robust CTL-responses. Co-delivery with TLR-ligands has proven a viable approach to enhance immunogenicity of vaccine formulations with combination of multiple ligands to work synergistically[5, 18, 21, 33].

In this work we conjugated (2-(4-((6-amino-2-(butylamino)-8-hydroxy-9H_purin_9-yl)methyl)benzamido)acetic acid), further denoted as CL264, to poly(HPMA-APMA). CL264 is a small molecule TLR7/8-agonist that is the synthetic analogue of single stranded RNA which is the natural ligand of TLR7/8. TLR7/8-agonists are known to induce expression of type I interferons which are potent activators of a TH1-type immune response, known to be crucial for the induction of strong anti-tumor immune responses. Interestingly, we and others have recently shown that lipid-, polymer- and nanoparticle-conjugation of small molecule ligands strongly reduces systemic inflammation and yields potent lymph node localized responses that enhance the adaptive immune response against co-delivered antigens[34-36].

CL264 was conjugated to the primary amine moieties of poly(HPMA-APMA) via standard amidation chemistry using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholin-4-ium chloride (DMTMM) as coupling reagent (**Figure 5**). ^{1}H NMR and SEC analysis of the resulting polymer is shown in Supporting Information (**Figure S1**) and confirmed successful substitution of the polymer. The extent of conjugation calculated from ^{1}H NMR analysis was estimated at 14 %. This was confirmed via quantitative UV-VIS spectroscopy, indicating that 12.5 % of the free amine groups originating from APMA were substituted with CL264. This polymer will further be denoted as CL264-poly(HPMA-APMA).

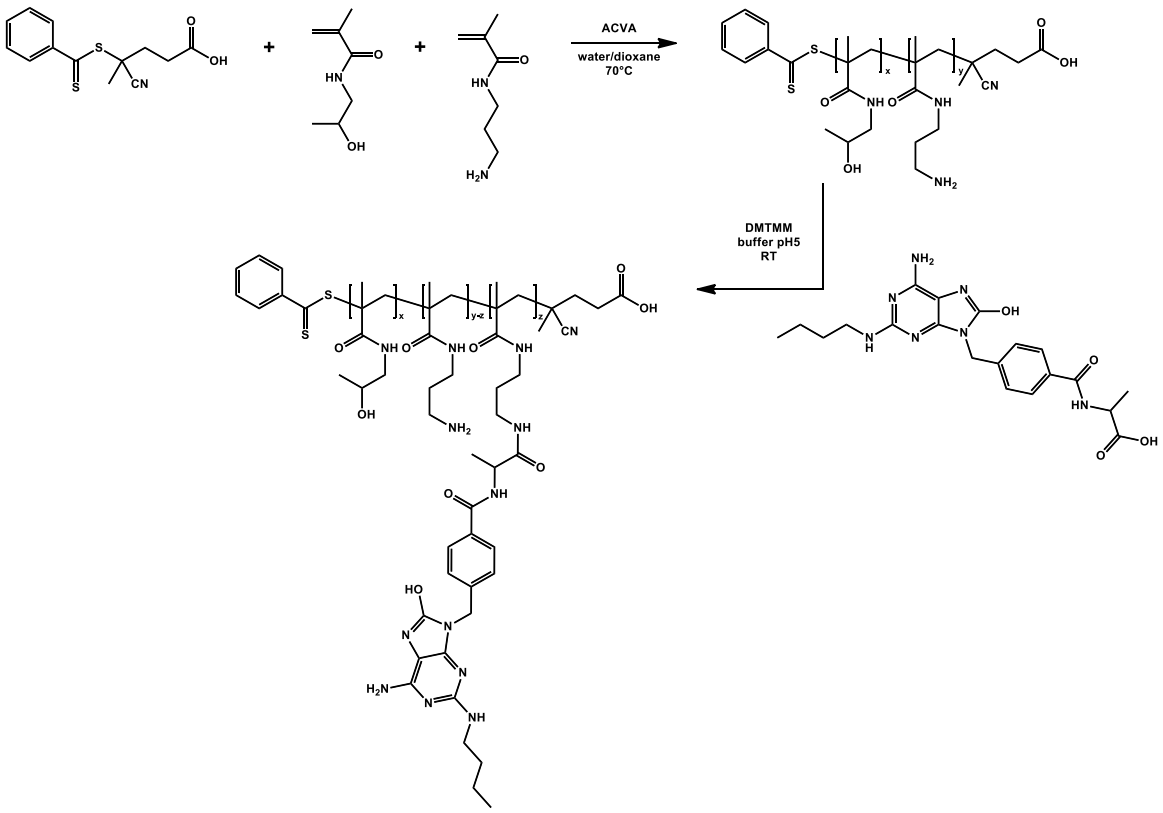

Figure 5. Synthesis of poly(HPMA-APMA) via RAFT polymerization and subsequent substitution of part of the APMA repeating units with the TLR7/8-agonist CL264.

Next, we investigated the influence of polymer-conjugation on the ability of the TLR-agonists to trigger receptor activation. For this purpose, we made use of RAW Blue TLR reporter cell line. The latter is derived from the murine RAW 264.7 cells with chromosomal integration of a secreted embryonic alkaline phosphatase (SEAP) reporter construct inducible by NF-κB and AP-1. Upon activation of the TLR-receptors, activation of NF-κB and AP-1 occurs leading to production of SEAP. The levels of SEAP can be easily monitored by spectrophotometry. RAW Blue cells were pulsed with increasing concentrations of soluble and polymer-conjugated TLR-agonist alongside unmodified polymer as control.

As shown in Figure 6A, the soluble TLR7/8-agonist CL264 and polymer-conjugated TLR7/8-agonist were potent activators of RAW Blue cells. A similar extent of activation was witnessed for both forms at concentrations starting from 0.5 µM, indicating that the polymer conjugation does not impair the binding of the TLR-agonist to its receptor above these concentrations. In contrast, no NF-kB activation could be observed for the polymer-conjugated TLR-agonist in concentrations below 0.5 µM. Note that blank polymer did not induce any activation.

To further support these findings, we investigated the effect of the soluble and polymer-conjugated TLR-agonists on the maturation of bone marrow derived DCs. For this purpose, DCs were pulsed overnight with a concentration range of the respective samples, followed by antibody staining against CD80 and analysis via flow cytometry. CD80 stimulates the CD28 receptor on T-cells, providing co-stimulatory signals for T-cell activation and skewing of TH1-immunity. The data obtained in these experiments (**Figure 6B**) followed the same general trends as observed in the RAW Blue activation studies. Whereas the blank polymers did not exceed the basal DC maturation level, both soluble and polymer-conjugated TLR7/8-agonist induced DC activation.

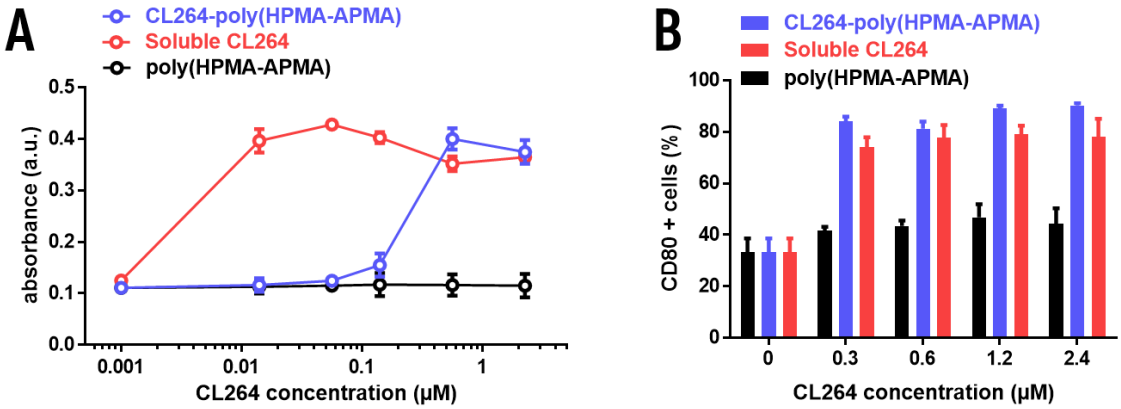

Figure 6. Screening of TLR-activation potency of CL264-poly(HPMA-APMA) on: (A) RAW Blue macrophages. (B) bone marrow derived DCs.

5. TLR-agonist engineering of antigen-loaded $CaCO_3$ microparticles

First we tested whether the presence of the TLR7/8-agonist on the polymer backbone influenced the adsorption behavior of the polymer onto OVA-loaded $CaCO_3$ microparticles. To do so, we repeated the experiment outlined in one of the previous sections (*vide supra – Polymer adsorption on antigen-loaded $CaCO_3$ microparticles*) and found no significant influence of the substitution of the primary amino groups of poly(HPMA-APMA) with the TLR7/8-agonist CL264. Data is summarized in **Figure S2** in Supporting Information. Interestingly, these findings point at the importance of electrostatic contributions due to the remaining cationic APMA moieties alongside non-electrostatic contributions, such as van der Waals interactions, on the adsorption behavior of the polymer onto $CaCO_3$ microparticles (*vide supra – Synthesis of a polymeric TLR7/8-agonist*).

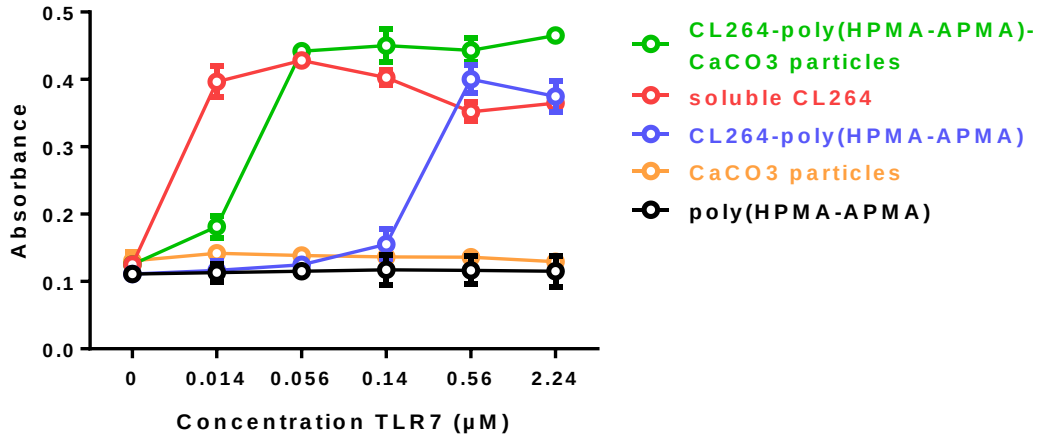

Figure 7. Screening of the potency of CL264-poly(HPMA-APMA) whether or not co-formulated with antigen-loaded $CaCO_3$ particles compared to the soluble TLR7/8-ligand via the RAW Blue assay.

Prior to determiniation of TLR-activation by polymeric TLR7/8-agonists adsorbed on $CaCO_3$ microparticles, we confirmed the absence of LPS contamination by a LAL assay (Supporting information, **Figure S3**). TLR-activation was then subsequently assessed via the RAW Blue reporter cell line. RAW blue cells were incubated with increasing concentrations of $CaCO_3$ microparticles engineered on their surface with polymeric TLR7/8-agonist up to a non-toxic particle concentration of 0.3 mg/mL. As shown in **Figure 7** the polymeric TLR-agonist could still trigger its receptor when adsorbed onto $CaCO_3$ microparticles. Interestingly, the TLR-ligand was more potent when adsorbed onto $CaCO_3$ microparticles than unbound. The increase in potency can be explained by the more efficient uptake by the RAW Blue cells of the TLR-ligand when adsorbed onto microparticles, which leads to enhanced interaction of the TLR-ligand with the receptor upon cell uptake. Note that as a control uncoated $CaCO_3$ particles were included which resulted in no TLR-activation.

6. Formulation of cancer cell lysate

As a model for patient-derived tumor cell lysate we used the Lewis Lung cancer cell line expressing ovalbumin (LLC.OVA). First, we compared the two most widespread techniques to induce cell lysis, i.e. osmotic shock in deionized water and repeated freeze-thaw cycles, in terms of efficiency with respect to the yield of free protein in solution. Bradford assay revealed that repeated freeze-thaw cycles yield a higher protein concentration which can likely be attributed

to the harsher circumstances the cells were exposed to compared to osmotic shock (Figure 8A1).

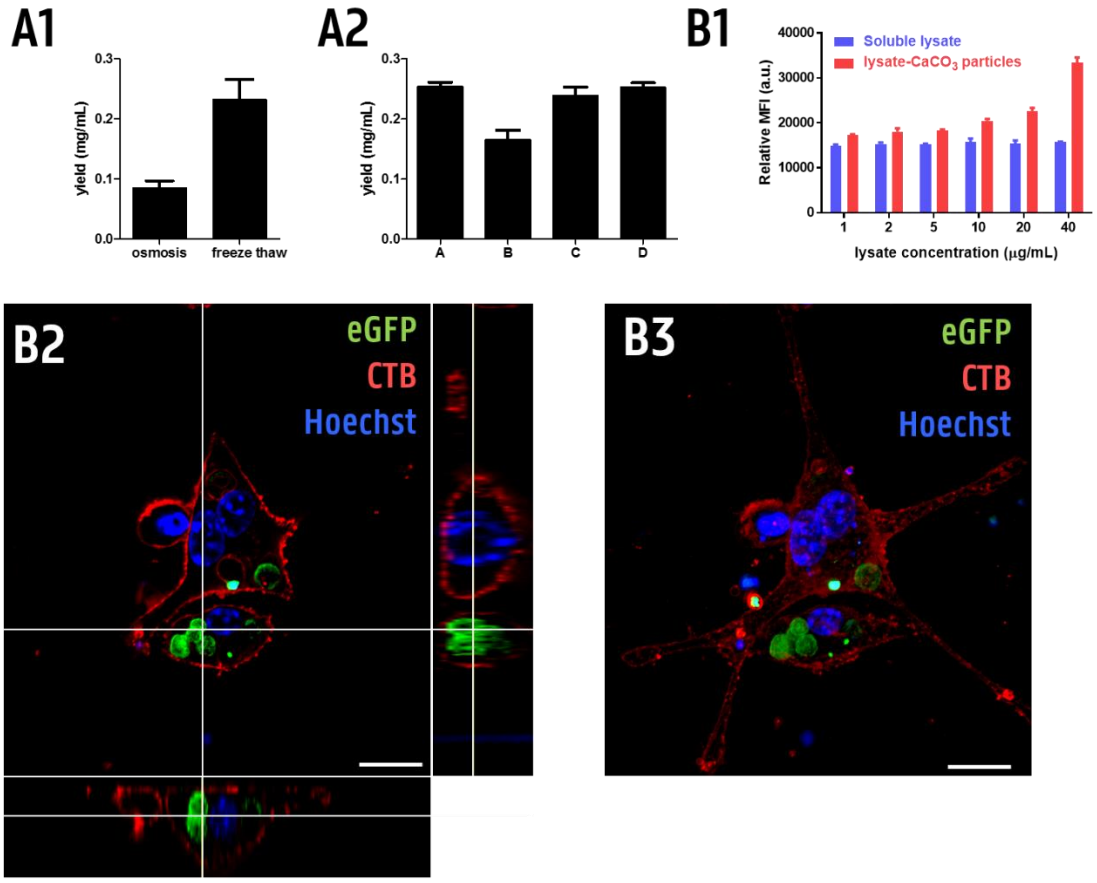

Figure 8. (A) Cell lysis protocol optimization by assessment of the extent of protein recovery in the final lysate measured via Bradford assay: (A1) osmosis compared to freeze thaw cycles and (A2) comparison of additional steps to increase protein recovery e.g. sonication (sample A) or not (sample B); followed by centrifugation (sample C); whether or not followed by filtration over a 100 µm filter (sample D). (B) *In vitro* uptake of lysate-loaded $CaCO_3$ particles by: (B1) flow cytometry analysis and (B2) confocal imaging. The cell membrane is stained with AF555-labeled cholera toxin B (CTB-AF555) and the cell nuclei are stained with Hoechst. Scale bar represents 15 µm.

Second, we assessed the influence of sonication and filtration on the amount of protein that can be recovered. After cell lysis by freeze-thaw, the samples were subsequently: sonicated (sample A); or not (sample B); followed by centrifugation (sample C); whether or not followed by filtration over a 100 µm filter (sample D). As shown in Figure 8A2, this revealed that sonication yields higher protein recovery potentially due to increased release of cellular contents caused by disruption of the cell membranes. Filtration after centrifugation did not have an additive value over centrifugation alone. Overall, we concluded six repetitive freeze

thaw cycles followed by sonication and centrifugation to be an optimal protocol for the preparation of cell lysate.

In a next step, the encapsulation efficiency of LLC.OVA lysate – prepared via the above outlined method – in $CaCO_3$ microparticles was assessed. UV-VIS analysis of the supernatant revealed an encapsulation efficiency of 77 % (+/-0.6), which was similar to the encapsulation efficiency obtained for soluble OVA (*vide supra – Synthesis of antigen-loaded $CaCO_3$ microparticles*). The presence of eGFP lysate inside the microparticles was visualized via confocal microscopy, shown in Figure 3B.

To investigate the influence of microencapsulation on the uptake efficiency of cell lysate by DCs, we prepared cell lysate of the enhanced green fluorescent protein (eGFP) expressing colon tumor-derived cell line (CT5.3-eGFP) to obtain fluorescent microparticles that can be tracked by fluorescence-based techniques. Flow cytometry demonstrated a clear dose-dependent uptake of cell lysate and importantly showed a dramatic increase in uptake efficiency of $CaCO_3$-encapsulated cell lysate compared to cell lysate in solution. Furthermore, confocal microscopy confirmed the presence of the fluorescent cell lysate loaded microparticles inside the DCs and not merely bound the cell membrane (Figure 8B).

To further explore the potential of the lysate-loaded $CaCO_3$ microparticles as vaccine carriers, we investigated whether the model tumor-associated antigen OVA can still be processed and cross-presented by DCs when the cell lysate is encapsulated in $CaCO_3$ microparticles. For this purpose, DCs (note that the DC2.4 cell line was used for this assay) were pulsed with a concentration range of respectively soluble and encapsulated LLC.OVA cell lysate. Subsequently, the DCs were stained with an antibody recognizing SIINFEKL, the OVA-CD8+ epitope, complexed to MHC class I H-2Kb molecules and analyzed by flow cytometry. As depicted in Figure S4, LLC.OVA lysate was not subjected to cross-presentation. In contrast, the results obtained for encapsulated lysate into $CaCO_3$ microparticles indicated enhanced cross-presentation. Further *in vivo* proof is however needed to investigate the therapeutic potential of this strategy.

CONCLUSION

In summary, we demonstrated that $CaCO_3$ microparticles hold potential as vaccine carriers for cancer cell lysates. $CaCO_3$ particles exhibited lower cytotoxicity and strongly enhanced cellular uptake of the cell lysate by DCs, leading to an improvement of the cross-presentation efficiency. In addition, the microparticles could be engineered with polymer-ligated TLR7/8-agonists to increase their immunogenicity. Future studies will focus to unravel the cytokine spectra that are induced by the TLR7/8-agonist and *in vivo* studies will need to be undertaken to assess the potential of our strategy to evoke tumor-specific immune responses.

SUPPORTING INFORMATION

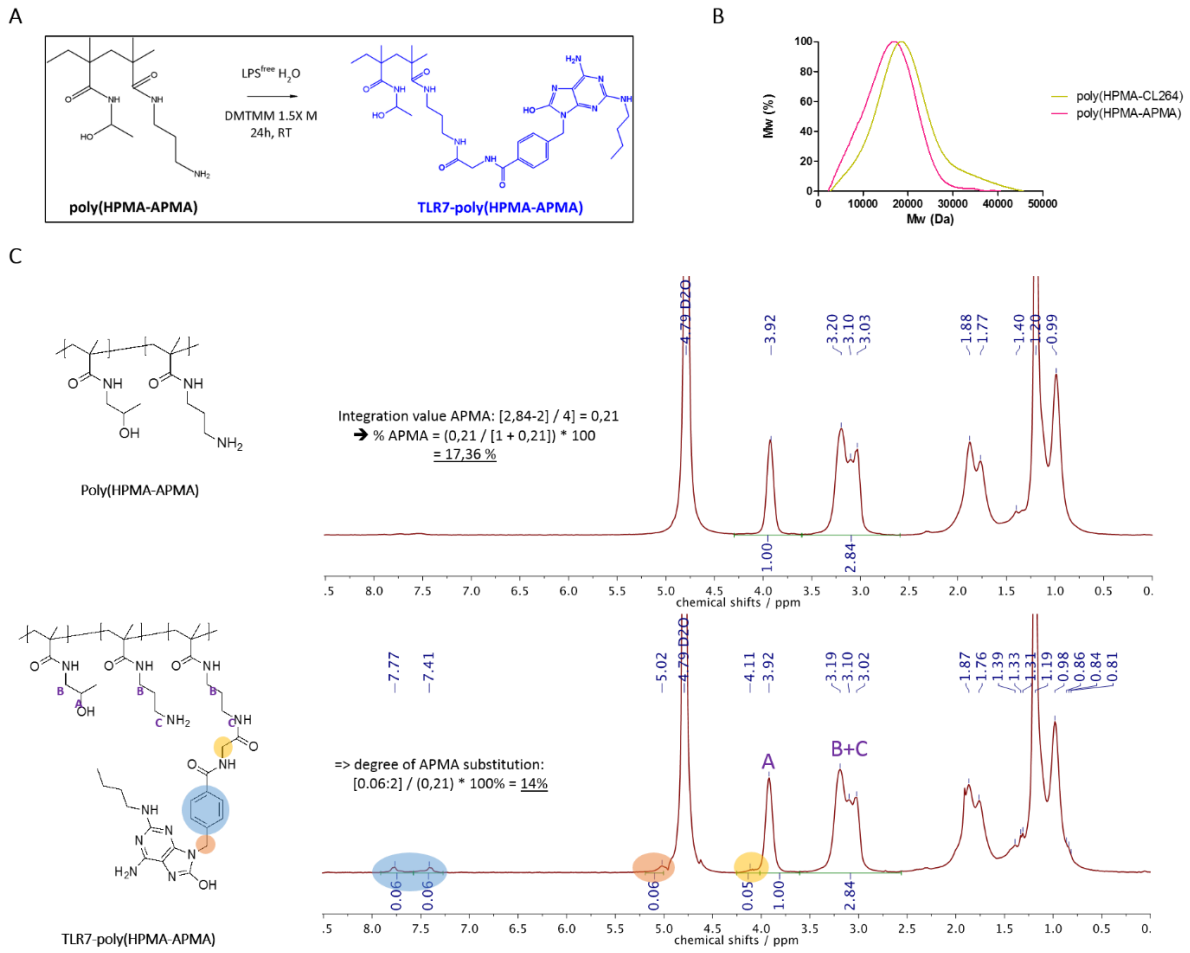

Figure S1. Characterization of the substitution of poly(HPMA-APMA) with the TLR7 agonist CL264 yielding TLR7-poly(HPMA-APMA): (A) Reaction scheme. (B) Size exclusion chromatography in DMA. (C) ^{1}H NMR analysis in D_2O.

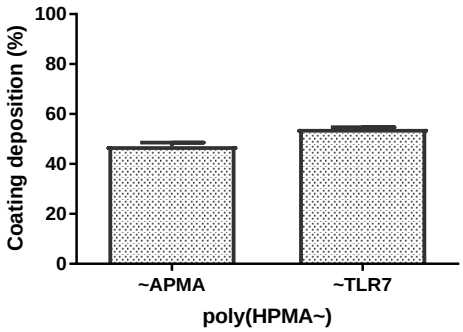

Figure S2. Coating deposition efficiency of poly(HPMA-APMA) on $CaCO_3$ particles substituted with the TLR7-agonist CL264 compared to unsubstituted poly(HPMA-APMA) determined by UV-VIS spectroscopy.

Figure S3. Endotoxin LAL assay result of the $CaCO_3$ particles and the separate components used for the synthesis.

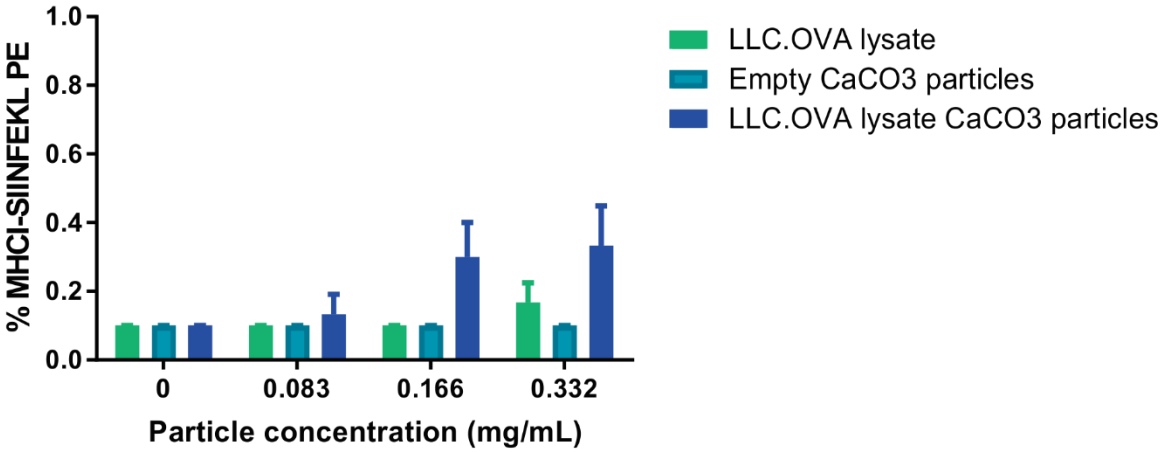

Figure S4. MHC-I presentation *in vitro* by dendritic cells of the lysate-loaded $CaCO_3$ particles.

EXPERIMENTAL SECTION

Materials. Calcium chloride ($CaCl_2$), sodium carbonate (Na_2CO_3), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent, sodium dodecyl sulfate (SDS), dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholin-4-ium chloride (DMTMM), succinic anhydride, NaOH, DMF, $NaHCO_3$ and paraformaldehyde (PFA) were obtained from Sigma Aldrich. Ovalbumin (OVA) was obtained from Worthington. Hydrochloric acid (HCl) 37 % v/v, rhodamine-isothiocyanate were purchased from Fischer Scientific. Dulbecco's Modified Eagle Medium (DMEM), RPMI 1640 medium, fetal bovine serum (EU qualified), penicillin/streptomycin (5000 U/mL), sodium pyruvate (100 mM), L-glutamine (200 mM), red blood cell lysis buffer, cell dissociation buffer (PBS based), PBS buffer (pH 7.2), Hoechst, cholera toxine B conjugates to AlexaFluor555 (CTB-AF555) and Zeocin were obtained from Invitrogen. Bradford reagent was obtained from Biorad whereas the pretreated Spectra/Por 7 dialysis membrane were purchased from Spectrumlabs. The anti-Mouse OVA257-264 (SIINFEKL) peptide bound to H-2Kb PE antibody and the anti-Mouse CD80 PE antibody were purchased from eBioscience and Quanti blue stain was obtained from Invivogen. Deuterated H_2O (D_2O) and d_6-DMSO were purchased from Deutero. The ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit was obtained from Genscript whereas the TLR7-agonist was purchased from Invivogen.

Immortalized cell lines

DC2.4 cell line. The DC2.4 cell line was a kind gift from Dr. Kenneth Rock (University of Massachusetts, Boston, US). The cells were cultured in RPMI medium supplemented with 10 % fetal bovine serum, 1 % penicillin/streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate and incubated at 37 °C with 5 % CO_2 saturation.

RAW Blue cell line. The RAW Blue cell line was purchased from Invivogen. The cells were cultured in DMEM medium supplemented with 10 % heat-inactivated fetal bovine serum, 1 % penicillin/streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate and 0.01 % Zeocin and incubated at 37 °C with 5 % CO_2 saturation.

LLC. OVA cell line. The LLC.OVA cell line was a kind gift from Prof. Karim Vermaelen (University of Ghent, Belgium). The cells were cultured in RPMI medium supplemented with 10 % fetal

bovine serum, 1 % penicillin/streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate and incubated at 37 °C with 5 % CO_2 saturation.

CT5.3-eGFP cell line. The CT5.3-eGFP cell line[37] was cultured in DMEM medium supplemented with 10 % fetal bovine serum, 1 % penicillin/streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate and incubated at 37 °C with 5 % CO_2 saturation.

Electron microscopy

Scanning electron microscopy. Scanning electron microscopy (SEM) was performed on a Quanta 200 FEG FEI instrument. Samples were deposited onto a silicon wafer and dried under a gentle nitrogen stream at ambient temperature. Prior to imaging, the samples were sputtered with a palladium/gold coating.

Transmission electron microscopy. Transmission electron microscopy (TEM) was performed on a JEOL 1010 instrument. Prior to imaging, samples were subjected to series of fixation (0.1 M Na cacodylate buffer (pH 7.2) containing 4 % paraformaldehyde and 2.5 % glutaraldehyde) and dehydration steps, embedded in epoxy resin and cut into ultrathin section using an ultramicrotome.

Synthesis $CaCO_3$ particles. The synthesis of calcium carbonate particles was based on Volodkin *et al*. In brief, 650 µL of a 1 M $CaCl_2$ solution and subsequently 650 µL of a 1 M Na_2CO_3 solution is added to 5 mL of a 1 mg/mL solution of ovalbumin (OVA) or a 1 mg/mL solution of cancer cell lysate under vigorous stirring for 30 seconds on ice. The resulting suspension is centrifuged at 300 G for 5 minutes at 4 °C followed by removal of the supernatant and two washing steps with deionized water. Samples were visualized on a Leica DM2500P microscope equipped with a 40X (NA 0.75) objective, DIC filters and a DFC360FX camera.

Encapsulation efficiency. Encapsulation efficiency of both OVA and LLC.OVA lysate was performed by UV–VIS spectrophotometry. The absorbance of the supernatant after synthesis of the particles was compared to a standard curve of OVA and LLC.OVA lysate respectively. The UV–VIS spectra were recorded on a spectrophotometer (Shimadzu UV-1650PC) in 1 cm × 1 cm quartz cells.

Conjugation of TLR7-ligand CL264 to poly(HPMA-APMA)

Synthesis CL264-poly(HPMA-APMA). Poly(HPMA-APMA), dissolved in LPS free water, was incubated with an equimolar amount of TLR7-ligand CL264 to APMA units overnight under continuous stirring at room temperature in the presence of 1.5 M excess of DMTMM. After 24 h incubation the reaction mixture was dialyzed against LPS free water (MWCO 3.5 kDa) for 1 day and lyophilized.

^{1}H-NMR and SEC analysis. The obtained polymers were all analyzed by ^{1}H-Nuclear Magnetic Resonance (NMR) and Size Exclusion Chromatography (SEC). NMR spectra were recorded on a Bruker 300 MHz FT NMR in D_2O. Chemical shifts (δ) are provided in ppm relative to TMS. SEC elugrams were recorded on a Shimadzu 20A system in dimethylacetamide (DMAc) as solvent containing 50 mM LiBr. The system was equipped with a 20A ISO-pump and a 20A refractive index detector (RID). Measurements were recorded at 50 °C with a flow rate of 0.7 mL/min. Calibration of the 2 PL 5 µm Mixed-D columns was done with poly(methyl methacrylate) (PMMA) standards obtained from PSS (Mainz, Germany).

Polymer deposition efficiency. After synthesis and two washing steps the particles were incubated with different concentrations of polymer for 5 minutes under continuous shaking to ensure optimal interaction. The particles were centrifuged at 300 G for 5 min. followed by measurement of UV absorbance of the supernatant compared to a standard curve of the respective polymer. The zeta potential was measured after two washing steps on a Zetasizer Nano S (Malvern Instruments Ltd., Malvern, U.K.) with a HeNe laser (λ = 633 nm) at a scattering angle of 173 °.

Fluorescent labeling of poly(HPMA-APMA). Poly(HPMA-APMA) was incubated with an equimolar amount of rhodamine-isothiocyanate in 0.1 M $NaHCO_3$ buffer overnight under continuous stirring. Subsequently the obtained mixture was dialyzed against deionized water for 3 days (MWCO 3.5 kDa) and lyophilized.

Isolation of bone marrow derived dendritic cells. Mouse bone marrow-derived DCs (bmDCs) were isolated by flushing femurs of C57BL/6 mice with complete RPMI with a 26 G needle. The cell suspension was filtered through a 100 µm cell strainer and incubated for 3−5 min in red blood cell lysis buffer on ice. The cells were subsequently seeded into a 24 well plate at a density

of 1.5 × 10^5 cells/ mL in complete RPMI containing 20 ng/mL of GM-CSF and incubated at 37 °C/5 % CO_2 for 7 days. To ensure optimal bmDC growth, fresh medium containing 20 ng/mL GM-CSF (provided by the VIB Protein Service Facility, Ghent, Belgium) was added on day 3, and on day 6 the medium was refreshed. On day 6 or 7, depending on the experiment, the bmDCs were isolated, seeded and pulsed with the test compounds.

MTT assay. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Bone marrow derive dendritic cells were seeded in 96 well plates at a density of 50000 cells/mL (total volume 100 µL) in six-fold on day 6. Subsequently, the cells were incubated with different concentrations of $CaCO_3$ particles and cultured for 24 h and for 48 h followed by addition of 40 µL of the MTT reagent (1 mg/mL). After an incubation period of 2–3 h the formed formazan crystals were dissolved in 100 µL of a 10 % m/v SDS/0.01 M HCl solution overnight protected from light. The absorbance was measured by a microplate reader at 570 nm. As a negative and positive control PBS buffer and DMSO respectively were added to the wells.

In vitro uptake assay. Bone marrow derived dendritic cells were pulsed at day 7 with fluorescent particles at different concentrations. After 24 h of incubation, the cells were dissociated using cell dissociation buffer followed by centrifugation for 5 min at 200 G at 0 °C. After resuspension, the samples were stored on ice and measured on a BD Accuri C6 flow cytometer. The data was analyzed using FlowJo.

Confocal microscopy imaging. DC2.4 cells were seeded at a density of 0.4 × 10^6 cells/mL in a glass bottom Will-co dish and incubated overnight. Next, the fluorescent particles were added, incubated for 24 h, and fixated in a 2 % paraformaldehyde solution for 10–15 min. The cells were subsequently washed and simultaneously stained by CTB-AF555 and Hoechst for 1 h at room temperature. Finally the samples were washed with PBS and imaging on a confocal microscope (Leica DMI6000 B inverted 241 microscope) equipped with an oil immersion objective (Zeiss, 63 ×, 242 NA 1.40) and attached to an Andor DSD2 confocal scanner.

In vitro MHC-I presentation assay. DC2.4 cells were seeded at a density of 0.2 × 10^6 cells/mL in a 24 well culture plate and incubated overnight followed by incubation with different concentrations of the samples. After 48 h at 37 °C the positive control (SIINFEKL) was added in

triplicate in a concentration of 1 µg/mL, 1 hour prior to staining with SIINFEKL-MHC-I PE-labeled antibody for 30 minutes on ice protected from light. Subsequently the samples were centrifuged for 5 minutes at 200 G at 4 °C, resuspended in PBS and analyzed with flow cytometry.

In vitro RAW Blue assay. RAW Blue macrophages are seeded in a 96 well round bottom plate at a density of 0.5 x 10^6 cells/mL and immediately pulsed with the desired concentrations of the test compounds in six-fold. As a negative control PBS is added. After 24 h incubation, 50 µL of the supernatant is transferred into a 96 well flat bottom plate and incubated with 150 µL of Quanti blue solution. After 3 to 6 h incubation at 37 °C the color change absorbance is measured with a plate reader at 620-655 nm.

In vitro BM-DC maturation assay. The bone marrow derived dendritic cells are pulsed on day 6 with different concentrations of the test compounds in triplicate and PBS or DMSO as negative control. After 24 h incubation the cells are dissociated with cell dissociation buffer followed by centrifugation for 5 minutes at 200 G at 4 °C and resuspension in 50 µL of antibody cocktail. After 30 minutes of incubation on ice protected from light, the samples were centrifuged (5 minutes, 200 G) at 4 °C, resuspended in PBS and subsequently measured by flow cytometry.

LAL test. A Limulus Amebocyte Lysate (LAL) assay was performed to verify that samples were not contaminated with endotoxins. The Toxinsensor Chromogenic LAL Endotoxin Assay Kit is performed according to the manufacturer's instructions. In brief, the samples are diluted based on the maximum valid dilution (MVD) in order to overcome interference of the test compounds while still allowing detection of the endotoxin limit. A standard curve is prepared by dissolving the lyophilized endotoxin standard in LAL reagent water and diluting the resulting stock in 5 concentrations ranging from 0.01 to 0.1 endotoxin unit per mL. Further on, all samples and standard are incubated with the LAL lysate for 40 to 60 minutes at 37 °C, followed by addition of the chromogenic substrate and incubation for 6 minutes at 37 °C. Next, the reaction is stopped by addition of the color stabilizers #1, #2 and #3. Finally the samples are measured at 545 nm by UV-VIS spectrophotometry.

Lysate preparation. First, the cells are suspended in deionized water at a density of 20 x 10^6 cells/mL and sequentially lyzed via different methods. On the one hand, the cell suspension is

frozen in liquid nitrogen and thawed in a warm water bath at 37 °C in six fold or incubated for 60 minutes on ice to allow osmotic lysis, on the other. Then, the samples are sonicated for 10 seconds followed by 50 seconds rest on ice in six fold, centrifuged for 5 minutes at 2000 G at 4 °C. The supernatant was subsequently isolated and lyophilized.

Bradford assay. The efficiency of the lysate preparation protocols is determined via measuring the protein content in the supernatant after cell lysis. Therefore, serial dilutions were prepared of a Bovine Serum Albumin (BSA) standard solution (2 mg/mL). The Bradford reagent was prepared following the manufacturer's instructions. In brief, the reagent was diluted 1 to 5 with deionized water followed by filtration over a Whatmann #1 filter. Next, 10 µL of the standards and the test samples were transferred in a 96 well plate and incubated with 200 µL of the Bradford reagent for 10 minutes on ice and measured at 590 nm with a multiplate reader.

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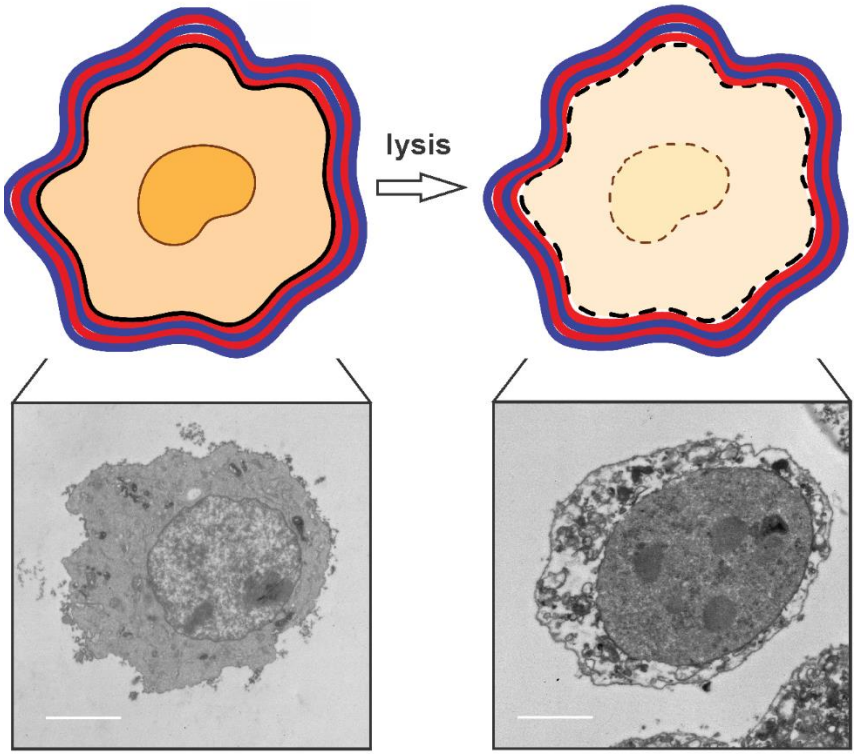

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Bio-hybrid tumor cell templated capsules: A generic formulation strategy for tumor-associated antigens in view of immune-therapy

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CHAPTER 5

BIO-HYBRID TUMOR CELL-TEMPLATED CAPSULES

ABSTRACT

For the development of effective anti-cancer vaccines, tumor-associated antigens need to be internalized by antigen presenting cells alongside specific co-stimulatory signals. Interestingly, relative to soluble antigens, nano- and micro-particulate antigens are much better presented to CD8+ T-cells, a crucial step in the induction of cytotoxic T-cells that can eliminate malignant cells. In this regard, a strategy to encapsulate cancer cell-derived proteins into a particulate delivery system would be of high interest. Here we present a versatile approach to incorporate cancer cell proteins into polymeric capsules using the cells themselves as templates for Layer-by-Layer assembly of complimentary interacting species. After coating, the cells are killed by hypo-osmotic treatment leading to bio-hybrid capsules loaded with cell lysate. Particular focus is devoted in this chapter on choosing the optimal coating components and conditions to maximize cell membrane integrity during the coating process, to minimize pre-mature protein release and to achieve optimal encapsulation of cell lysate upon lysis of the cells. To further underline the potential of our approach, we demonstrate that heat shock proteins, important immune-activators, can be induced and encapsulated into the bio-hybrid capsules.

INTRODUCTION

Despite major effort in cancer research, there remains a great need for more specific and targeted therapies to combat metastatic disease and to reduce the need for traditional chemo- and radiotherapies that are both prone to serious side effects[1-3]. Anti-cancer immune-therapy involves priming the patient's own immune system to recognize and eliminate malignant cells[4]. To achieve targeted treatment, cancer antigens have to be internalized by dendritic cells and presented to CD8+ T-cells in combination with the appropriate cytokine spectrum and co-stimulatory signals[1,2,5-10]. Recent advances at the interface between immunology and materials chemistry have elucidated that formulating protein-based antigens into particulate carriers in the range of 50 nm – 10 µm strongly promote cross-presentation by DCs to CD8+ T-cells[9-15]. These findings provide a clear rationale to design strategies for the delivery of antigens in particulate form to DCs.

Unfortunately, encapsulation strategies for tumor-associated antigens are limited as antigens of many cancer types are still unidentified on the one hand and are also prone to continuous mutation on the other[16,17]. To overcome these limitations, we detail an approach to encapsulate tumor-associated antigens by templating a synthetic membrane onto the surface of cancer cells followed by lysis of the cancer cells retaining cell lysate within the hollow void of the obtained capsules (**Figure 1A**). Cell encapsulation has not yet been used for anti-cancer immune-therapy but has found applications in several biomedical applications including tissue engineering and diabetes treatment[18-21]. Current progress on implementing whole cell lysates as anti-cancer vaccine is mostly based on *ex vivo* DC therapy, involving electroporation of DCs derived from the cancer patient's own blood monocytes[22,23]. This is a highly costly, labor-intensive procedure and does not take advantage of the physiological stimuli that occur in direct *in vivo* vaccination and therefore urges towards viable alternatives[4,24].

By employing *in vivo* targeting of autologous tumor cell lysate, the patient's individual tumor-specific and/or tumor-associated antigens can be delivered to the immune system in an immunogenic fashion, which should enable the induction of broader immune responses specifically tailored to the patient's unique tumor mutanome[16,17]. However, one might argue that immunization of cancer patients with a vaccine containing autologous tumor cell lysate could lead to induction of autoimmunity against cellular proteins that are shared with normal

cells. However, this has, at least to our knowledge, not yet been reported in both preclinical and clinical studies[25-28].

Layer-by-Layer (LbL) assembly of complementary interacting components is an attractive technique to deposit a semi-permeable membrane on the surface of non-planar substrates[29.] It allows an easy, all-aqueous mild encapsulation of a wide variety of species, mostly polymeric or inorganic template particles that are used to design hollow capsules. Several studies have already been reported on LbL coating of cells mainly focusing on encapsulation of living yeast cells or bacteria[20,30-32]. However these microbial cells are more robust due to their rigid cellular wall whereas mammalian cells are more fragile[33]. Here we present the LbL encapsulation of murine melanoma B16.F10 cells, as model cancer cell line, aiming at high preservation of the cell membrane integrity and good protein retention contained within the deposited multilayer coating.

RESULTS AND DISCUSSION

1. Screening the interaction between cells and oppositely interacting species

Due to the overall negative charge of the cell membrane, we attempted at first to coat B16.F10 melanoma cells based on electrostatic interaction of the oppositely charged polyelectrolytes poly-L-arginine (P_LARG) (**Figure 1B1**; polycation) and dextran sulfate (DEXS) (**Figure 1B2**; polyanion). This choice is based on our previous work where we showed multilayer capsules composed of these polyelectrolytes are biocompatible, degradable *in vitro*[34] and *in vivo*[35] and induce broad cellular and humoral immune responses against encapsulated antigen[36-38]. However, whereas in the form of a polyelectrolyte complex poly-L-arginine is not inducing acute cytotoxicity[39], we observed that incubation of live cells in a poly-L-arginine solution at a relevant concentration for Layer-by-Layer assembly (i.e. 0.1 to 1 mg/mL in isotonic HEPES buffer) induces instantaneous aggregation, cell lysis and cell death (*vide infra – Figure 2 and Figure 3*).

Therefore, we were prompted at investigating an alternative for electrostatic assembly to assure a better preservation of cell viability during cell coating. In this regard, hydrogen bonding is attractive because it involves non-ionic species and particularly hydrogen bonded thin films

composed of neutral charged hydrophilic polymers such as e.g. poly(vinylpyrrolidone) (PVP, **Figure 1B3**) and tannic acid (TA; **Figure 1B4**) which have recently attracted interest[31,32,40]. Although PVP and TA form strong complexes, PVP/TA multilayers have been reported to gradually disassemble over time, likely due to oxidation, and are therefore an attractive system for delivery purposes[41,42]. Additionally we have recently reported the design of porous microparticles based on TA/PVP via spray drying[43]. These particles were used to encapsulate protein antigens and we successfully demonstrated that the antigens, after cellular uptake, could still be processed together with a dramatic increase in cross-presentation. This suggests that in intracellular conditions, proteases are still granted access to the payload that is firmly entrapped within the particles under extracellular conditions. Tannic acid is a safe food-grade compound that is applied in biomedical applications including hemostatic coatings, nano-capsules and cell encapsulation. Besides via hydrogen-bonding, tannic acid has also shown its capability to form thin films via multiple mechanisms[44,45]. PVP is a non-ionic polymer that is approved by the FDA for pharmaceutical applications.

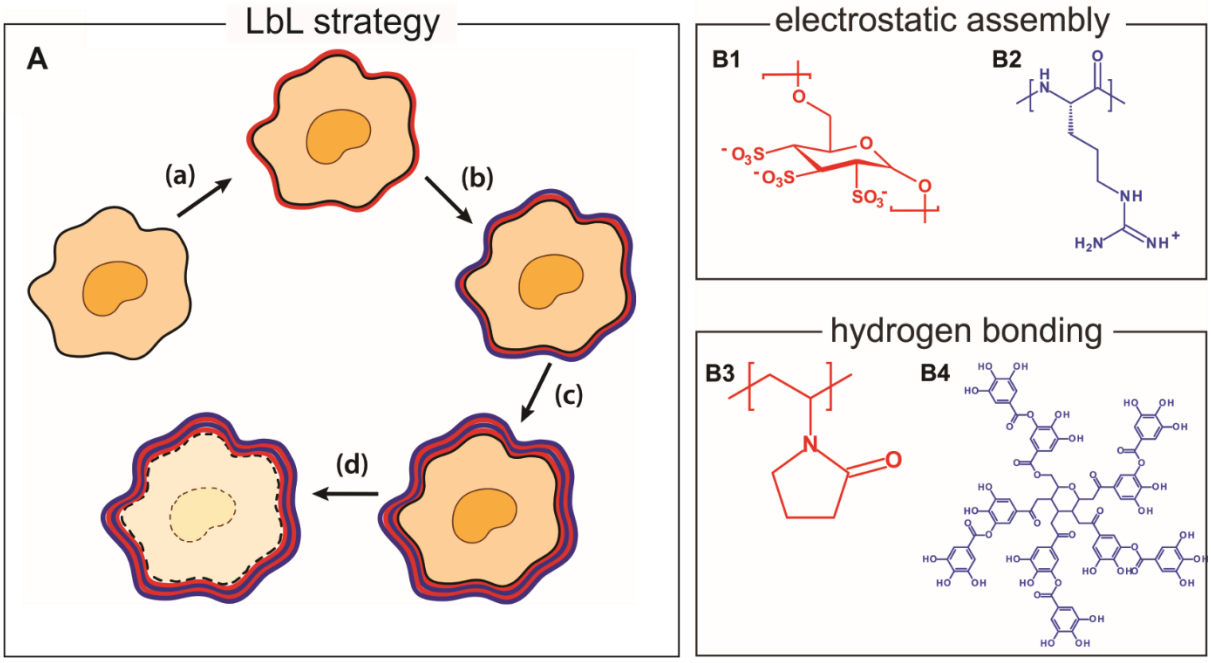

Figure 1. Schematic representation: **(A)** of the design of LbL-coated bio-hybrid cancer cell-templated capsules with alternating layers (step a-c) of either the electrostatically interacting polyelectrolytes dextran sulfate **(B1)** and poly-L-arginine **(B2)** or poly(N-vinylpyrrolidone) **(B3)** and **(B4)** tannic acid that interact via hydrogen bonding followed by lysis of the cells upon hypo-osmotic treatment (step d).

As the aim of our work is to encapsulate whole cancer cells, it is important to preserve cell integrity as much as possible while affecting cell viability as little as possible in order to retain a maximum amount of cellular proteins within the LbL coating. Therefore, in a first series of experiments, we investigated the effect of incubating cells in isotonic aqueous solution of respectively DEXS, P_LARG, TA and PVP on cell integrity and cell viability. For this purpose B16.F10 cells were incubated in 1 mg/mL HEPES-buffered solutions of either P_LARG or DEXS while on the other hand the B16.F10 cells were incubated in PBS buffered 1 mg/mL solutions of either TA or PVP. HEPES buffer is used to solubilize DEXS and P_LARG as the latter is not soluble in PBS due to ionic crosslinking of the cationic guanidinium moieties of the P_LARG by the trivalent phosphate anions of the PBS buffer. To avoid active phagocytosis of these components during the coating process, all handlings were performed on ice to block energy-dependent internalization pathways.

The effect of the respective test components on cell viability and membrane integrity was subsequently assessed by inline flow cytometry (FACS) in presence of a live/dead stain (**Figure 2**). This technique comprises continuous sampling/measuring of a cell suspension while adding the test component of choice at a certain point to probe for the effect on cell viability. In addition, the flow cytometry observations were supported by visualization of the cytotoxicity by staining the B16.F10 cells with calcein-AM (C-AM) and propidium iodide (PI). C-AM is a cell-permeable dye that is converted by esterases into a non-cell-permeable state upon cellular uptake. As such, it strongly stains live cells. PI intercalates with double-stranded DNA, as found in the cell nucleus, but is cell impermeable. PI will thus only stain the nuclei of cells that have a damaged cell membrane and is therefore used as a probe for cell membrane integrity.

The gating strategy applied to analyze the inline flow cytometry experiments is shown in **Figure 2A**. Here we deliberately did not gate out (by plotting FSC-A vs. FSC-H and SSC-A vs SSC-H) doublets or multiplets (i.e. clusters of multiple cells instead of single cells) thus allowing us to probe possible induction of cell aggregation upon addition of the test component to the cells during the flow cytometry measurements. The first gate (forward scatter versus side scatter)

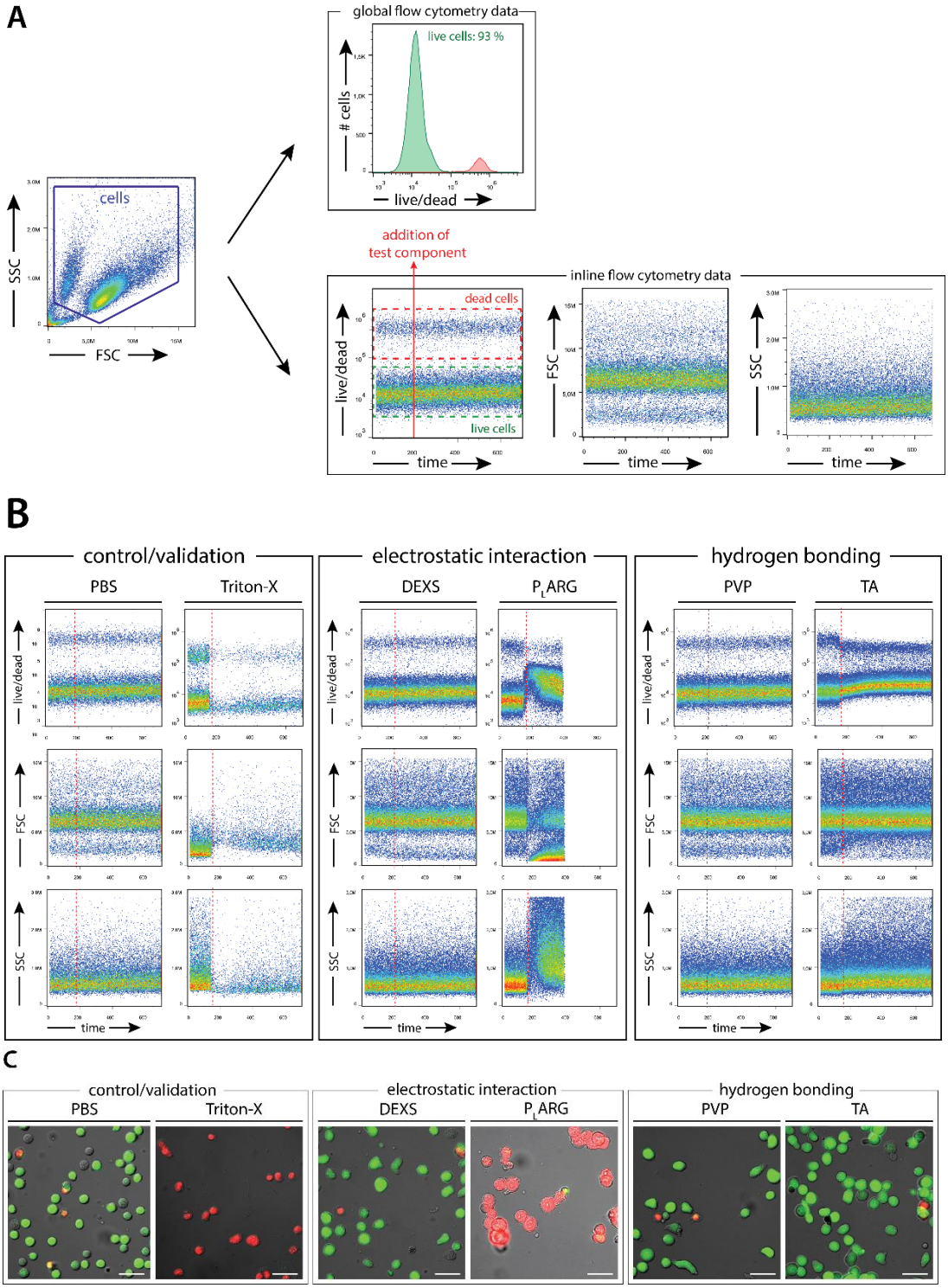

Figure 2. (A) Flow cytometry gating strategy for determination of B16.F10 cell viability offline and inline. **(B)** Inline flow cytometry data representing the response to the addition of the respective test components in the FL-3 fluorescence channel (live/dead staining and the forward (FSC), respectively side (SSC) scatter channels. The dashed red line indicates the time points at which the test components were added. **(C)** Fluorescence microscopy images of B16.F10 cells staining with C-AM/PI after 10 min. incubation with the respective test components on ice. Overlay of the DIC, green and red fluorescence channels. Scale bar is 50 µm.

serves to select both live and dead cells and to exclude cell debris. These two populations are marked by respectively a green (live cells) and a red (dead cells) dashed rectangle. In this regard, as shown in the second gate, it is important to realize that when performing *in vitro* cell culture experiments, there is always a small amount of dead cells present. The next step in the gating strategy is plotting the fluorescence channel used for the detection of live/dead staining (i.e. FL-3) in function of time. To gather additional information on the cellular response to the addition of the respective test components, we also plotted the forward (FSC) and the side (SSC) scatter channel as function of time. The panels B in Figure 2 represent the evolution over time of the live/dead signal of cells present in the gate that contains both live and dead cells. The measurement was started at time point zero and the test components were added after 2 minutes. To validate the set-up, we used PBS and Triton-X (i.e. a detergent that solubilizes the cell membrane) as controls. PBS is expected to not affect the cell viability, while Triton-X should immediately kill the cells. Indeed, the inline flow cytometry plots in Figure 2B, do not indicate any alternation in the signal in function of time when PBS is added. By contrast, Triton-X dramatically changes the response in FL-3, FSC and SSC channels combined with a strong decrease in numbers of events immediately after addition of Triton-X, indicating massive cell death and cell lysis.

Subsequently, we evaluated the influence on cell membrane integrity upon addition of the components that are of interest for Layer-by-Layer coating. DEXS did not influence the fluorescence signal. This is also confirmed by the corresponding fluorescence microscopy images in Figure 2C, recorded from an identically treated cell suspension as for the inline flow cytometry. Clearly, the majority of the cells exhibit strong green fluorescence by C-AM staining and only a few are stained red by PI. By contrast, addition of P_LARG causes an immediate distortion of the fluorescence and scattering signals. Observation of clogging of the tubing due to aggregating cells (evidenced by the sharp decrease in FSC and increase in SSC signal), forced us to abort the measurements. The corresponding fluorescence microscopy images (Figure 2C) confirmed the aggregation and the nuclear staining with PI suggests that the majority of the cells have indeed lost their membrane integrity. When the inline flow cytometry experiments were performed with TA or PVP, no loss of cell viability/integrity was observed by either flow cytometry or microscopy. These data point out the superior performance of TA and PVP, compared to polyelectrolytes, to encapsulate cells in a polymeric multilayer coating while

maintaining membrane integrity as much as possible and affecting cell viability as little as possible. Remarkably, in case of TA, a slight increase in the fluorescence signal from the live cell population was noted whereas the dead cell population slightly decreased in signal upon addition of this component. Moreover, the FSC and SSC signals appear slightly altered. Although it is most likely that tannic acid interacts via hydrogen bonding with cell surface proteins or the live/dead stain, the exact reason for this subtle shift in fluorescence remains unclear.

Next, we also assessed the cellular viability/integrity with (common offline) flow cytometry (Figure 2A) comprising live/dead measurements after deposition of the test components and two washing steps to remove non-adsorbed material (Figure 2A). However, one might argue that in case cells completely lose their integrity or in case cells are lost during the multiple steps of pipetting, washing and centrifugation, they can no longer be stained by any of the dyes used in the previous experiments (i.e. live/dead, C-AM and PI). By consequence, these would neither be included in the amount of dead cells nor in the total cell number, and thus give rise to an overestimation of the cell viability. In order to address this issue we performed a MTT cell viability assay on B16.F10 cells that were incubated for 10 min on ice with the respective test components. MTT assay probes for the metabolic activity of cells by measuring the enzymatic conversion (which can only by performed by viable cells) of the substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide into the purple-colored formazan. As such, relative to a blank control, this assay quantifies the percentage of live cells taking into account the possible lost cells due to induced lysis by the test component or the multiple washing/centrifugation steps the cells are exposed to. Figure 3 summarizes these measurements and compares the values obtained by live/dead staining and MTT assay. Clearly, the observed trends correspond well for both techniques, with only a slightly lower cell viability/integrity measured by MTT assay. Besides being independent of fully disintegrated cells and a small amount of cells that were aspirated when pipetting during washing and centrifugation, another difference between the live/dead measurement and the MTT assay is that the the latter requires, after pulsing and removal of the test component, an additional 2 h incubation period of the cells with the MTT substrate. As such, the cell viability/integrity measured by MTT assay probes for longer-term effect than the online FACS live/dead assay that depicts the immediate cellular response to the respective test compounds. Deeper investigation into this phenomenon is beyond the scope of this work. However, generally we

can conclude that the inline flow cytometry set-up is a straightforward method to assess the instantaneous cellular response to a specific test component.

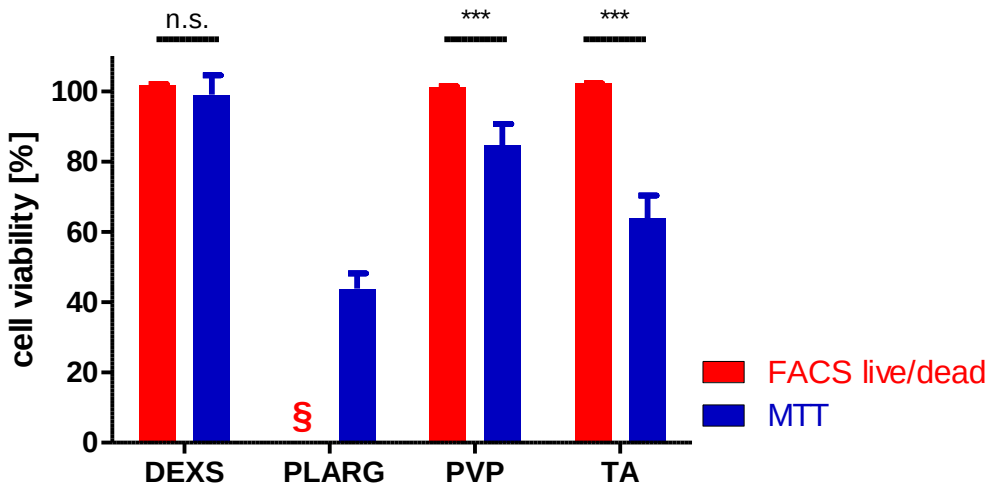

Figure 3. Cell viability/integrity, measured via MTT and FACS live/dead assay, measured after 10 min incubation of B16.F10 cells on ice with the respective test components. n=6. ***: $p < 0.001$. §: FACS live/dead analysis was not possible due to massive cell aggregation.

2. Characterization of the LbL coating

Based on our initial flow cytometry and microscopy findings, we next attempted to coat the B16.F10 cells with a multilayer film by alternated assembly of TA and PVP. For this purpose, cells were sequentially incubated in 1 mg/mL solutions of respectively TA or PVP in PBS buffer. Also in these experiments, all handlings were performed on ice to block energy-dependent internalization pathways. After deposition of each layer, the cells were centrifuged and washed two times with PBS buffer to remove non-absorbed material. An important aim in this chapter is to measure the effect of these handlings on cell viability and more importantly on cellular integrity. Therefore, before dispersion of the cells in the respective coating solutions, cell viability/integrity was monitored by optical microscopy (C-AM/PI staining), FACS (live/dead staining) and MTT assay.

As repeated incubation on ice, pipetting, washing and centrifugation steps might have an effect, we also performed control experiments by subjecting cells to the same regime of handlings, but fully in PBS instead of using the respective coating components. The evolution of the cellular integrity/viability (monitored by either MTT or FACS live/dead) of these control groups

is represented by the dashed curves in Figure 4A. FACS live/dead assay does not point out any significant cell death during repeated washing/centrifugation, whereas MTT assay does indicate a significant loss. Again, as discussed in the previous paragraph, this can likely be attributed to the longer time that is required to perform the MTT assay, to the small fraction of cells that is aspirated during the centrifugation/washing cycles and to possible induced cell lysis by the test component. In view of these findings future research will focus on novel strategies, thereby reducing loss of cell viability and membrane integrity, that allow one-step coating of cells based on novel concepts that were recently introduced into the field of colloidal engineering[44,46].

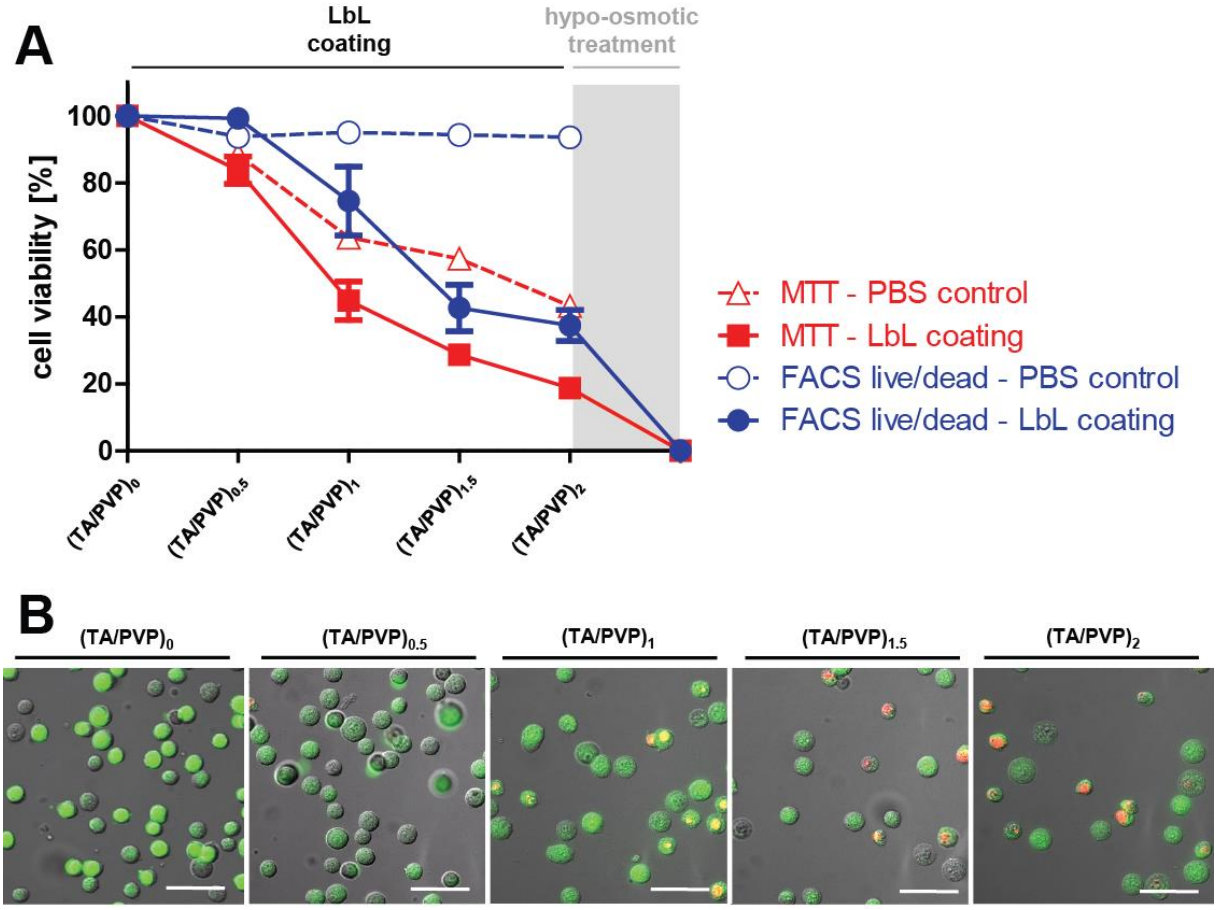

Figure 4. (A) Cell viability, measured via MTT and FACS live/dead assay, measured after each deposition cycle during LbL coating of B16.F10 cells. The images are an overlay of the DIC, green and red fluorescence channels. N=3. (B) Fluorescence microscopy images of B16.F10 cells staining with C-AM/PI after each deposition cycle during LbL coating of B16.F10 cells. The images are an overlay of the DIC, green and red fluorescence channels. Scale bar is 50 µm.

The non-dashed curves in Figure 4A, represent the evolution of cell viability/integrity during LbL coating of the B16.F10 cells and indicate a gradual decrease as function of time. MTT and FACS

live/dead assay show a similar trend that is confirmed by the microscopy images, as shown in **Figure 4B**, indicating increasing amounts of dead cells when depositing more layers. Importantly, no massive cell lysis or aggregation is observed during LbL coating of the cells. **Figure 4A** also represents the effect of a hypo-osmotic treatment on the cell viability/integrity. Previous studies on LbL coating of living cells describe cell survival of approximately 80 % up to 95%.[32, 47]. Our results show however, a dramatic reduction in cellular viability/integrity after deposition of 2 bilayers. We assume this can be attributed to the different approach we elaborated on in our present work to assess the effect of LbL coating on cell integrity. So far in literature either yeast cells or bacteria, both having a rigid cell wall, have been used for LbL coating or cell viability/cell membrane integrity has been monitored solely after deposition of the entire LbL coating[32, 47, 48]. The latter thus excludes the extent of cell death or lysis that occurs during the coating process itself, which appears, at least in our findings, to play an important role.

3. Assessing the influence of the starting layer on cellular integrity

Next, we also aimed at investigating the effect of the starting layer (i.e. TA or PVP) on cellular integrity. Interestingly, when the Layer-by-Layer coating was initiated from PVP rather than TA, maintenance of the membrane integrity was improved as measured both by flow cytometry and MTT assay (**Figure 5A**). This suggests that using TA as starting material for cell-templated LbL assembly gives rise to an increased cytotoxicity upon further LbL coating. Although PVP is known for its non-fouling behavior, exhibiting only very low interaction with the cell surface, we observed that starting Layer-by-Layer assembly with PVP afforded a better preservation of the membrane integrity during the coating process. The possibility of decreased penetration of the MTT dye should however be considered due to increasing thickness and strength of the polymer coating that is deposited on the cells. In this respect, we were interested in gaining further insight in how the cell surface is affected by incubation of the cells with a bilayer coating either initiated with PVP or TA, by transmission electron microscopy (TEM). For this purpose, cells were coated with a $(PVP/TA)_2$ or a $(TA/PVP)_2$ coating, fixated and embedded in epoxy resin. **Figure 5B** shows the TEM images recorded from ultrathin sections cut from the epoxy-embedded cells. These images clearly demonstrate the formation of an electron dense layer surrounding the cells and thereby confirm successful deposition of the coating on the cell surface. Interestingly, when comparing cells coated with a $(PVP/TA)_2$ versus a $(TA/PVP)_2$

membrane, a dramatic difference in morphology of the resulting cell-templated capsules is observed. In case of $(PVP/TA)_2$ coated cells, the electron dense coating was confined to the surface of the cells, whereas in case of a $(TA/PVP)_2$ coating, electron dense material was found to be spread throughout the interior of the capsules as well (indicated by the red arrows).

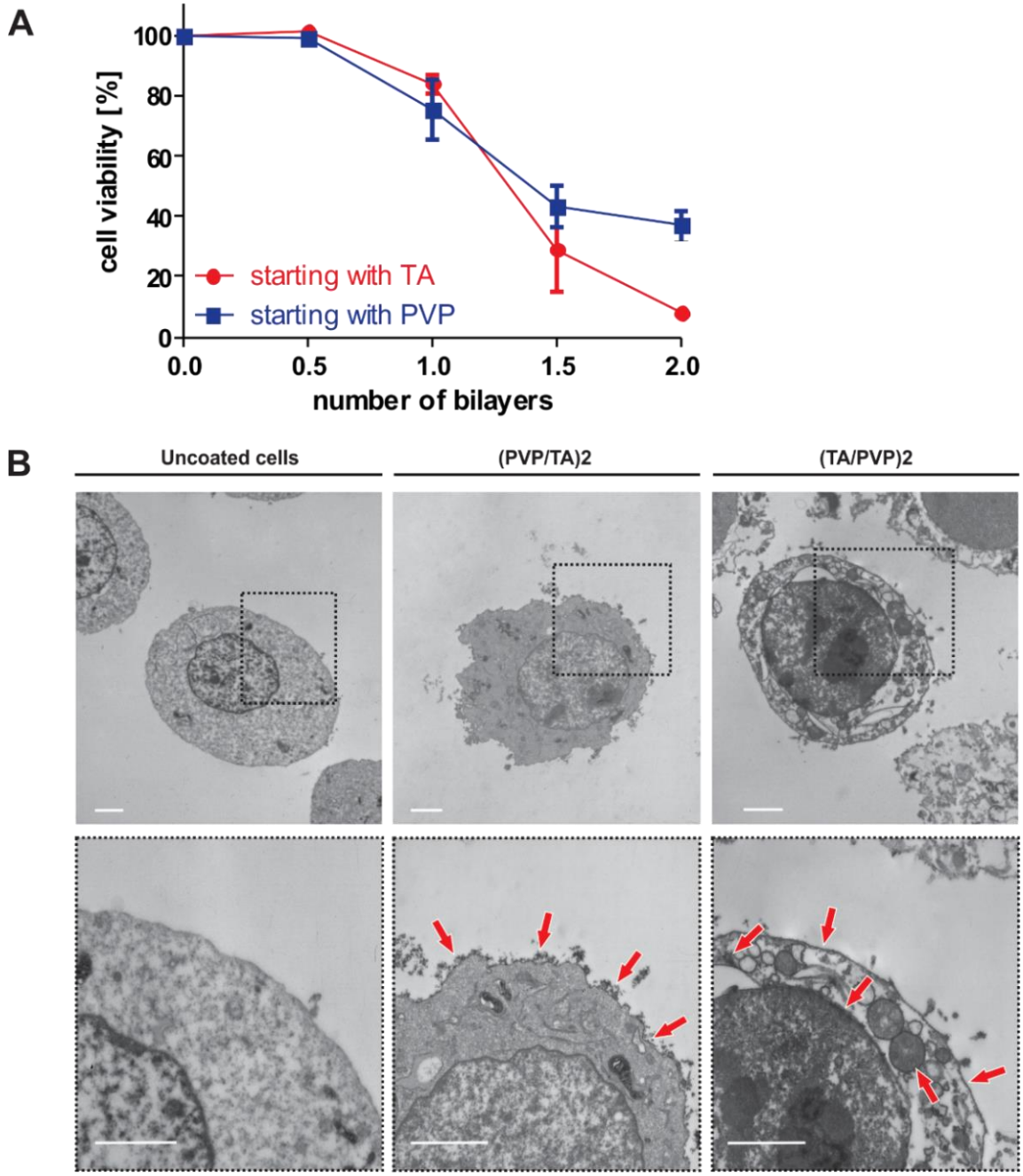

Figure 5. (A) Cell viability, measured by a FACS live/dead assay, after each deposition cycle during LbL coating of B16.F10 cells. LbL coating was started with either TA (red curves) or PVP (blue curves). N=3. **(B)** TEM images of B16.F10 cells coated with 2 bilayers initiated with either TA or PVP compared with uncoated cells. The dashed rectangles show a zoomed area. Scale bar is 2 µm. The red arrows indicate the LbL coating.

These findings corroborate the MTT data and the live/dead flow cytometry data, suggesting that TA partially crosses the cell membrane thereby creating pores in the cell membrane and thus enabling the uptake of the live/dead dye PI. In contrast, when cells are first incubated with PVP, only a coating on the cell surface is deposited, suggesting that adsorption of PVP onto the cell surface in a first step prevents cellular infiltration of TA in subsequent steps of the cell-coating process.

To gain further high resolution morphological insight into the cellular response to TA and PVP during Layer-by-Layer coating of the B16.F10 cells, TEM images (Figure 6) were recorded after deposition of each layer, either starting LbL assembly with PVP or TA. When LbL coating is started with TA, the morphology of the cells changes already after addition of the first TA layer, witnessed by the presence of an abnormal amount of vesicles (orange arrows). This indicates immediate cellular toxicity, likely attributed to tannic acid that crosses the cell membrane. In addition, the extent of swollen ER (blue) and mitochondria (yellow arrows) gradually increases as function of the number of deposited layers. Finally, after two TA/PVP bilayers, the cells exhibit a high amount of lysosomes (green arrows), i.e. degeneration remnants of the mitochondria, along with a high extent of degeneration of the cytoplasm and the cell nucleus (purple arrows). This can probably be attributed by the presence of additional pores in the plasma- and nuclear membranes together with the presence of the electron dense PVP/TA material (red arrows) on the cell surface and inside the cell.

In contrast, when the LbL coating is started with PVP, the cells exhibit a normal morphology after deposition of the first (i.e. $(PVP/TA)_1$) bilayer as the morphology of the cells appears to be unchanged compared to uncoated cells. However, during the position of the third layer (i.e. $(PVP/TA)_{1.5}$) the cell morphology starts to change, witnessed by the presence of swollen endoplasmic reticulum (ER), swollen mitochondria and lysosomes, indicated by respectively blue, yellow and green arrows. After deposition of two PVP/TA bilayers, the cellular degeneration process appears less pronounced than when LbL coating was started from TA and no PVP/TA complexes are observed inside the cells. However, also in this case, an increased amount of abnormal cell structures together with initial degeneration of the cytoplasm and the cell nucleus (depicted by the purple arrows) are observed. These findings support our previous

observations on membrane integrity (Figure 5A), where we observed a gradual increase of PI uptake in function of the alternating deposition cycles.

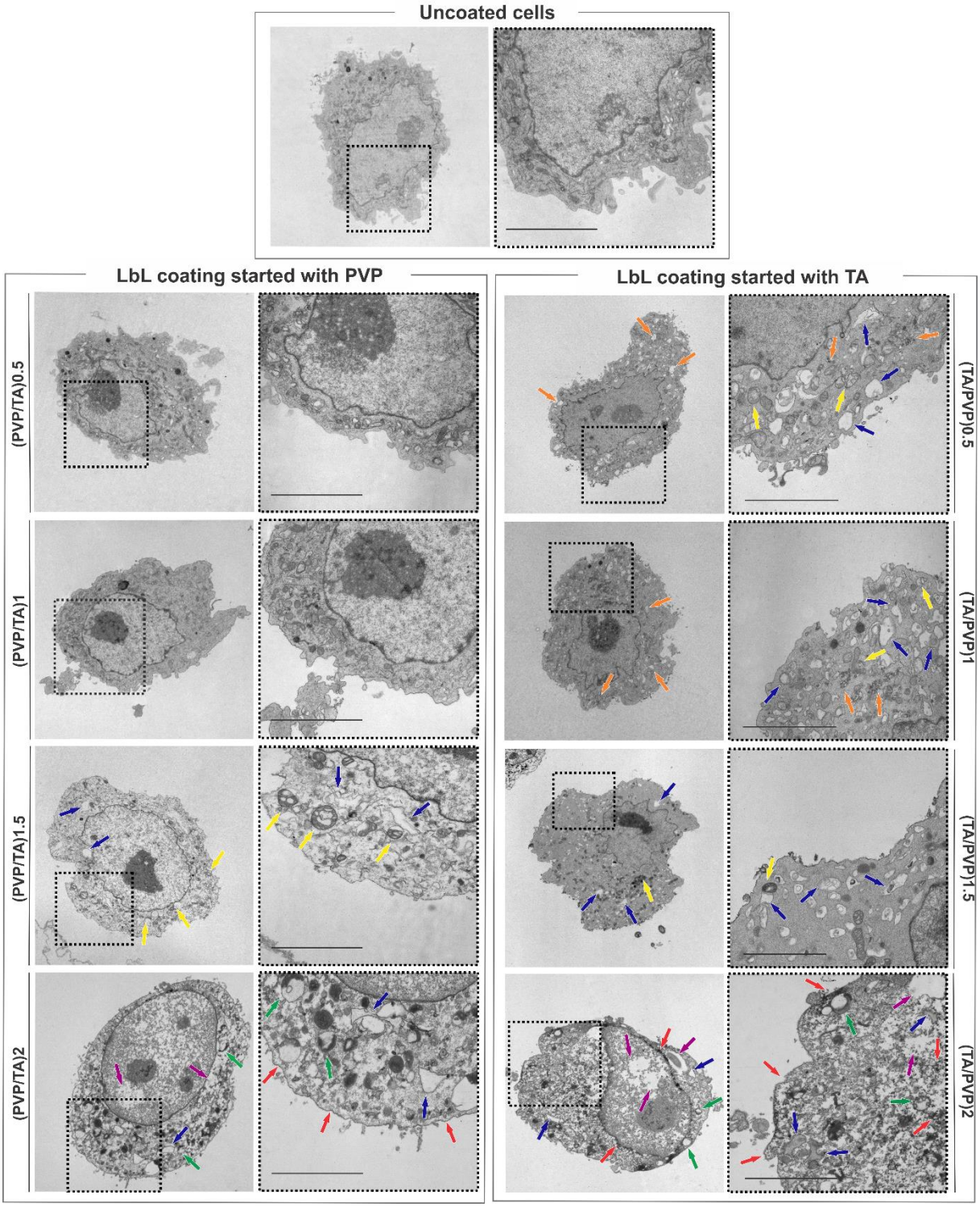

Figure 6. TEM images of B16.F10 cells coated with 1 to 4 alternating layers of PVP and TA, either started with PVP (left panels) or TA (right panels). The dashed rectangles show a zoomed area. Scale bar is 3 µm. The arrows indicate different cell structures: Orange = vesicles, Yellow = swollen mitochondria, Blue = swollen ER, Green = lysosomes, Purple = cell degeneration. The red arrows indicate the LbL coating.

4. Determination of the optimal amount of layers for maximum protein retention

After establishing the feasibility of coating B16.F10 cells with a PVP/TA coating, starting with PVP as a first layer, we aimed at investigating the number of layers that is required to minimize protein release from the bio-hybrid capsules after lysis of the coated cells. For this purpose, after deposition of each layer, we exposed the cells to a hypo-osmotic medium by incubating them in deionized water. This treatment is intended to induce cell death in a facile and elegant way without addition of specific chemicals. As shown in **Figure 4A**, both MTT and FACS live/dead assay prove that the hypo-osmotic treatment effectively kills the cancer cells as no viable cells were detected anymore by both techniques. The effect of this treatment on cell integrity (i.e. by C-AM/PI staining) and on the morphology of the bio-hybrid capsules was subsequently evaluated by fluorescence microscopy (**Figure 7A**). In addition, the diffusion of cellular proteins through the multilayer membrane upon exposure to hypo-osmotic treatment was measured by gel electrophoresis (SDS-PAGE) (**Figure 7B**). The latter was performed by centrifuging the coated cells after each deposition cycle followed by analysis of the supernatant by gel electrophoresis. In general, the optimal amount of layers is considered as the number of layers that provide a stable capsular structure upon cell lysis, accompanied by minimal release of the cell lysate through the multilayer membrane.

From the fact that all cells became stained by PI, shown in **Figure 7A**, one can conclude that the cells are efficiently killed and lose their plasma membrane integrity upon hypo-osmotic treatment, independent of the number of deposited layers. However, only if the multilayer coating was composed of at least 3 layers (i.e. $(PVP/TA)_{1.5}$), the cells retained their spherical morphology after hypo-osmotic treatment. SDS-PAGE (**Figure 7B**, middle panel) performed on the supernatant of centrifuged hypo-osmotic treated cells revealed that a two bilayer coating (i.e. $(PVP/TA)_2$) allows maximum retention of cellular proteins within the capsules without further improvement when additional layers were deposited. The encapsulation efficiency obtained with two bilayers of PVP/TA was considered to be high compared to the initial amount of cellular proteins before coating (results not shown).

Next, we performed SDS-PAGE on the pellet of centrifuged LbL-coated cells that were exposed to hypo-osmotic treatment in order to determine to which extend proteins are encapsulated in the bio-hybrid cell-templated capsules. In this regard, we verified that $(PVP/TA)_2$ cell-

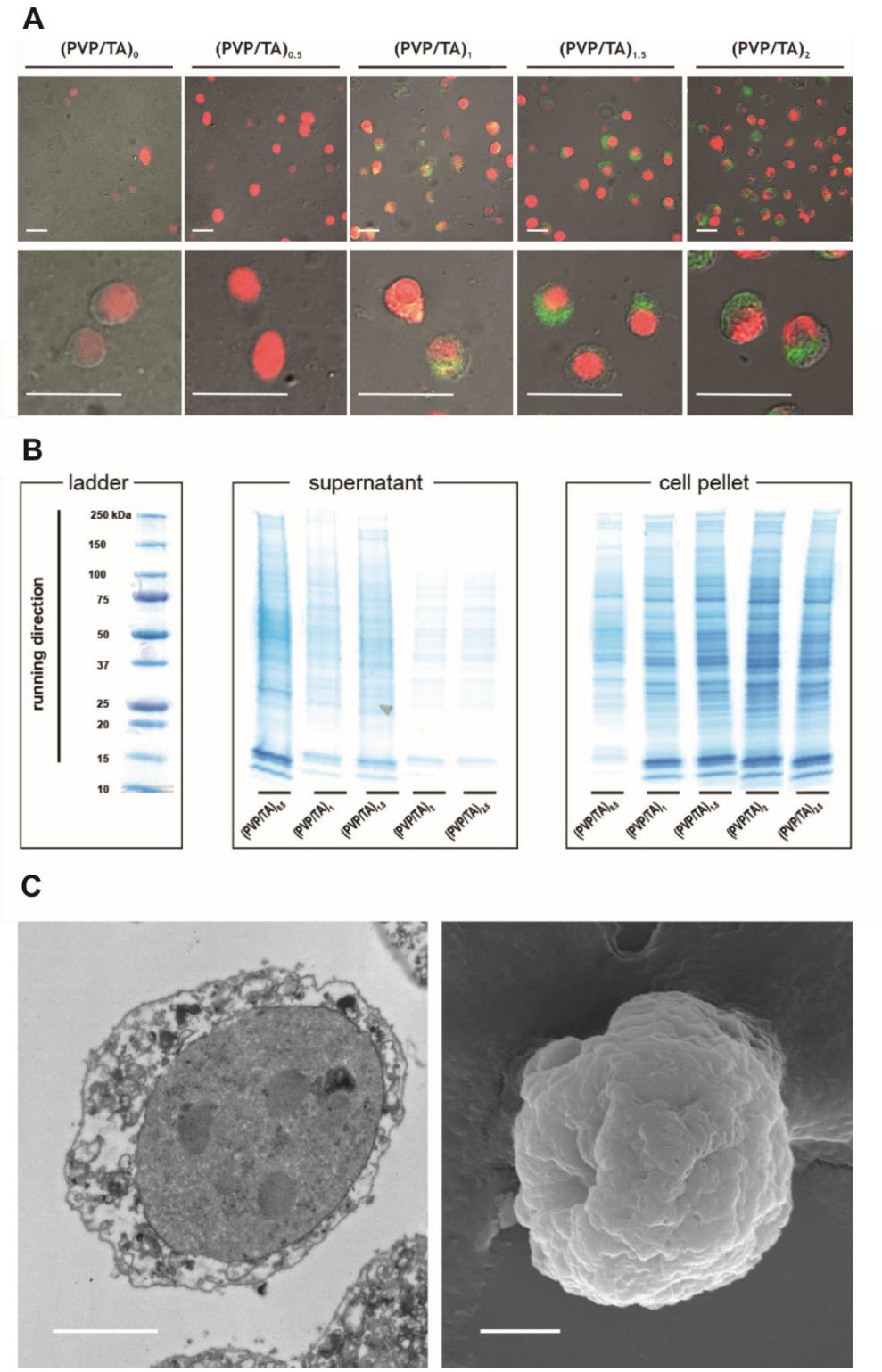

Figure 7. (A) Fluorescence microscopy images of B16.F10 cells stained with C-AM/PI upon hypo-osmotic treated after Layer-by-Layer deposition of PVP/TA. The images, shown at different magnification, are an overlay of the DIC, green and red fluorescence channels. The dashed rectangles show a zoomed area. Scale bar is 20 µm. **(B)** SDS-PAGE recorded from the supernatant and the cell pellet after hypo-osmotic treatment of the PVP/TA Layer-by-Layer coated B16.F10 cells. **(C)** Transmission (left panel) and scanning (right panel) electron microscopy images of bio-hybrid cell-templated capsules obtained by hypo-osmotic treatment of $(PVP/TA)_2$ coated B16.F10 cells. Scale bar is 3 µm.

templated capsules disintegrate upon exposure to the combination of SDS, electric field and the convective flow (data not shown). Figure 7B (right panel) shows a high amount of cellular proteins in the pellet from the deposition of 1 PVP/TA bilayer onwards. Overall, one can conclude that 2 bilayers of PVP/TA the best solution when aiming for optimal retention, after cell lysis, of the cellular protein content into stable bio-hybrid cell-templated capsules. Figure 7D depicts electron microscopy (TEM and SEM) of these capsules, showing the presence of a continuous $(PVP/TA)_2$ membrane surrounding remnants of the cellular cytoplasm and the cell nucleus that appeared to have retained at least in parts its morphology. SEM was performed under high vacuum and further underlines the physical stability of these capsules.

5. Proof of concept: Modulating the immunogenicity via heat shock

Although demonstrated for inducing cell death by hypo-osmotic shock, our approach for encapsulating cell lysate can be broadly applied and also offers the opportunity to induce cell death via a number of different ways which can be attractive to modulate the immune-stimulatory properties of the cell lysate. Indeed, compelling evidence has emerged that the initiation of tumor-specific immune responses depends on how tumor cells are killed prior to uptake by antigen presenting cells[49-51]. In particular, a process called immunogenic cell death has been reported to strongly boost anti-tumor immunity[52,53]. Immunogenic cell death is evoked by strong cellular stress responses and causing the dying cells to emit so-called Damage-Associated Molecular Patterns (DAMPs). As a proof of concept, we aimed at exploring whether our cell encapsulation strategy also allows to modulate cell death and to induce DAMPS. To address this, we exposed the cells prior to coating to a heat shock by incubating them during 1 h at 42 °C. Exposing cells to such heat treatment should lead to the induction of so-called heat shock proteins (HSP) that can be sensed by the immune system as a danger signal. Subsequently, the cells were cultured for an additional 24 h and then coated with a $(PVP/TA)_2$ membrane. To determine the presence of HSPs within the capsules, SDS-PAGE was performed on the capsule suspension followed by western blot analysis. As depicted in Figure 8, HSP 90 is detected both in control cells and heat shock treated cells suggesting HSP 90 is basally present in B16.F10 cells and expression is not increased upon exposure to heat shock. In contrast, HSP 70 was found only in the case of pre-treated cells indicating that pre-treatment of the cells with heat shock allows us to up-regulate certain heat shock proteins and to encapsulate the induced DAMPs within the capsules.

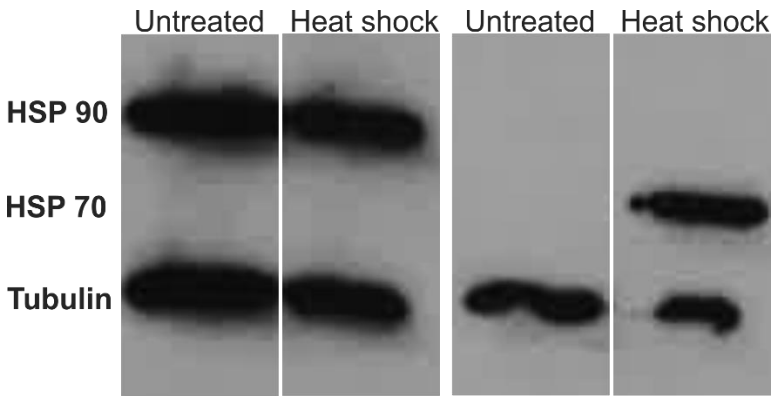

Figure 8. Western blot result of LbL coated B16.F10 cells either untreated or treated with 1 h heat shock at 42 °C prior to coating. Presence of HSP within the capsules is determined by using antibodies against HSP 70 and HSP 90. Tubulin staining is included as a loading control.

CONCLUSION

To summarize, we have demonstrated in this chapter that live cancer cells can be encapsulated within a synthetic membrane composed of PVP and TA via hydrogen bonding. Fine-tuning of the assembly conditions allowed us to obtain cell-templated bio-hybrid capsules containing a high amount of encapsulated proteins. We also demonstrated, in a proof of concept study, that heat shock prior to cellular encapsulation can be employed to potentially modulate the immunogenic properties of the capsules. In our ongoing research, we are currently aiming for modification of the cell-templated capsules via exposure to salt[54] or high temperature[55] in order to induce shrinkage of the capsules and to allow more efficient uptake by immune cells. Additionally, we plan to engineer the capsule surface with immune-stimulating cues and to investigate the potential of these capsules and other cell lysate formulation strategies as delivery system for cancer antigens in view of anti-cancer immune-therapy.

EXPERIMENTAL SECTION

Materials. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (EU qualified), penicillin/streptomycin (5000 U/mL), sodium pyruvate (100 mM), L- glutamine (200 mM), cell dissociation buffer (PBS based), PBS buffer (pH 7.2), Calcein-AM (CAM) and Propidium Iodide (PI) were purchased from Invitrogen. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent, sodium dodecyl sulfate (SDS), bovine serum albumin (BSA), Poly-L-arginine hydrochloride (Mw > 70 kDa), dextran sulfate (10 kDa) and DMSO were obtained from Sigma Aldrich. Poly(vinylpyrrolidone) (PVP) K16-18 and hydrochloric acid (HCl) 37% v/v was obtained from Fischer Scientific. Tannic acid (TA) was purchased from Fluka. 2-Mercaptoethanol, laemli sample buffer (4x), Coomassie blue stain (G-250) were purchased from Bio-rad. For western blot staining the monoclonal antibodies HSP 70 and HSP 90α/β were respectively obtained from Enzo Life Sciences and Santa Cruz Biotechnology.

Cell lines. B16.F10 cells (ATCC CRL-6475) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum, 1 % penicillin/streptomycin, 1 mM sodium pyruvate and 2mM L-glutamine and incubated at 37 °C with 5 % CO_2 saturation. Isolation of the cells for experiments was performed by incubation of the cells in a PBS-based dissociation buffer for 10 minutes.

Fabrication of bio-hybrid cell-templated capsules. B16.F10 cells were harvested and diluted to a concentration of 20 x 10^6 cells/mL. Cells were coated at 0°C in a isotonic phosphate buffered saline (PBS) buffer or HEPES buffer depending on the coating components. The cell suspension was incubated during 10 min with either one of the coating components (dextran sulfate and poly-L-arginine or tannic acid and poly(vinylpyrrolidone)) dissolved in PBS or HEPES respectively at 1 mg/mL. The cells were subsequently centrifuged at 200 G for 5 minutes and washed two times with PBS or HEPES buffer. Subsequently, the complimentary interacting component was added and again incubated for 10 min followed by centrifugation and washing. This was repeated until the desired amount of layers was assembled. After coating, the live cells were incubated for 30 min in deionized water at room temperature in order to kill the cells.

MTT assay. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Coated B16.F10 cells were seeded in 96 well plates

at a density of 50000 cells/mL in complete DMEM medium (total volume 100 µL) in six-fold. Subsequently the cells were cultured for 24 h followed by addition of 40 µL of the MTT reagent (1 mg/mL). After an incubation period of 2-3 h, the formed formazan crystals were dissolved in 100 µL of a 10 % m/v SDS/0.01 M HCl solution overnight protected from light. The absorbance was measured by a microplate reader at 570 nm. As a negative and positive control PBS or HEPES buffer and DMSO respectively were added to the wells.

Online flow cytometry. Online flow cytometry was performed on a BD Accuri C6 flow cytometer using the Live/dead fixable far red dead cell stain kit purchased from Invitrogen. For assessing the cell membrane integrity, cells were suspended at a density of 1 x 10^6 cells/mL followed by the addition of 1 µL live/dead reagent. Subsequently, the cells were placed under continuous gentle agitation on ice and the sip of the flow cytometer was immersed into the cell suspension. Next, flow cytometry was started by measuring the cells for 2 min followed by addition of the respective test components. Data were collected over 12 min on ice and processed via FlowJo.

Live/dead assay (offline). Cells were suspended in a concentration of 1 x 10^6 cells per mL in PBS and incubated with 1 µL of live/dead reagent (purchased from Invitrogen) for 30 minutes on ice. The particles were collected by centrifugation and residual reagent was removed by washing once with 1 % BSA in PBS. The percentage live versus dead cells was determined by flow cytometry.

Fluorescence microscopy analysis of cell viability. A double staining with calcein-AM (C-AM; live cell staining) and propidium iodide (PI; dead cell staining) was performed. Cells, at a density of 1 x 10^6 cells/mL, were incubated for 30 minutes with a mixture of C-AM and PI at a concentration of respectively 3 µM and 0.25 nM. After removal of the excess reagent, the cells were visualized by fluorescence microscopy using a Leica DM2500P equipped with a 40X (NA 0.75) objective, DIC filters and a DFC360FX camera.

Electron microscopy

Transmission electron microscopy (TEM) was performed on a JEOL 1010 instrument. Prior to imaging, samples were subjected to series of fixation (0.1 M Na cacodylate buffer (pH 7.2) containing 4 % paraformaldehyde and 2,5 % glutaraldehyde) and dehydration steps, embedded in epoxy resin and cut into ultrathin section using an ultramicrotome.

Scanning electron microscopy (SEM) was performed on a Quanta 200 FEG FEI instrument. Samples were deposited onto a silicon wafer and dried under a gentle nitrogen stream at ambient temperature. Prior to imaging, the samples were sputtered with a palladium/gold coating.

Gel electrophoresis (SDS-PAGE). To visualize potential protein release during the LbL coating and after lysis or to evaluate the presence of proteins within the final bio-hybrid cell-templated capsules, gel electrophoresis was performed respectively on the supernatant or on the cell suspension. The samples were diluted with a 1:9 β-mercaptoethanol:laemli sample buffer solution (4x), incubated for 5 minutes at 95 °C and loaded on 4-20 % precast gels. After the run (150 kV), visualization of the protein bands was obtained by incubation of the gels into Coomassie blue stain.

Western Blot. To visualize expression of heat shock protein 70 (HSP 70) and heat shock protein 90 (HSP 90), the samples were separated by gel electrophoresis as described above. After the run, the gels were transferred to nitrocellulose membranes and quenching was performed by incubation for 30 minutes in a blocking solution containing 5 % non-fat milk and 0.5 % Tween 20 in PBS. The membranes were stained with primary antibody anti-HSP90α or anti-HSP70 and anti-α-tubulin antibody as a loading control for at least 1 hour. After rinsing the membranes three times with the blocking solution the membrane was incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody for 1 hour. The membranes were subsequently rinsed three times with the blocking solution followed by rinsing three times with a 0.5 % tween-20 solution in PBS. The proteins were visualized by the enhanced chemiluminescence (ECL) procedure.

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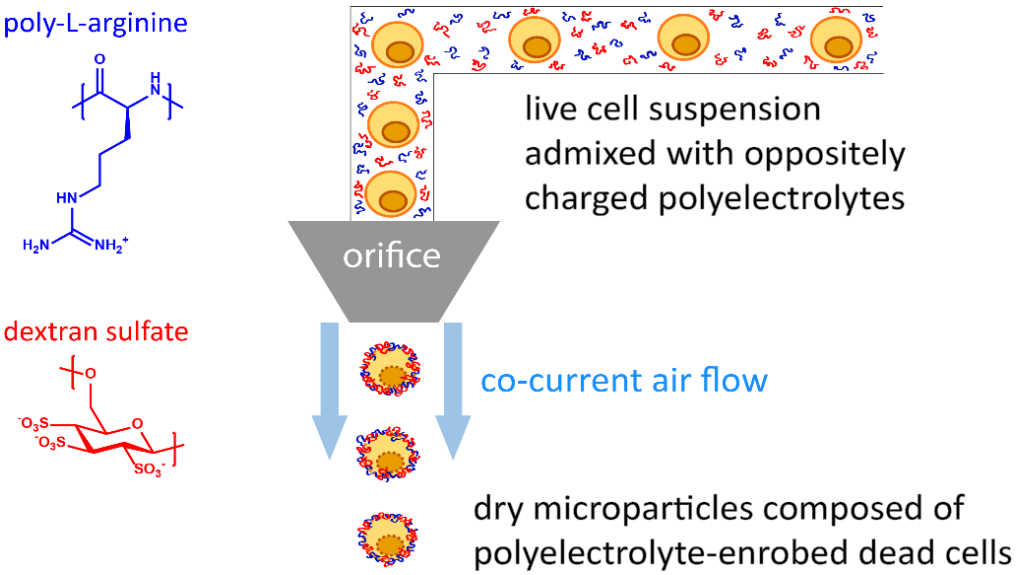

Parts of this chapter were submitted in:

Polyelectrolyte-enrobed cancer cells in view of personalized immune-therapy.

L. Lybaert, K. Ryu, R. De Rycke, A. Chon, O. De Wever, K. Y. Vermaelen, A. Esser-Kahn and B. G. De Geest, *in preparation*

CHAPTER 6

POLYELECTROLYTE-ENROBED CANCER CELLS

ABSTRACT

Targeting the immune system with a personalized vaccine containing cues derived from the patient's malignancy might be a promising approach in the fight against cancer. It includes neo-antigens as well as non-mutated tumor antigens, preferentially leading to an immune response that is directed to a broader range of epitopes compared to strategies involving a single antigen. Here, we report on an elegant method to encapsulate whole cancer cells into polyelectrolyte particles. Porous and non-aggregated microparticles containing dead cancer cells were obtained by admixing mannitol and live cancer cells with oppositely charged polyelectrolytes, dextran sulfate (anionic polysaccharide) and poly-L-arginine (cationic polypeptide) prior to atomization into a hot air stream. We showed that the polyelectrolyte-enrobed cancer cells, upon redispersion in PBS buffer, are stable and do not release cell proteins in the supernatant. *In vitro* experiments revealed that the particles are non-toxic and strongly increase uptake of cell lysate by dendritic cells. *In vitro* assessment of antigen presentation by dendritic cells revealed the potential of the polyelectrolyte-enrobed cancer cells as promotors of antigen cross-presentation. Finally, we demonstrated that the immunogenicity could be enhanced by surface adsorption of a polymer-substituted TLR7-agonist.

INTRODUCTION

Anti-tumor therapy that involves dendritic cells (DCs) to evoke a tumor-specific immune response is an attractive alternative to classic chemo- and irradiation therapy as it avoids the side-effects associated to the latter therapies[1-5]. Unfortunately, several hurdles remain between laboratory practice and successful clinical translation. One approach – termed personalized anti-tumor immune-therapy – involves the formulation of patient's own tumor-derived components into an anti-cancer vaccine[6,7].

Personalized immune-therapy implementing patient's own tumor tissue of the patient, collected from a biopsy or from surgery, might hold promise to raise the potency and tumor-specific immunity of cancer vaccines. The design of patient-derived cancer cell vaccines can involve three different methods, i.e. identification of neo-antigens, preparation of tumor cell lysate or the use of intact cancer cells. The first approach requires analysis of the genome of patient-derived cells to identify proteins that are absent from the normal human genome and exclusively rise from tumor-specific mutations[8,9]. This method however is complex, labor intensive and costly. In contrast to this first approach, preparation of cancer cell lysate from the cancer tissue of the patient is less complex and includes neo-antigens as well as non-mutated tumor antigens, preferentially leading to a broader immune response[7,10]. Third, incorporation of intact tumor cells can be an interesting approach as in this case all cell components such as cell membrane proteins are also involved and when translated to whole tumor tissue, also offer the possibility to co-encapsulate stromal proteins. Vaccines comprising autologous cell material can be an alternative for exisiting vaccines based on allogenic tumor cell lines involving GVAX encountering human leucocyte antigen (HLA) mismatch resulting in an anti-HLA reponse rather than a tumor antigen-directed reponse[11].

In this chapter we describe a simple, yet efficient, strategy to formulate whole cancer cell lysate into microparticles and demonstrate that this process enhances antigen cross-presentation by DCs. By admixing live cells in aqueous medium with oppositely charged polypeptides and polysaccharides followed by atomization into a hot air stream, a complex coacervate is formed surrounding the cells through spray drying. Evaporation of the water phase during the atomization process yields a dry powder composed of polyelectrolyte-enrobed cancer cells. This approach is schematically illustrated in **Figure 1**. Our method generates a whole cell-based

lysate within a single polyelectrolyte complex coacervate microparticle, and ensures – owing to the atomization/drying step – all cells to be dead in the final formulation. The latter avoids, upon administration, regrowth of new tumors due to residual living cells, as often the case with whole cell based lysates. In addition, our spray drying approach yields a dry particle formulation which can easily be stored over prolonged times and is highly attractive if one envisions multiple administrations over longer periods of time[12-14].

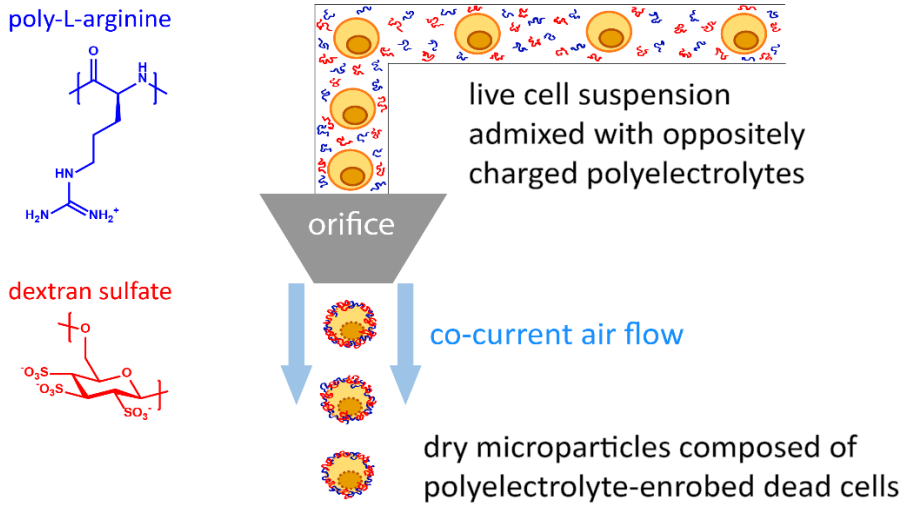

Figure 1. Schematic illustration of the production of polyelectrolyte-enrobed whole cell microparticles. Live cells are mixed in aqueous solution with dextran sulfate (negatively charged polysaccharide) and poly-L-arginine (positively charged polypeptide). Atomization of this suspension in a heated air flow produces dry microparticles composed of single dead cells enrobed with a polyelectrolyte matrix.

RESULTS AND DISCUSSION

As a model cancer cell line we used the murine Lewis lung carcinoma cell line (LLC.OVA) that is stably transfected with a non-secreted, truncated form of ovalbumin (tOVA). The latter will act as tumor-associated model antigen and allows a straightforward read-out of the immunological response by OVA-based assays.

1. Preparation of polyelectrolyte-enrobed cancer cells

In a first series of experiments we examined whether spray drying of living cancer cells was feasible applying similar conditions as previously determined in our laboratories for spray-drying of soluble proteins and polymers[15-18]. An LLC-OVA cell suspension, at a density of

60 x 10^6 cells per 10 mL deionized water, was stirred on ice to minimize cell lysis and aggregation. Subsequently mannitol, dextran sulfate (DEXS) and poly-L-arginine (P_LARG) were added in a 40:4:5 (w/w) ratio. The role of mannitol is to enhance the microparticle recovery yield after the atomization step, to reduce protein denaturation and to generate porosity in the polyelectrolyte coacervate matrix which enhances protease influx and degradation of the matrix upon uptake by dendritic cells as shown in our earlier work[16]. As control, microparticles were prepared without cells. Both formulations comprised a dry powder with an average recovery yield (calculated from the initial solid mixture amount) of approximately 50 %. Scanning electron microscopy (SEM) imaging (**Figure 2 – panel 1**) revealed that the cell-containing particles exhibited a slightly more irregularly shape compared to the empty control particles. Redispersion of the microparticles in phosphate buffered saline (PBS; pH 7.4, 150 mM NaCl) results both in case of cell-containing microparticles and the empty control microparticles in the formation of a non-aggregated suspension, as confirmed by optical microscopy (**Figure 2 – panel 2**).

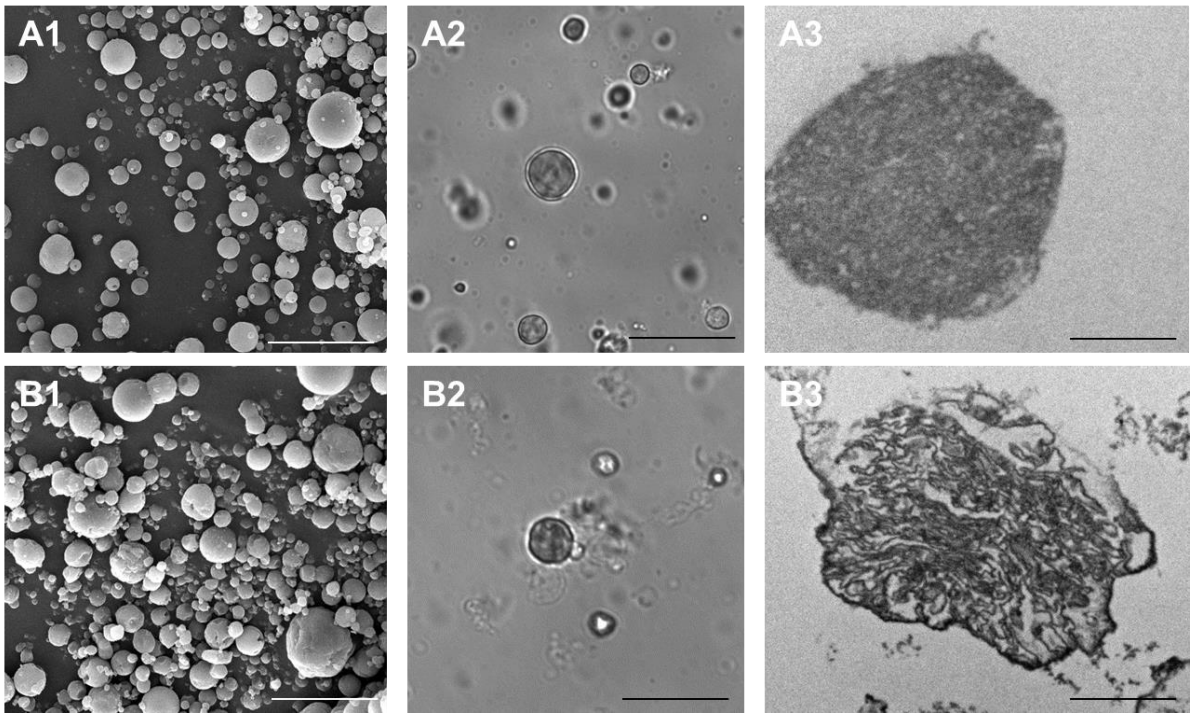

Figure 2. Optical (1), scanning electron (2) and (3) transmission electron microscopy images of (A) empty microparticles and (B) LLC.OVA containing microparticles. Scale bar is 20 micron in (1) and (2) and 2 micron in (3).

Transmission electron microscopy (TEM) imaging (**Figure 2 – panel 3**) revealed that the control particles had a relatively homogenous porous interior. In contrast, microparticles produced from LLC.OVA cells exhibit a more complex internal structure, which can likely be attributed to the presence of cellular components that yield high contrast on TEM, such as lipid-rich domains, ER, and the nuclear envelope.

To assess whether each component in the formulation is required to obtain a stable microparticle suspension in PBS, we prepared control samples containing only cells, cells mixed with mannitol but no polyelectrolytes, and cells mixed with mannitol and only DEXS or P_LARG. As illustrated in **Figure 3**, none of these conditions was successful. Spray drying of cells only resulted in an extremely low yield, similar to what we previously observed for spray drying of proteins and polymers in absence of mannitol[16]. Moreover, the recovered amount of material could not properly be redispersed again in PBS. Samples containing mannitol but no polyelectrolytes and samples containing mannitol and either one of both polyelectrolytes had decent yield (i.e. approximately 50 %), but only resulted in microparticles upon redispersion in PBS in case of cells/mannitol/P_LARG. However, the latter was highly aggregated, which we attribute to ionic gelation of the P_LARG by the divalent phosphate anions in PBS. When redispersion was performed in deionized water, again no microparticles were found. These findings clearly demonstrate the need for mannitol, DEXS and P_LARG to prepare microparticles at a sufficient recovery yield and with the ability to be properly redispersed in PBS.

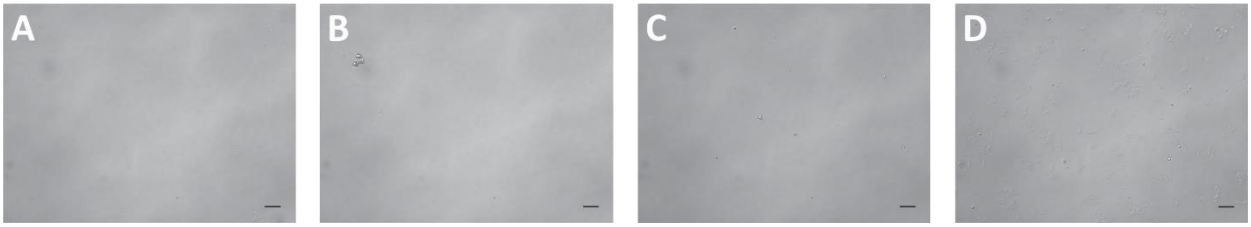

Figure 3. Microscopic imaging of spray dried control samples containing. (A) only cell material. (B) cell material and mannitol. (C) cell material, mannitol and dextran sulfate. (D) cell material, mannitol and poly-L-arginine. Scale bar is 10 µm.

Next, SDS-PAGE was used to analyze, whether upon redispersion in PBS, protein release from the microparticles occurred. We monitored the particle suspension itself and the supernatant comparing the cell-containing microspheres with a lyophilized cell suspension containing the

same amount of cells as used for the preparation of the microparticles. As shown in Figure 4A, relative to the lyophilized cell control sample, no protein release was detected in the supernatant of the centrifuged microparticle suspension and very little protein was detected when the microparticle suspension itself was loaded onto the SDS-PAGE gel. As control, we also included empty microparticles (i.e. without cells) to exclude interference of the microparticle components. As shown in lane 1 of Figure 4A, these microparticles indeed cannot be detected on the SDS-PAGE gel. Further visual proof of successful encapsulation of cellular proteins into polyelectrolyte coacervate microparticles was gained by using the fluorescent eGFP expressing cell line CT5.3, a murine colon tumor-derived cell line. A microparticle suspension prepared from this cell line exhibited a homogeneous green fluorescence throughout the microparticle volume (Figure 4B), without the presence of fluorescence in the surrounding medium. This was confirmed by fluorimetry and indicated an encapsulation efficiency of nearly 100 %.

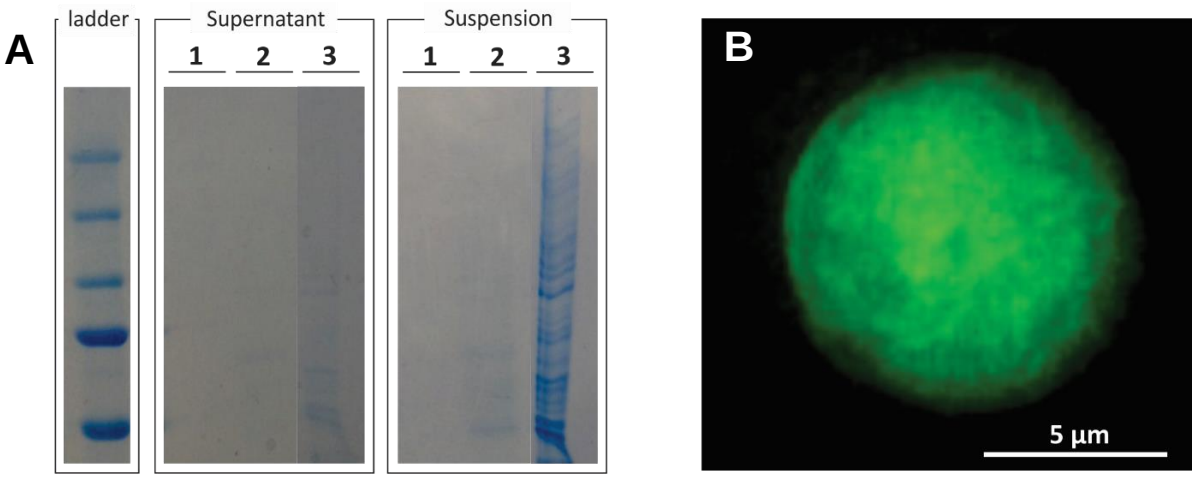

Figure 4. (A) Assessment of the encapsulation efficiency upon redispersion in PBS via SDS-PAGE recorded from the supernatant and suspension of: (1) empty microparticles; (2) LLC.OVA containing microparticles; (3) lyophilized LLC.OVA cells. (B) Fluorescence microscopy image of a microparticle produced from the eGFP expressing CT5.3 cells.

2. *In vitro* evaluation of polyelectrolyte-enrobed cancer cells

Firstly, cytotoxicity of the microparticles was evaluated by MTT assay. This revealed the particles to be non-toxic up to a concentration of 0.5 mg/mL as depicted in Figure 5A. Next, we assessed the *in vitro* uptake of the microparticles by the murine dendritic cell line DC2.4. For this purpose, microparticles produced from eGFP-positive CT5.3 cells were used to allow for straightforward detection by fluorescence based methods. Flow cytometry was used to

compare microparticle formulated cells with lyophilized cells. Note that both samples contained the same concentration of cell-based material. From these data (Figure 5B) it was clear that formulated microparticles resulted in a more efficient cellular association of cell lysate in a dose-dependent manner. Subsequently, confocal microscopy (Figure 5C) on similarly treated DC2.4 cells verified that the microparticles were indeed internalized by the DCs and were not merely bound to the cell membrane.

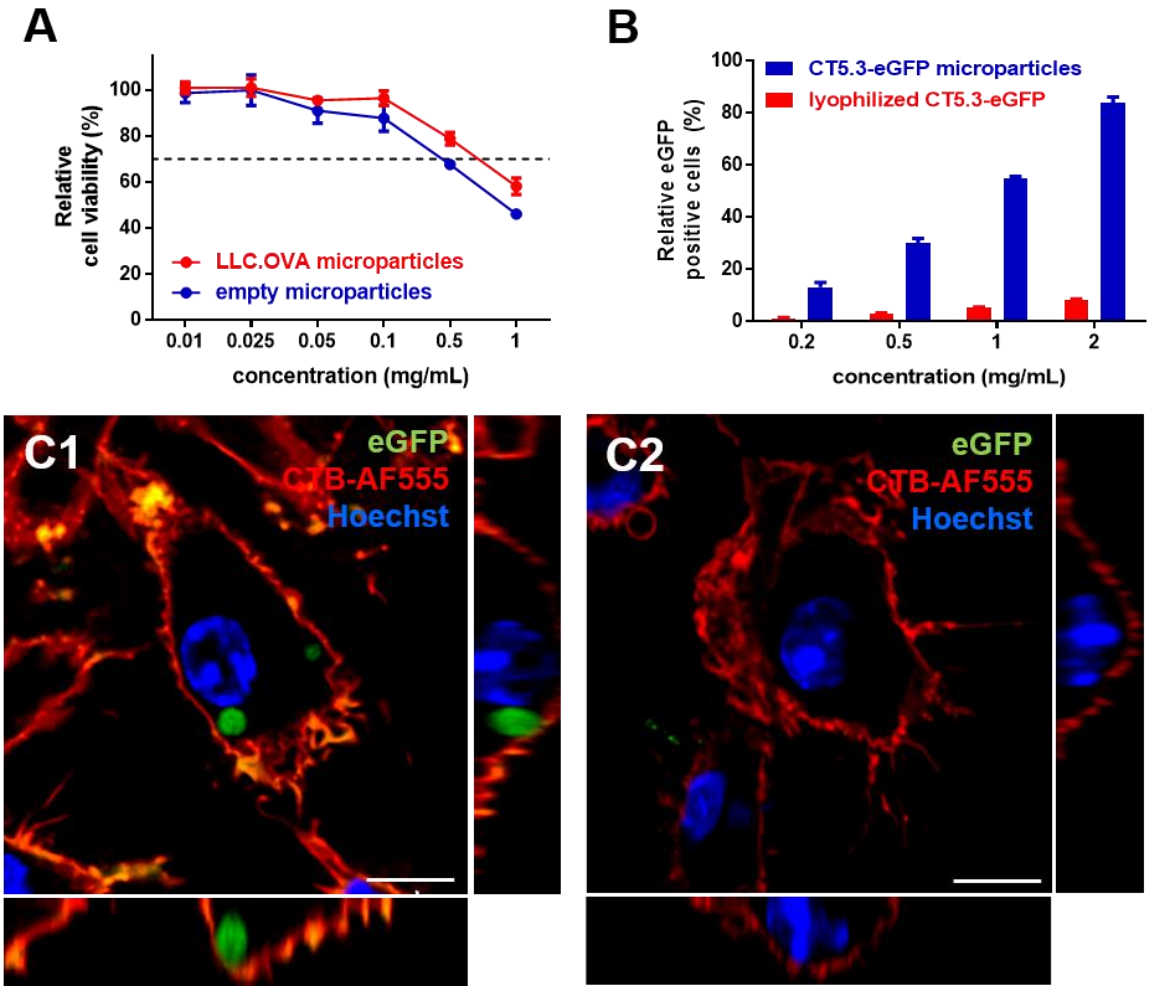

Figure 5. *In vitro* evaluation of spray dried cell-derived polyelectrolyte microspheres on DC2.4 cells: (A) MTT assay (n-6). (B) Flow cytometry analysis of uptake efficiency (n-3). (C) Confocal microscopy imaging of the interaction of the cell-containing microspheres compared to lyophilized cells with DCs. The cell membrane is stained with AF555-labeled cholera toxin B (CTB-AF555) and the cell nuclei are stained with Hoechst. Scale bar represents 15 µm.

Whereas soluble antigens are predominantly presented via MHC-II peptide complexes by DCs to CD4+ T-cells, formulation of soluble antigens into microparticles is known to enhance cross-presentation via MHC-I by DCs. The latter is essential for the priming of cytotoxic CD8+ T-cells

that hold the capacity to recognize tumor cells and eliminate these via secretion of perforin and granzymes[17-19]. Here we investigated whether encapsulation of cell lysate into polyelectrolyte microparticles also promoted antigen presentation via MHC-I. In this experiment, microparticles containing LLC-OVA cells were used. Successful processing and MHC-I presentation of the ovalbumin, as model tumor-associated antigen, in the cell lysate would enable a flow cytometric detection of the SIINFEKL OVA-CD8+ epitope presented by MHC class I H-2Kb molecules via antibody staining. After 48 h of incubation with different particle concentrations, DC2.4 cells were stained and analyzed by flow cytometry. Figure 6 clearly shows a dose-dependent increase of the cross-presentation efficiency when LLC-OVA cells were encapsulated in microparticles, whereas control experiments with lyophilized LLC.OVA cells did not show any significant cross-presentation. Neither was this the case for empty microparticles.

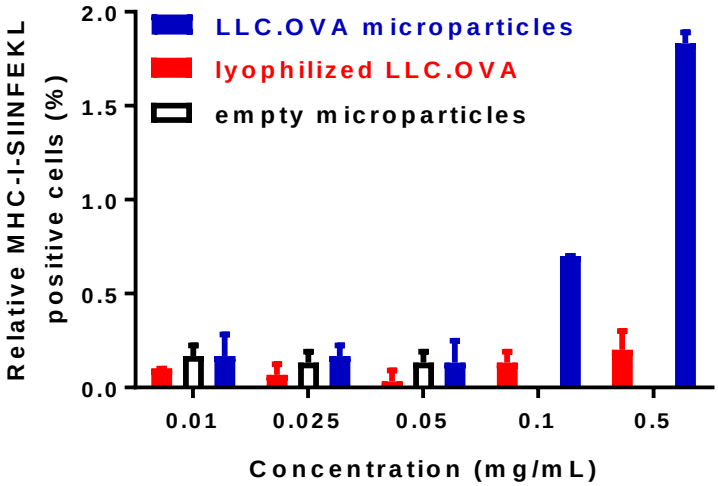

Figure 6. *In vitro* assessment of the MHC-I cross-presentation efficiency by DC2.4 cells.

3. Co-formulation of polyelectrolyte-enrobed cancer cells with immune-stimulating cues

After formulation of tumor antigens into particles to augment cross-presentation, a potent cancer vaccine additionally requires co-formulation of particulate antigens with immune-stimulating cues to enhance the immunogenicity of the vaccine formulation[20-24]. This is necessary because particle-based formulation alone is insufficient for potent CTL-induction as this process requires three signals: [1] interaction between the T-cell receptor and the MHC-I presented antigen on the DC surface; [2] interaction between the CD28 T-cell receptor and CD80 or CD86 on the DC surface; and [3] cytokine stimulation of T-cells by DCs. The latter two signals can be mounted by triggering pathogen recognition receptors (PRRs) present at

different location in DC, including cell surface and endosomal membranes and the cytoplasm. Amongst the multiple PRRs, Toll-like receptors (TLRs) have been widely explored as target for molecular adjuvants to skew TH1-driven immune responses and augment their amplitude and persistency. TLR7/8-triggering is in particular attractive in the context of tumor vaccination as these receptors are present on the cell endosomal membrane in a wide range of both human and murine DC subsets. Triggering leads to elevated levels of type I IFN and IL-12, which are key cytokines to promote TH1- and CTL-responses required for potent anti-tumor immune responses[25-27]. Interestingly, small molecule agonists of TLR7/8 based on guanosine analogues and imidazoquinolines have been identified and polymer-conjugation of these molecules has recently been shown by us and others[28-30] as an ideal strategy to reduce their systemic dissemination, thereby greatly enhancing their toxicity profile, and to enhance their adjuvanticity towards co-administered antigens.

Here we used a polymer backbone composed of N-(hydroxypropyl) methacrylamide (HPMA) and N-(3-aminopropyl) methacrylamide (APMA). Poly(HPMA-APMA)) containing 80 HPMA and 20 APMA repeating units was synthesized via RAFT polymerization as earlier reported[31]. The primary amine moieties of this polymer were substituted with the TLR7/8-agonist 2-(4-((6-amino-2-(butylamino)-8-hydroxy-9H_purin_9-yl)methyl)benzamido)acetic acid yielding TLR7-poly(HPMA-APMA) (*vide supra – Figure 5: Chapter 4*).

The ability to co-formulate this polymer with cell lysate into microparticles was tested using CT5.3-eGFP cells and rhodamine labeled polymers that were synthesized by converting a small fraction of the poly(HPMA-APMA) amino groups with rhodamine-isothiocyanate. Confocal microscopy of the redispersed microparticles in PBS (**Figure 8A**) clearly demonstrated the presence of the polymer, predominantly in a dotted pattern, likely due to complexation with the polyelectrolytes, whereas the eGFP signal was clearly visible throughout the whole microparticle volume. Subsequently, microparticles were produced containing TLR-poly(HPMA-APMA). A TLR-reporter cell assay (i.e. RAW Blue) was performed to determine whether upon formulation into microparticles TLR-triggering is still possible. Note that RAW Blue cells are engineered RAW 264.7 macrophages that express a broad range of PRRs and upon stimulation of these receptors produce secreted embryonic alkaline phosphatase (SEAP) which can easily be detected by UV-VIS spectrophotometry. As shown in **Figure 8B**, LLC.OVA

containing particles as well as poly(HPMA-APMA) did not evoke any activation evidenced by the lack of increase in absorbance relative to the negative PBS control. This suggested that the microparticles and the polymer on their own are poorly immunogenic.

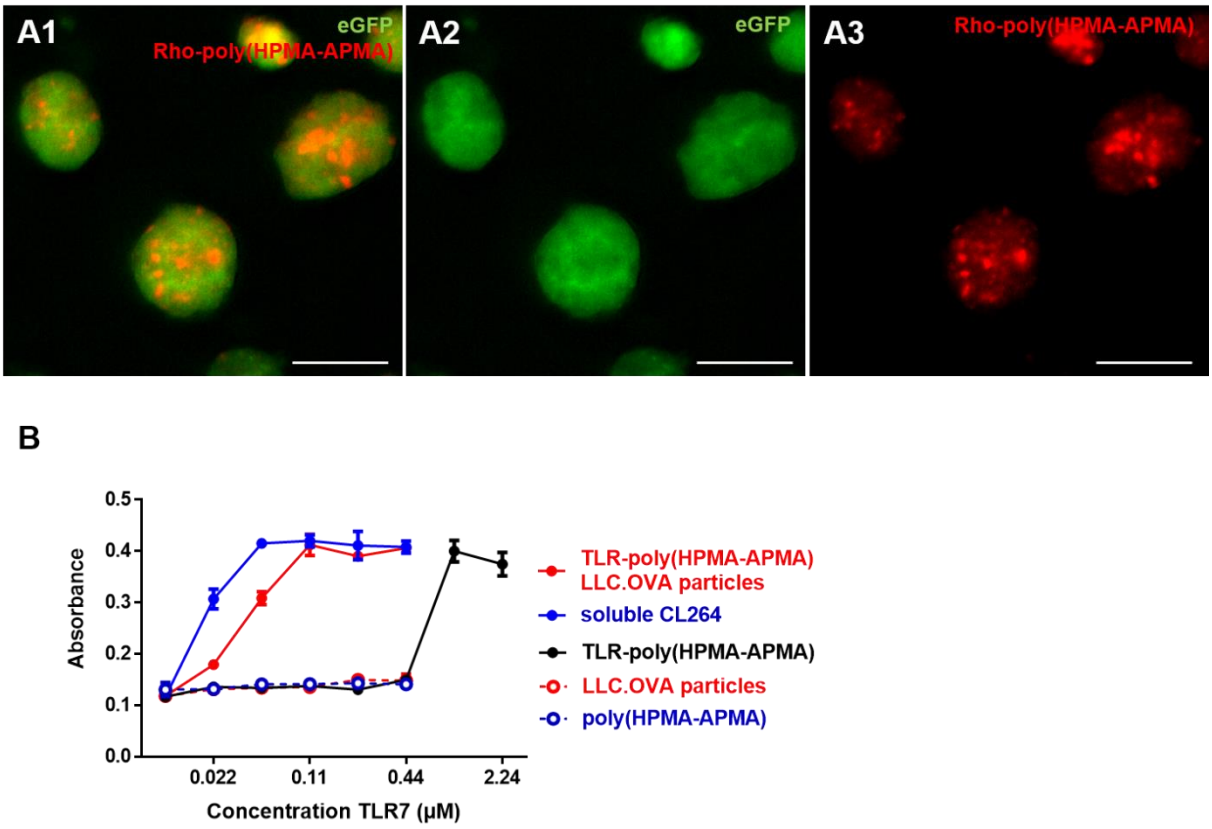

Figure 8. (A) Confocal microscopy images of spray dried microspheres containing CT5.3-eGFP cell material and rhodamine-labeled poly(HPMA-APMA). Scale bar represents 10 µm. (B) RAW blue assay comparison of the soluble TLR7-agonist, the polymer-ligated TLR7-agonist and the polyelectrolyte microspheres whether or not co-formulated with TLR7-poly(HPMA-APMA).

In addition, TLR-poly(HPMA-APMA) only induced strong maturation above molar concentrations of 0.44 µM in contrast to soluble TLR7/8-agonist which evokes maturation at significantly lower concentrations. This decrease in efficiency of the polymer-linked TLR7/8-agonist can be attributed to steric hindrance of the polymer upon binding with the TLR-receptor and/or partial shielding of the active site of the TLR7/8-agonist. However, the latter is unlikely as both CL264 as well as other imidazoquinoline analogues have been conjugated at a similar position without fully abrogating their potency[28,29,32]. Most interestingly, microparticles containing TLR-poly(HPMA-APMA) strongly promoted TLR-activation almost equally efficiently as the soluble TLR7/8-agonist. This clearly shows the beneficial effect of particulate formulation of antigens together with TLR-agonists as attractive vaccine carriers. The increase in potency

can be attributed to more efficient uptake of the particulate vaccine formulation opposed to soluble vaccine which enables enhanced interaction of the TLR-ligand with its receptor upon cell uptake.

CONCLUSION

In this chapter we have reported on the formulation of whole cancer cells into solid polyelectrolyte-based coacervate microparticles formed by the oppositely charged biologically-inspired polyelectrolytes. Relative to cell lysate produced by lyophilization, microparticle-formulated cells were internalized by DCs to a much larger extent. Using a cancer cell line that stably expresses OVA as model tumor-associated antigen, we found that the antigen cross-presentation efficiency by DCs was significantly enhanced in case of microencapsulated cells. We further demonstrated the ability to co-encapsulate TLR7-agonist-ligated polymers into the microparticles and verified that TLR-triggering can still occur. These findings pave the road for the development of whole cell based cancer vaccines that avoid the issue of tumor regrowth observed when using conditioned cancer cells and are more potent than soluble cell lysate based vaccines. In addition, since dry polyelectrolyte-enrobed cancer cells were obtained via spray drying, this formulation is highly stable and does not require cold chain preservation prior to administration into the patient unlike current liquid vaccine formulations. Further experiments will involve *in vivo* assessment of the potency of the vaccine particles in triggering a robust anti-tumor immune response.

EXPERIMENTAL SECTION

Materials. Mannitol, dextran sulfate (10 kDa), poly-L-arginine hydrochloride (Mw > 70 kDa), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent, sodium dodecyl sulfate (SDS), ethanol, dimethyl sulfoxide (DMSO), $NaHCO_3$ and paraformaldehyde (PFA) were obtained from Sigma Aldrich. Hydrochloric acid (HCl) 37 % v/v, rhodamine-isothiocyanate were purchased from Fischer Scientific. Dulbecco's Modified Eagle Medium (DMEM), RPMI 1640 medium, fetal bovine serum (EU qualified), penicillin/streptomycin (5000 U/mL), sodium pyruvate (100 mM), L-glutamine (200 mM), cell dissociation buffer (PBS based), PBS buffer (pH 7.2), Hoechst, cholera toxine B conjugates to AlexaFluor555 (CTB-AF555) and Zeocin were obtained from Invitrogen. Laemli buffer (4x), 2-Mercaptoethanol, Coomassie blue stain (G-250) and 4–90 20 % mini-protean TGX gels were purchased from Bio-rad whereas the pretreated Spectra/Por 7 dialysis membrane were purchased from Spectrumlabs. Quanti blue stain was obtained from Invivogen and anti-Mouse OVA257-264 (SIINFEKL) peptide bound to H-2Kb PE antibody was purchased from eBioscience.

Cell lines.

LLC. OVA cell line. The LLC.OVA cell line was a kind gift from Prof. Karim Vermaelen (University of Ghent, Belgium). The cells were cultured in RPMI medium supplemented with 10 % fetal bovine serum, 1 % penicillin/streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate and incubated at 37 °C with 5 % CO_2 saturation.

CT5.3-eGFP cell line. The CT5.3-eGFP cell line[33] was cultured in DMEM medium supplemented with 10 % fetal bovine serum, 1 % penicillin/streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate and incubated at 37 °C with 5 % CO_2 saturation.

DC2.4 cell line. The DC2.4 cell line was a kind gift from Dr. Kenneth Rock (University of Massachusetts, Boston, US). The cells were cultured in RPMI medium supplemented with 10 % fetal bovine serum, 1 % penicillin/streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate and incubated at 37 °C with 5 % CO_2 saturation.

RAW Blue cell line. The RAW Blue cell line was purchased from Invivogen. The cells were cultured in DMEM medium supplemented with 10 % heat-inactivated fetal bovine serum, 1 %

penicillin/streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate and 0.01 % Zeocin and incubated at 37 °C with 5 % CO_2 saturation.

Synthesis of cancer cell polyelectrolyte microspheres. Prior to cell count, mannitol and dextran sulfate (DEXS) were dissolved in 10 mL of LPS free water to a concentration of 20 mg/mL or 2 mg/mL respectively and a 5 mg/mL solution of poly-L-arginine (P_LARG) was prepared in LPS free water. Next 60 x 10^6 LLC.OVA cells were suspended in 15 mL of LPS free water and added to the mannitol-DEXS solution under stirring on ice. All handlings were performed in sterile conditions in a biohood to avoid endotoxin contamination. Subsequently 5 mL of the poly-L-arginine solution was added dropwise under stirring to the cell suspension on ice prior to spray drying. Spray drying of the mixtures was performed on a lab-scale Buchi B2902 spray-dryer under sterile conditions. The latter involved pre-sterilization of the spray dryer with ethanol and LPS free water prior to spray drying of the test samples. The spray-dryer operated in co-current air flow at drying air temperature of 130 °C. After spray drying the yield was determined and the obtained powder was stored at -20 °C. The samples were visualized on a Leica DM2500P microscope equipped with a 40X (NA 0.75) objective, DIC filter and a DFC360FX camera after reconstitution in water. The initial weight of the LLC.OVA cell suspension was determined after lyophilization to calculate the yield after spray drying and of the amount of lyophilized cells needed as control.

Electron microscopy.

Scanning electron microscopy. Scanning electron microscopy (SEM) was performed on a Quanta 200 FEG FEI instrument. Samples were deposited onto a silicon wafer and dried under a gentle nitrogen stream at ambient temperature. Prior to imaging, the samples were sputtered with a palladium/gold coating.

Transmission electron microscopy. Transmission electron microscopy (TEM) was performed on a JEOL 1010 instrument. Prior to imaging, samples were subjected to series of fixation (0.1 M Na cacodylate buffer (pH 7.2) containing 4 % paraformaldehyde and 2.5 % glutaraldehyde) and dehydration steps, embedded in epoxy resin and cut into ultrathin section using an ultramicrotome.

Gel electrophoresis (SDS-PAGE). To analyze cell lysate encapsulation efficiency upon reconstitution of the particles after spray drying in LPS free water, gel electrophoresis was

performed. The samples were diluted with a 1:9 2-mercaptoethanol:Laemli sample buffer solution (4x), incubated for 5 minutes at 95 °C and loaded on 4-20 % precast gels. After the run (150 kV), visualization of the protein bands was achieved by incubation of the gels into Coomassie blue stain.

MTT assay. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. DC2.4 cells were seeded in 96 well plates at a density of 5.10^4 cells/mL (total volume 100 µL) in six-fold. Subsequently the cells were incubated with different concentrations of microspheres and lyophilized LLC.OVA cells and cultured for 24 h followed by addition of 40 µL of the MTT reagent (1 mg/mL). After an incubation period of 2–3 h the formed formazan crystals were dissolved in 100 µL of a 10 % m/v SDS/0.01 M HCl solution overnight protected from light. The absorbance was measured by a microplate reader at 570 nm. As a negative and positive control PBS buffer and DMSO respectively were added to the wells.

In vitro cell uptake assay. DC2.4 cells were seeded in a 24 well plate at a density of 0.15 x 10^6 cells/mL one day before the cells are pulsed with fluorescent particles at different concentrations. After 24 h of incubation, the cells were dissociated using cell dissociation buffer followed by centrifugation for 5 min at 200 G at 0 °C. After resuspension, the samples were stored on ice and measured on a BD Accuri C6 flow cytometer. The data was analyzed using FlowJo.

Confocal microscopy. DC2.4 cells were seeded at a density of 0.4 × 10^6 cells/mL in a glass bottom Will-co dish and incubated overnight. Next, the fluorescent particles were added, incubated for 24 h, and fixated in a 2 % paraformaldehyde solution for 10–15 min. The cells were subsequently washed and simultaneously stained by CTB-AF555 and Hoechst for 1 h at room temperature. Finally the samples were washed with PBS and imaging on a confocal microscope (Leica DMI6000 B inverted 241 microscope) equipped with an oil immersion objective (Zeiss, 63 ×, 242 NA 1.40) and attached to an Andor DSD2 confocal scanner.

In vitro MHC-I presentation assay. DC2.4 cells were seeded at a density of 0.2 × 10^6 cells/mL in a 24 well culture plate and incubated overnight followed by incubation with different concentrations of the samples. After 48 h at 37 °C the positive control (SIINFEKL) was added in

triplicate in a concentration of 1 µg/mL, 1 hour prior to staining with SIINFEKL-MHC-I PE-labeled antibody for 30 minutes on ice protected from light. Subsequently the samples were centrifuged for 5 minutes at 200 G at 4 °C, resuspended in PBS and analyzed with flow cytometry.

Fluorescent labeling of poly(HPMA-APMA). Poly(HPMA-APMA) was incubated with an equimolar amount of rhodamine-isothiocyanate in 0.1 M $NaHCO_3$ buffer overnight under continuous stirring. Subsequently the obtained mixture was dialyzed against deionized water for 3 days (MWCO 3.5 kDa) and lyophilized.

Polymer synthesis and conjugation of CL264. Poly(HPMA-APMA), dissolved in LPS free water, was incubated with an equimolar amount of TLR7-ligand CL264 to APMA units overnight under continuous stirring at room temperature in the presence of 1.5 M excess of DMTMM. After 24 h incubation the reaction mixture was dialyzed against LPS free water (MWCO 3.5 kDa) for 1 day and lyophilized.

In vitro RAW Blue assay. RAW Blue macrophages were seeded in a 96 well round bottom plate at a density of 0.5 x 10^6 cells/mL and immediately pulsed with the desired concentrations of the test compounds in six-fold. As a negative control PBS was added. After 24 h incubation, 50 µL of the supernatant was transferred into a 96 well flat bottom plate and incubated with 150 µL of Quanti blue solution. After 3 to 6 h incubation at 37 °C the color change absorbance was measured with a plate reader at 620-655 nm.

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PART III

FUTURE PERSPECTIVES AND GENERAL CONCLUSIONS

CHAPTER 7

TOWARDS THE IDEAL PERSONALIZED THERAPEUTIC VACCINE:

A PATIENT-SPECIFIC MULTISTEP APPROACH

INTRODUCTION

Active anti-tumor immune-therapy has emerged as one of the most promising strategies in the fight against cancer as it primes the patient's own immune system to recognize and ultimately destroy the malignancy. Since active anti-tumor immune-therapy relies on the patient's immune system, it takes advantage of the natural defense mechanism of the patient thereby enabling the development of immunological memory[1-3]. Induction of immunological memory is essential for prolonged protection against relapse and/or metastasis, which are often more aggressive and resistant and thus harder to treat. This is the reason why immune-therapy has dramatically changed the oncology field and is nowadays extensively studied in the search for more efficient and specific therapies. Cancer vaccination targeting dendritic cells *in vivo* is, to our opinion, a very appealing strategy to tackle cancer[4-7]. The reason why *in vivo* therapeutic vaccination holds promise is fourfold: [1] the cancer vaccine can be personalized, in other words specifically tailored to the patient's mutanome containing neo-antigens or patient-derived cancer tissue; [2] cancer vaccination targets dendritic cells which are the most potent class of antigen presenting cells, the crucial link between the innate and the adaptive immunity and are able to induce a strong anti-tumor CTL- and TH1-response; [3] *in vivo* targeting takes advantage of the physiological stimuli that occur upon vaccination leading to a more broad and profound anti-tumor response alongside immunological memory; and [4] relatively mild side effects occur such as local erythema and flu-like symptoms.

Despite extensive research, *in vivo* cancer vaccination has not lived up to its expectations yet. This can likely be attributed to the immune-suppressive tumor microenvironment and tumor stroma, which physiologically and physically protect the malignancy against attack by the immune system. The dual role and the complex relationship between the immune system and cancer has been more and more elucidated over the past decade evincing similarities in cancer pathogenesis and immune-escape, thereby unraveling the paradoxical role of the immune system in cancer progression[8-10]. The combination of all the different immune-suppressive strategies cancer cells use in order to evade the immune system together with the inter-tumoral and intra-tumoral heterogeneity of malignancies[11,12] points out the evident need for a multistep approach that should likely be tailored and personalized for every single patient. We

detail three parameters, that need to be optimized in order to obtain the ideal anti-tumor immune-therapy: immunogenicity, efficacy and personalized medicine.

TUMOR-RELATED IMMUNOGENICITY ISSUES

Tumor cells themselves can be low or non-immunogenic which forms one of the biggest challenges in cancer immune-therapy in general. Cancer cells with altered immunogenicity have adapted their antigen presentation machinery which involves in 50 % of the cases downregulation or lack of immunogenic antigen expression. In this way, the cancer cells cannot be recognized anymore by the adaptive immune system and escape immune-destruction[13-15]. In addition, an immunogenic tumor can revert to a non-immunogenic malignancy through immune-selection and escape from the immune system over time[16,17]. This involves the initial strong immune selection process by a TH1-type anti-tumor response in primary tumors, often highly MHC-I positive, which leads to immune-escape of the remaining MHC-I negative clones through immunoediting[18,19]. This leads to low or non-immunogenic variants with high capability of progressing into metastatic lesions. On one hand, non-immunogenic tumors comprise cells with total MHC class I loss and are therefore susceptible to NK-cell-mediated lysis. However, the immune-suppressive TME often induces tolerogenic or anergic NK-cells resulting into failure of cytotoxic eradication. Low-immunogenic tumors, on the other, have only partial loss of MHC class I expression which allows the tumor cells to escape cytotoxic attacks for both CTLs and NK-cells[18,20]. Tumor cells exhibiting downregulation of MHC class I expression (low-immunogenic) are considered to be soft reversible lesions in contrast to malignancies that lack MHC-I expression (non-immunogenic) which are hard irreversible lesions and are present in 30-40 % of human cancers[18,21,22]. As active cancer immune-therapy relies on the immunogenicity of the tumor to elicit anti-tumor immune responses, non- and low-immunogenic malignancies form a major hurdle.

Non- or low-immunogenic tumors can however be reverted into immunogenic tumors via low dose immunogenic chemo- or radiotherapy and sensitize anti-tumor immunity. As described earlier, chemo- and radiotherapy can be used to evoke immunogenic cell death (ICD) in patient derived cancer cells prior to vaccine design (v*ide supra – Chapter 2: DAMP-based adjuvants*). This can also be used to evoke ICD *in vivo* in the tumor mass of the patient suggesting that

chemo- and radiotherapy can improve the efficacy of cancer immune-therapy via enabling uptake of antigens by DCs and providing the necessary co-stimulatory signals to activate T-cell expansion through DAMPs[23,24]. For conventional radio- and chemotherapies the maximum tolerated dose is administrated to reduce tumor growth leading to severe adverse events such as myelosuppression, neutropenia and thrombocytopenia. In contrast, immunogenic radio- and chemotherapy requires low doses thereby drastically reducing the side effects[25,26]. This indicates that the role of conventional antitumor therapies needs to be revisited and re-evaluated as a key factor in clinical efficacy of cancer immune-therapy through reestablishment of the tumor immunogenicity. Another strategy known to enhance tumor immunogenicity of low immunogenic tumors, via upregulation of MHC-I antigen presentation, involves IFN-γ therapy[27,28]. However, some tumors lack or have an abnormal functioning IFN-γ receptor pathway signaling and escape the immune system [29,30]. An overview of strategies that can be used to increase the tumor immunogenicity is illustrated in **Figure 1**.

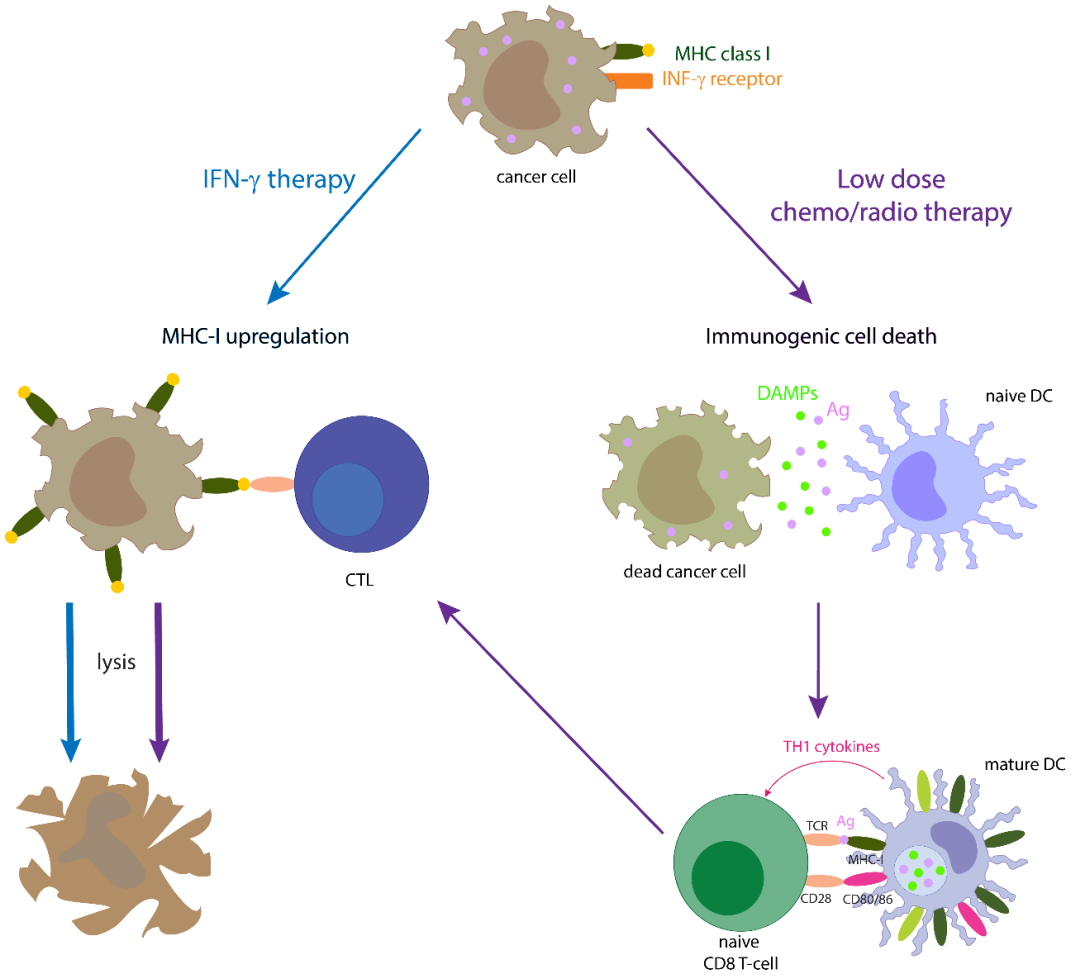

Figure 1. Strategies to enhance tumor immunogenicity.

Taken together, the immunogenicity of the cancer itself is an important factor in the balance between immune-evasion and immune-destruction by anti-tumor immunotherapies. Recent discovery of the potential of immunogenic radio- and chemotherapy is an important step in developing more potent anti-tumor immunotherapies through enhancing the immunogenicity of tumors.

EFFICACY: COUNTERING T-CELL EXHAUSTION AND T-CELL EXCLUSION

Besides issues regarding immunogenicity, the biggest hurdle for anti-tumor therapeutic vaccination comprises the lack of efficacy due to the high immune-suppressive burden surrounding the target tissue. Tumor cells suppress the immune system in order to avoid CTL-mediated eradication through two mechanisms involving T-cell exhaustion and T-cell exclusion in T-cell inflamed or non-T-cell inflamed cancer tissue respectively[31].

1. T-cell inflamed tumor: avoid T-cell exhaustion

T-cell inflamed tumors involve tumors that are invaded by tumor-infiltrated T-cells (TILs). In response, cancer cells and tolerogenic immune cells apply different strategies in order to paralyze T-cells resulting in T-cell exhaustion[32,33]. These tumors, immunogenic or not, are non-responding and resistant to immune-destruction. T-cell exhaustion was originally identified during chronic infections as a natural defense mechanism of the host to avoid cell damage due to prolonged inflammation inherent to increased cytotoxicity through CTL overstimulation[34-37]. This immune-intrinsic feedback loop is however overstimulated in cancer. T-cell exhaustion is a result of: [1] chronic and continuous stimulation of cancer antigens[38]; [2] immune-suppressive cytokines (IL-10, TGF-β); [3] depletion of nutrients; and [4] impaired CD4+ TH1-cell function. Exhausted T-cells are hypo-responsive and over-express inhibitory receptors (immune checkpoints) resulting in downregulation of cytokine production (IL-2, TNF-α, IFN-γ), defective proliferative and cytolytic capacity and increased apoptosis[34,39-42]. Exhausted T-cells are however reversible and can be reactivated through different strategies that recently have gained a lot of interest as potent inducers of the efficacy of therapeutic vaccines. Strategies to reverse the hypo-responsive state of exhausted T-cells aim to eliminate the direct cause of T-cell exhaustion, i.e. physiological changes and immune-suppressive cells, or counteract the inherent result of T-cell exhaustion, i.e. immune checkpoint up regulation[41,43].

1.1 Reversing nutrient depletion and hypoxia

One of the mechanisms responsible for T-cell exhaustion relies on physiological changes in the tumor microenvironment regarding depletion of tryptophan and glucose or hypoxia. Tryptophan depletion is related to expression of IDO, a naturally induced enzyme by IL-10, TGF- β, PGE-2 and type I IFN in chronic viral exposure to protect the host against tissue damage as a result of prolonged inflammation. Expression of IDO results in metabolization of tryptophan into kynurenine and depletes the local environment from tryptophan which is essential for T-cell proliferation. Moreover, kynurenine primes naive CD4+ T-cells to differentiate into Treg cells[44,45]. This immune-suppressive mechanism is abused by immune-suppressive immune cells (such as MDSCs and tol-DCs), stromal cells as well as the cancer cells themselves to avoid CTL-mediated destruction. By consequence inhibition of IDO-mediated metabolism of tryptophan is proposed as a promising strategy to enhance to efficacy of anti-cancer immunotherapeutics. Currently several IDO-inhibitors are explored in clinical trials of which Indoximod® or 1-methyl-D,L-tryptophan (1-MT) is the most extensively studied and acts as a competitive inhibitor of the IDO enzyme alongside newer IDO inhibitors including INCB024360 and Ebselen[46-51].

Some tumor areas are hypoxic due to incomplete angiogenesis which impairs oxidative phosphorylation by T-cells for energy supply and thereby evokes T-cell exhaustion. Moreover, to sustain exponential growth, cancer cells consume glucose in large quantities to fuel their energy needs. The latter leads to hypoglycemia in the tumor environment which also alters the nutrient availability for TILs negatively. In addition, hypoglycemia and hypoxia both induce expression of immune checkpoint programmed-death 1 protein (PD-1) on the T-cell surface resulting in suppression of activated CTLs in the tumor microenvironment (*vide infra – Immune checkpoint inhibition*)[52]. As a result of hypoxia, several genes involved in oxygen signaling pathways are activated or altered in cancer cells among which hypoxia inducible factor 1α (HIF-1α) or mammalian target of rapamycin (mTOR) and unfolded protein response (UPR). Activation of the HIF-1 pathway results in induction of new blood vessels, glucose metabolism and invasion. In contrast the mTOR and UPR pathways, critical factors in basic cellular functions and cell survival respectively, are altered to allow tumor progression and evoke hypoxic tolerance[53]. It is therefore of interest to target these pathways in order to control the expression of the genes and contain tumor growth. Current research is focused on intervening

these pathways in order to control the expression of these genes to potentially contain tumor growth and further enhance the efficacy of cancer immune-therapy[54-56].

1.2 Elimination or redirection of immune-suppressive cells

In addition to physiological changes in the TME, another direct cause of T-cell exhaustion is the immune-suppressive environment created by MDSCs, tolerogenic DCs, TAMs and regulatory T-cells, driving tumor-infiltrating CTLs into hypo-responsive T-cells. Therapeutic targeting of these tolerogenic cells that aims to eliminate, deactivate or re-educate cells towards immunogenic cells holds promise to increase efficacy of current immunotherapeutics.

Among the immune-suppressive cells, MDSCs are one the most critical factors in T-cell exhaustion due to their multifaceted influence on the immune system enclosing nutrient deprivation, cellular toxicity induced by ROS and NO, recruitment of Treg cells and reprogramming of DCs and macrophages towards their tolerogenic phenotype. Therefore, targeting MDSCs is one of the most promising strategies to increase vaccine efficacy as their immune-suppressive function is extensive and diverse. Approaches that involve MDSC-targeting are threefold and aim: [1] to eliminate MDSCs; [2] to deactivate MDSCs; and [3] to lower MDSC levels[57-59].

Elimination of MDSCs can be achieved by low dose chemotherapy whereas inactivation is accomplished by inhibition of certain immune-suppressive mechanisms applied by MDSCs. This involves inhibition of ROS or NO production through up regulation of the Nf-E2-related factor 2 (NRF-2) which influences the secretion of antioxidant enzymes[58,60] or through phosphodiesterase-5 (PDE-5) inhibition by interfering with arginase1 and NO synthase expression[61,62]. Next to elimination and deactivation of MDSCs, approaches that aim to lower MDSC levels are also explored which comprise inhibition of the synthesis of MDSCs or induction of differentiation of the latter into immunogenic DCs or macrophages. On one hand, MDSC-synthesis can be blocked through bisphosphonate treatment[63,64] or STAT-3-inhibitors[65-67] which inhibit MDSC mobilization from bone marrow or inhibit MDSC proliferation respectively. Differentiation of MDSC into dendritic cells on the other hand can be obtained via all-trans retinoic acid (ATRA) treatment which activates genes that lead to maturation and differentiation[68,69]. Many of these approaches show potential and are currently tested in

clinical trials[70-72], however there is still a need for more detailed knowledge about the MDSC function and physiology to result in more targeted and effective therapies.

Two other populations of immune-suppressive cells that can be targeted to avoid T-cell exhaustion are DCs and macrophages which both are polarized into their tumor-promoting phenotype due to immune-suppressive stimuli in the tumor microenvironment. Therefore, immunogenic reprogramming is a potential interesting strategy to target tol-DCs and TAMs. Immune-activation can be achieved by TLR-agonists which induces maturation and secretion of pro-inflammatory cytokines and leads to reprogramming of the tumor-promoting into the tumoricidal phenotype[73,74]. Another approach for re-education involves STING-agonists. The STING or stimulator of IFN genes pathway is a very important signaling pathway in triggering type I IFN production which is essential for cross-priming and thus a strong anti-tumor CTL-mediated immune response[75-77]. In this regard, other approaches to enhance type I IFN in the TME via reprogramming of DCs and TAMs also show potential such as direct delivery of type I IFN[78-80] or induction of type I IFN production via local radiotherapy[81,82].

In addition to reprogramming, other strategies to target TAMs are explored, similar to those applied for MDSC-targeting, that aim to block the pro-tumoral function and limit recruitment[83,84]. The pro-tumoral function can be blocked by apoptosis induction of TAMs or bone marrow mobilization using chemical or synthetic drugs such as Trabectedin or bisphosphonates. Recruitment prevention, on the other hand, can be acquired by blocking chemokines involved in recruitment through antibodies or genetic depletion[83,85]. However, these efforts are still at an early stage and further investigation is essential to further define the mechanisms involved in regulating tolerogenic DCs and TAMs to allow more specific targeting and avoid adverse events.

Regulatory T-cells are, next to MDSCs, another major influencing factor in T-cell exhaustion and lowering the levels or suppression of the functional activity of Treg cells is an absolute must in the attempt to increase the efficacy of therapeutic vaccines. Treg cell depletion can be obtained via unspecific and specific methods of which the latter are preferred for obvious reasons. Unspecific targeting of Treg cells can involve low dose chemotherapeutics such as cyclophosphamide, fludarabine and gemcitabine which favor regulatory T-cells over other T-cells potentially due their higher proliferative state[86,87]. In contrast, specific targeting

comprises targeting of specific receptors that are overexpressed on the cell surface. A few interesting targets are currently tested in the clinic including the high affinity IL2-receptor CD25, the glucocorticoid-induced tumor necrosis factor receptor (GITR), a member of the tumor necrosis factor receptor OX40 and chemokine receptors[86-93]. Although these targets all show potential and are tested in clinical trials, it is of interest to more finely tune the targeting through receptors that are exclusively expressed on Treg cells to increase the efficacy without compromising on adverse events due to lack of specificity[94].

1.3 Immune checkpoint inhibition

Exhausted T-cells express a variety of different immune checkpoints, such as programmed-death 1 (PD-1), lymphocyte activation gene 3 protein (LAG3), CD160, 2B4, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), T-cell immunoglobulin domain and mucin-containing protein 3 (TIM-3) and B-and T-lymphocyte attenuator protein (BTLA) among many others[42,95-97]. Extensive research has led to FDA-approval in 2011 of the CTLA-4 inhibitor Ipilimumab (Yervoy®)[98,99] and two PD1-inhibitors Nivolumab (Opdivo®)[100] and Pembrolizumab (Keytruda®)[101,102] in 2014 for the treatment of renal cell carcinoma and for the treatment of melanoma and non-small cell lung cancer respectively

1.3.1 Anti-CTLA-4

As described earlier CTLA-4 exhibits much higher overall affinity for CD80 and CD86, expressed on the surface of T-cells, compared to the CD28 receptor on the dendritic cell surface. The interaction of the latter with CD80 or CD86 on the T-cell surface provides a co-stimulatory signal necessary for optimal priming of T-cells. CTLA-4 on the other hand does not provide the naive T-cell with the required signal and counteracts the CD28 activity which leads to inhibition of T-cell activation, T-cell proliferation, T-cell survival and production of type I IFN and IL-2 in the initial stage of the immune response[103,104]. This immune control mechanism is a natural defense against overactive T-cells or prolonged T-cell activation to avoid autoimmunity[105,106]. However, cancer cells exploit this phenomenon through recruitment and polarization of CD4+ T-cells into Treg cells that highly express CTLA-4 in order to evade immune eradication.

Blocking CTLA-4 on Treg cells is therefore a highly attractive target to avoid T-cell suppression by Treg cells and has led to FDA-approval. The first FDA-approved immune checkpoint blockade

is the CTLA-4-inhibitor Ipilimumab which offers terminal stage melanoma patients with metastasis clinical significant survival benefits[107-109]. Although these results are very promising, Ipilimumab evokes in 70 to 85 % of the patients any grade of adverse events (AE) and in 20 to 35 % grade 3 to 5 immune-related adverse events which can be attributed to the lack of tumor specificity[110,111]. The most common AEs are low grade, endurable and reversible, however in a minority of patients these can be severe and sometimes irreversible and life-threatening[112-114]. In this regard, many research endeavors for new strategies that are more selective are ongoing to potentially achieve more efficient Treg suppression and decreased AE frequencies.

1.3.2 Anti-PD1

The PD1 inhibitory receptor is another attractive immune checkpoint receptor target as it is expressed on T-cells which, upon interaction with its ligand PDL1, leads to T-cell apoptosis and exhaustion. In contrast to CTLA-4, PDL1 is not exclusively expressed on the surface of regulatory T-cells but can also be present on the cancer cells themselves as well as tol-DCs, TAMs and MDSCs. In addition, the PD-1 pathway limits the activity of previously activated effector T-cells in response to infection or to limit autoimmunity and only interferes in later stages of the immune response opposed to the CTLA-4 pathway. This can potentially explain the lower toxicity profiles of FDA-approved Pembrolizumab en Nivolumab where the majority of the anti-PD-1 treated patients only suffer from low grade AEs[87,115]. Moreover, they have shown dramatic responses in clinical benefit for up to 35 % with advanced and terminal disease[116].

Summarized, immune checkpoint blockade is the most promising strategy that has been developed in the last decade and has significantly boosted the efficacy of cancer immune-therapy and cancer treatment in general. Although this field is expanding, there is still a lot more to discover involving other potential immune checkpoint targets that are currently investigated alongside improvement of toxicity profiles and management. An overview of the different strategies to avoid T-cell exhaustion is shown in Figure 2.

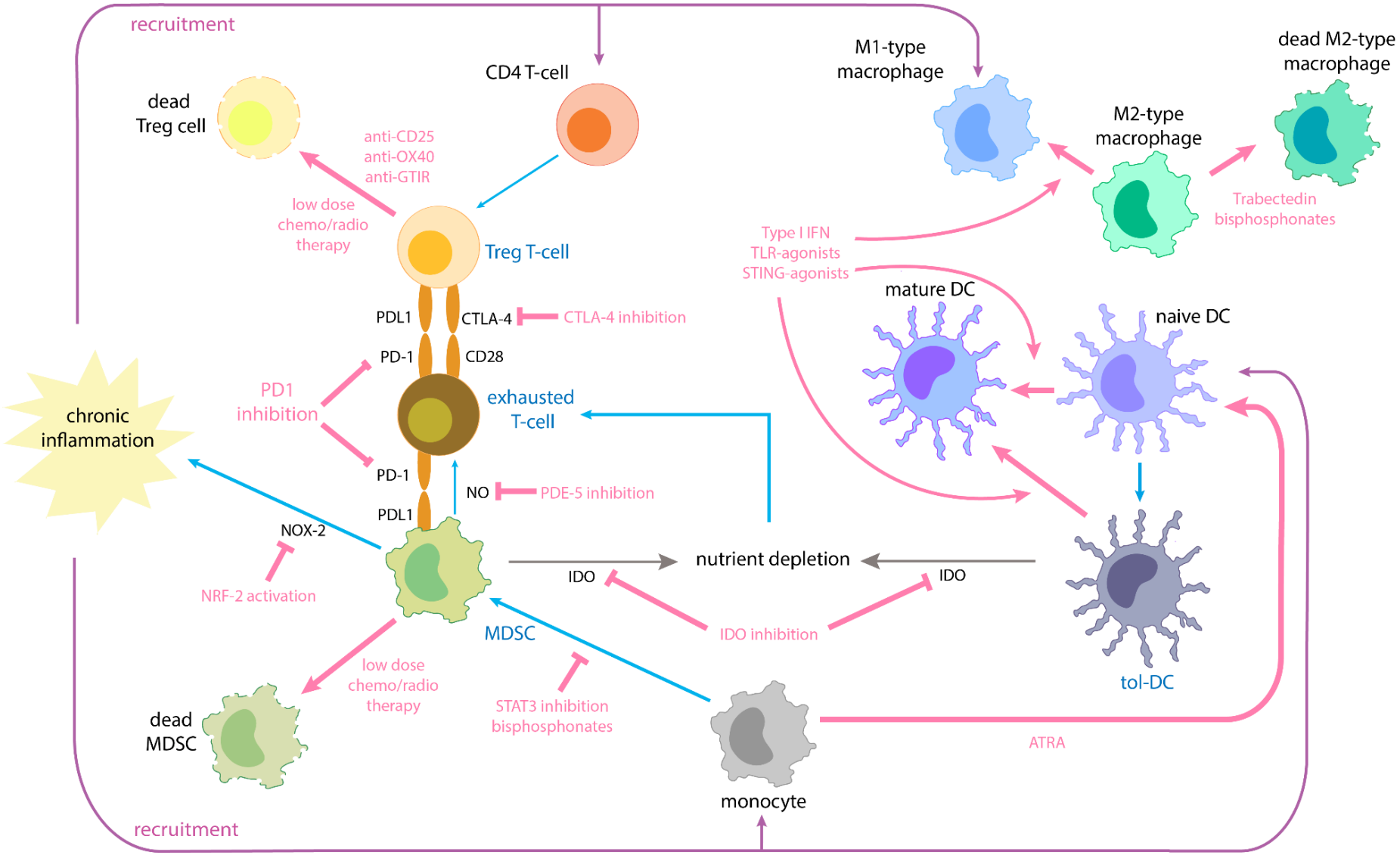

Figure 2. Overview of the main strategies to avoid T-cell exhaustion.

2. Non-T-cell inflamed tumor

Although many promising strategies have emerged to increase the efficacy of cancer immune-therapy through reversal of T-cell exhaustion in T-cell inflamed tumors, some tumors do not respond to these therapies due to T-cell exclusion. Escape through T-cell exclusion is mediated by impaired recruitment of dendritic cells and other innate immune cells and/or deficient T-cell recruitment. These malignancies are described as T-cell non-inflamed, lack inhibitory factors such as PD-1 and CTLA-4 and pose perhaps an even bigger hurdle to current cancer immunotherapeutics[117,118].

One of the main strategies of malignancies to exclude T-cells from the tumor microenvironment is related to DC-recruitment and the β-catenin pathway. In non-T-cell inflamed metastatic melanoma 48 % of the patients showed activation of the β-catenin pathway[119,120]. This clearly revealed an inverse correlation of the latter with recruitment of dendritic cells resulting in decreased chemokine expression and by consequence impaired T-cell recruitment. Next to the β-catenin pathway, additional molecular altered pathways in the TME contribute the non-T-cell inflamed environment of malignancies via reduced recruitment of DCs and T-cells including the PI3K-PTEN-AKT pathway, the STAT-3 signaling pathway and the p53 pathway[95,121-123].

Another point to consider, is the potential influence of the often dense architecture of stroma surrounding the tumor which can act as a physical barrier and potentially confining the T-cells in the stroma. The density and orientation of the stromal extracellular matrix has the ability to positively or negatively influence T-cell migration towards the tumor[124,125]. In addition, the abnormal vasculature due to incomplete angiogenesis can be another limiting factor for T-cell trafficking and by consequence the therapeutic potential of immunotherapies[126,127]. Therefore, targeting these T-cell exclusion strategies in order to reverse non-T-cell inflamed into T-cell inflamed tumors shows potential to enhance the efficacy of immunotherapeutics[128].

PERSONALIZED ANTI-TUMOR IMMUNE-THERAPY

Failure of anti-tumor immunotherapies is often related to lack of immunogenicity or efficacy due to the influence of a combination of different immune-suppressive strategies as discussed above. However, there is another important factor that can have a dramatic effect on therapy response involving personalized medicine. In general, one of the biggest hurdles faced by personalized anti-cancer medicine is affordability. However, the increasing use of personalized strategies in more and more tumor types and its proven potential will aid the cost reduction and implementation in the current practice. Personalized anti-tumor immune-therapy comprises the design of immunotherapeutics that are specifically tailored to the patient's mutanome and/or taking into account the patient's unique immunoscore to increase the probability to induce a durable response.

1. Patient specificity: unique mutanome

Every antigen pool of a certain type of cancer in different patients varies and is referred to as inter-tumoral heterogeneity. In addition, there is also intra-tumoral heterogeneity which involves the diversity in every individual tumor in the same patient. By consequence, traditional antigen vaccines that target one or more known tumor-associated non-mutated antigens (TAAs) often do not have the desired effect as these antigens are unlikely to be expressed in every patient due to mutation or downregulation of the respective antigen and immune-suppression as a result of thymic tolerance. In this regard, patient-derived cancer tissue vaccines and neo-antigen vaccines hold promise as they mind the patient's unique mutanome. Neo-antigen vaccines enclose only tumor-specific mutated antigens (TSAs) which are patient- and tumor-specific whereas patient-derived cancer tissue vaccines involve both TSAs and TAAs.

Strikingly, it has recently been elucidated that neo-antigens not always induce a strong anti-tumor immune response as a high neo-antigen burden does not always correlate with a clinical response due to neo-antigen intra-tumoral heterogeneity. This results in two different subsets of neo-antigens, i.e. clonal and subclonal neo-antigens, that are present in the majority of cancer cells or in only a subset respectively[129]. It is therefore not the quantity of the mutational burden (the total amount of neoantigens, i.e. clonal and subclonal neo-antigens) but the quality (the clonal antigen percentage) that is associated to increased prognosis and survival[116,130].

Interestingly, it has been shown that immunogenic active neo-antigens share peptide sequences that show similarities with pathogen sequences and subsequently can more efficiently prime a strong immune response[131-134].

Although the efficacy of TAA-based vaccines and of TSA- or neo-antigen-based vaccines has not been compared (yet), neo-antigen vaccines are likely to be more potent due to lack of thymic tolerance and the recent findings linking response rates of immune checkpoint inhibitors with clonal neo-antigen burden[129] and neo-antigen specific T-cells[132,135]. By consequence, strategies to specifically define immunogenic clonal neo-antigens show promise to increase the efficacy and immunogenicity of neo-antigen vaccines.

2. Patient selection: biomarkers

Despite the progress that has been made in optimizing the immunogenicity and efficacy of anti-tumor immunotherapeutics, it is imperative to decrease the efficacy variability and increase the probability of developing a durable response to avoid ineffective treatment accompanied with unnecessary toxicities and costs[1]. Screening of patients prior to treatment in order to determine which therapy is suitable and most likely to evoke a durable clinical response, is extensively studied and holds promise in improving response. Conventional screening involves determination of the tumor stage based on standard histopathological evaluation criteria, i.e. tumor burden (T), presence of cancer cells in lymph nodes (N) and evidence of metastasis (M) or the TNM classification[136]. Although TNM classification of a tumor estimates the response to therapy, it is inadequate to predict the clinical outcome of patients as clinical outcome can vary among the same TNM category[137,138]. Moreover, the TNM classification is incomplete as it is only focused on tumor cells and does not take the immune profile of the host into account[135].

Based on the important role of the immune system in tumorigenesis and tumor suppression, implementing immune-screening is essential to fully predict the clinical outcome of patients. Many different immune biomarkers are suggested as a valuable prognostic factor to help guide treatment choice and patient selection comprising the immune contexture, PDL1 and mutational load[139,140]. Due to the complexity of immune cells and their often contradictory effects on the immune response alongside patient variability, establishing an all-embracing screening platform that allows to completely predict the clinical outcome is still far from reality.

2.1 The immune contexture: Immunoscore

The immune contexture involves the type, location, density and functional orientation of the immune cell populations in the tumor, unique for every cancer and patient[141,142]. Analysis of the immune contexture or the determination of the 'immunoscore' of the patient's tumor contributes to characterize the tumor microenvironment and is a very strong prognostic factor[143-145]. On one hand, infiltration of lymphocytes in the tumor is a very important factor as it is related to T-cell exhaustion and non-T-cell inflamed tumors (*vide supra – Efficacy: countering T-cell exhaustion and T-cell exclusion*). Logically, high effector T-cell, TH1-helper cell and memory T-cell infiltration is strongly associated with improved outcome[146-152]. In addition, gene signatures involved in TH1-type responses (IFN-γ, IL-12) are also correlated with good prognosis as they are associated with increased effector and memory T-cell levels[153-156]. Infiltration of Treg cells, on the other, is correlated with poor prognosis due to their immune-suppressive function[157-159]. However, contradictory studies have shown the inverse correlation[160-162]. The same holds true for other potential biomarker immune cell populations, i.e. NK-cells, TH2-helper T-cells and B-cells[141]. This discrepancy requires further detailed analysis of the tumor microenvironment and the dual role of these immune cells alongside synthesis of more specific markers and assays for optimal phenotyping[163].

2.1.1 PDL1

PDL1-positive tumors were related to poor prognosis due to suppression of active T-lymphocytes through the immune checkpoint PD-1 pathway[164-168]. Due to the FDA-approval of PD1-inhibitors such as Nivolumab and Pembrolizumab, PDL1-positive tumors are now considered to have good clinical outcome as expression of PDL1 on tumor cells and immune infiltrates is found to be a potential biomarker for efficacy of immune checkpoint inhibitors[163,169-171]. Although the majority of the studies have shown good clinical outcome of immune checkpoint inhibitors in PDL1-positive patients, some reveal reverse correlation, i.e. PDL1-negative malignancies have shown good response or no response was observed for PDL1-positive phenotypes[172,173]. It is thereby important to consider that PDL1-expression can be induced and can have region-specific intensities which complicates screening[116].

2.1.2 Mutational load: neo-antigen load

Malignancies exhibiting high mutation rates are known to be more aggressive and tend to metastasize easier. However, higher frequency of DNA alterations increases the chance of inducing tumor-specific antigen mutations resulting in neo-antigens and thus recognition by the immune system. Indeed, a high mutational load is strongly correlated with neo-antigen expression and by consequence good clinical outcome[132,174-175]. This needs to be expanded, however, as a high neo-antigen load does not always results in favorable prognosis. It has been shown that only high clonal neo-antigen load will lead to response in contrast to subclonal neo-antigen burden[176].

2.1.3 Other biomarkers

In this regard, many other factors are currently investigated as potential biomarkers for tumor screening among which the most important are related to physiological tumor-specific parameters. These comprise serum prognostic markers such as IDO, C-reactive protein, hypoxia and MHC-I. IDO or indoleamine-2,3-dioxygenase metabolizes tryptophan into kynurenine which primes naive CD4+ T-cells to differentiate into Treg cells and depletes the local environment from tryptophan, essential for T-cell proliferation (*vide supra – Chapter 2: Tumor immune-escape*). IDO is correlated with poor prognosis, however, further research is needed to establish whether IDO can be used as an independent biomarker[177-180]. C-reactive protein is induced by cytokines in response to tumor inflammation and is associated with poor clinical outcome[181-183] just like intra-tumoral hypoxia[184-186] which impairs oxidative phosphorylation by T-cells for its energy supply. Another interesting biomarker involves expression of MHC-I which is positively correlated with good clinical outcome[187-189]. As earlier stated, downregulation or lack of expression of MHC-I by tumors results in immune-escape because the cancer cells cannot be recognized anymore by the immune system (*vide supra – Chapter 2: Tumor immune-escape*). However, this correlation can also be reversed in tumors that completely lack MHC-I expression probably due to more efficient NK-cell mediated lysis[190,191].

Although progress had been made in defining biomarkers to aid patient and treatment selection, it is clear that further optimization of the parameters is needed and more detailed knowledge needs to be gained about the tumor microenvironment to fully understand the mechanisms. Moreover, inter-tumoral and intra-tumoral heterogeneity further complicates

the search for independent biomarkers. Nevertheless, the use of biomarkers has dramatically improved the efficacy of anti-tumor immunotherapies and will undoubtedly continue this in the future.

TOWARDS THE IDEAL THERAPEUTIC VACCINE?

Anti-tumor immune-therapy has gained more and more interest in the last decade and has shown it can be an added value as anti-tumor strategy due to the importance of the immune system in tumorigenesis. The FDA approval of the first therapeutic DC-based vaccine Sipuleucel-T (Provenge®) for metastatic castration-resistant prostate cancer in 2010 and the checkpoint inhibitors Ipilimumab (Yervoy®) in 2011 for renal cell carcinoma, Nivolumab (Opdivo®) and Pembrolizumab (Keytruda®) in 2014 for melanoma and non-small cell lung cancer, has boosted the field of anti-tumor immune-therapy drastically.

Cancer vaccines targeting dendritic cells *in vivo* is an attractive strategy as it can be personalized and tailored to the patient's mutanome containing neo-antigens or patient-derived cancer tissue and it can induce immunological memory while inducing relatively mild side effects (*vide supra – Chapter 2: Defined synthetic antigen vaccines*). The paradoxical role of the immune system in promotion and prevention of tumor growth is now more and more unraveled and has improved and boosted the development of more immunogenic, specific, more efficient therapeutic vaccines. Moreover, it is evident that optimal treatment of patients requires a personalized and multistep approach specifically tailored to every single patient in order to fully eradicate the malignancy and induce a prolonged protective effect. Combining different strategies to attack the malignancy holds promise due to the induction of synergistic effects that will weaken the malignancy on different levels more efficiently. In addition, implementing a personalized approach will evoke patient-specific immune reactions and will consider tumor-specific biomarkers as well to further increase the efficacy and potency of the vaccine. Personalized cancer vaccination involves primarily formulation of the patient's mutanome to avoid lack of efficacy due to mutation, MHC-I downregulation or lack of expression and thymic tolerance. Moreover, the incorporation of multiple antigens (neo-antigens, cancer cell lysate or cells) into vaccine particles in combination with multiple PAMPs and DAMPs will increase the probability of evoking a broader and all-embracing cytotoxic and memory T-cell response.

Nevertheless, therapeutic cancer vaccination has not lived up to its expectations yet which can be likely attributed to the immune-suppressive tumor microenvironment and stroma, physiologically and physically protecting the malignancy against attacks from the immune system. In this regard, it is necessary to combine cancer vaccination with strategies that tackle these immune-suppressive mechanisms. Therefore, personalized cancer vaccination also requires consideration of tumor-specific biomarkers to select the most optimal combination of treatments. The latter ensures better efficacy and avoids unnecessary costs and toxicity.

Involving the immunoscore, in case of low infiltration of lymphocytes or T-cell exclusion, it is advisable to proceed the treatment with a strategy that reverses non-T-cell inflamed tumors into T-cell inflamed tumors. In contrast, tumors that exhibit high infiltration of immune-suppressive cells such as Treg cells[192-196], MDSCs[197-200], TAMs and tol-DCs[201] and are vaccinated with cancer vaccines combined with additional strategies that will block, eliminate or reprogram the respective cells to lower the probability of T-cell exhaustion show promise. Next to the immunoscore, other interesting biomarkers such as PDL1, IDO and lack of MHC-I expression can be implemented to enhance the efficacy of cancer vaccines involving immune checkpoint inhibitors, IDO-inhibitors and low dose chemotherapy or IFN-γ therapy respectively. Indeed, combination of immune checkpoint inhibitors with cancer vaccines have shown enhanced clinical outcome without increase of adverse events[202-207]. Furthermore, promising results have also been reported revealing the additive effect of combining IDO inhibitors with therapeutic vaccines[208,209]. Of note is the combination with low dose chemo-and radiotherapy or IFN-γ therapy where caution is needed as in some cases lack of MHC-I can also be correlated with good prognosis due to increased susceptibility to NK-cell recognition (*vide infra – Chapter 1: The innate immune system*). Nevertheless, promising data is obtained in several cancer types, demonstrating the advantage over monotherapy[210-214].

Summarized, the future of anti-cancer immune-therapy is bright and will further contribute to the fight against cancer. Personalized therapeutic vaccination combined with other anti-tumor immunotherapies, taking into account patient- and tumor specific markers, holds major promise. However, further research and more clinical studies are necessary to confirm and corroborate these findings indicating it is better to invade the tumor microenvironment with an army of highly specialized ‘soldiers’ instead of only one.

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CHAPTER 8

SUMMARY AND GENERAL CONCLUSIONS

Today cancer remains one of the main causes of death worldwide and it is predicted that the incidence rate of men and women diagnosed with cancer in their lifespan will increase up to 40 percent. Fortunately, extensive research has dramatically reduced the mortality rate due to improvement of the existing therapies together with the emergence of new therapeutic strategies in the immuno-oncology field. As stated in Chapter 1, the immune system is designed to recognize and eliminate infections through a smart, combined attack of immune cells of the innate and adaptive immune system. Interestingly, it has been elucidated that the immune system plays a dual role in cancer development through protection of the host against tumorigenesis (immunosurveillance) and, in contrast, promotion of tumor growth (tumor immune-escape).

This complex relationship is discussed in detail in Chapter 2 and unravels the immune-suppressive strategies used by cancer cells in order to evade immune recognition and eradication. In this regard, it is clear that subtle differences in immune cell populations can drastically change the action of the immune system from immunosurveillance to immune-escape. This points out the potential of cancer immune-therapy to shift the balance of a pro-

tumoral environment towards an unfavorable setting for cancer cells via manipulation of the immune-suppressive cells and the tumor microenvironment.

This work aims to contribute to the development of cell-lysate based anti-cancer vaccines. The potential advantage over conventional or other immunotherapeutic strategies are the less severe side effects and the induction of immunological memory to provide prolonged protection against metastasis or relapse. Although cancer vaccination is a promising immunotherapeutic strategy in the battle against cancer and despite extensive research in this field, it has not lived up to the expectations. There is still an unmet need for formulation strategies that allow easy, mild and efficient incorporation of cancer antigens into immunogenic vaccines.

In Chapter 2, the requirements for vaccines to efficiently target and activate dendritic cells *in vivo* are described and the hypothesis is raised that cancer vaccines need to resemble to pathogens in order to evoke a strong anti-tumor response. This involves formulation of cancer antigens into particulate carriers, to enhance uptake efficiency by dendritic cells, engineered with pathogen- and/or damage-associated molecular patterns (DAMPs and/or PAMPs), to evoke DC maturation via stimulation of pathogen recognition receptors (PRRs). PRR-stimulation is essential for the priming of cytotoxic CD8+ T-cells that hold the capacity to recognize and eliminate tumor cells. In addition, another important hurdle in cancer vaccine design is the lack of efficacy and the low applicability of many of the current approaches. This can be attributed to the use of single or multiple tumor-associated antigens that are limited in use as antigens of many types of cancer are still unidentified and are more prone to treatment failure due to mutation or lack of expression. This can be circumvented with personalized cancer vaccination that primes the patient's immune system not only against tumor-associated antigens but also against the patient's individual tumor-specific antigens potentially leading to more potent immune responses tailored to the patient's unique tumor mutanome. Note that personalized cancer vaccines contain cancer antigens obtained from patient-derived tissue and requires sufficient amount of tumor and thus is only applicable for solid tumors that can be surgically resected. On one hand, neo-antigen vaccines involve individual tumor-specific antigens identified via genomic analysis which is costly, labor intensive and complex. Patient-derived

cancer cells or cell lysate containing vaccines, on the other, could have an important advantage in terms of cost and labor burden.

In this regard, in this thesis four different strategies are developed that allow for the formulation of potent personalized cell-derived cancer vaccines as illustrated in **Figure 1**. **Chapter 3** and **Chapter 4** deal with the formulation of soluble cancer cell lysates whereas **Chapter 5** and **6** focus on the formulation of intact cancer cells into microparticles which additionally comprise cell membrane components, and when translated to whole tumor tissue, also offer the possibility to co-encapsulate stromal proteins.

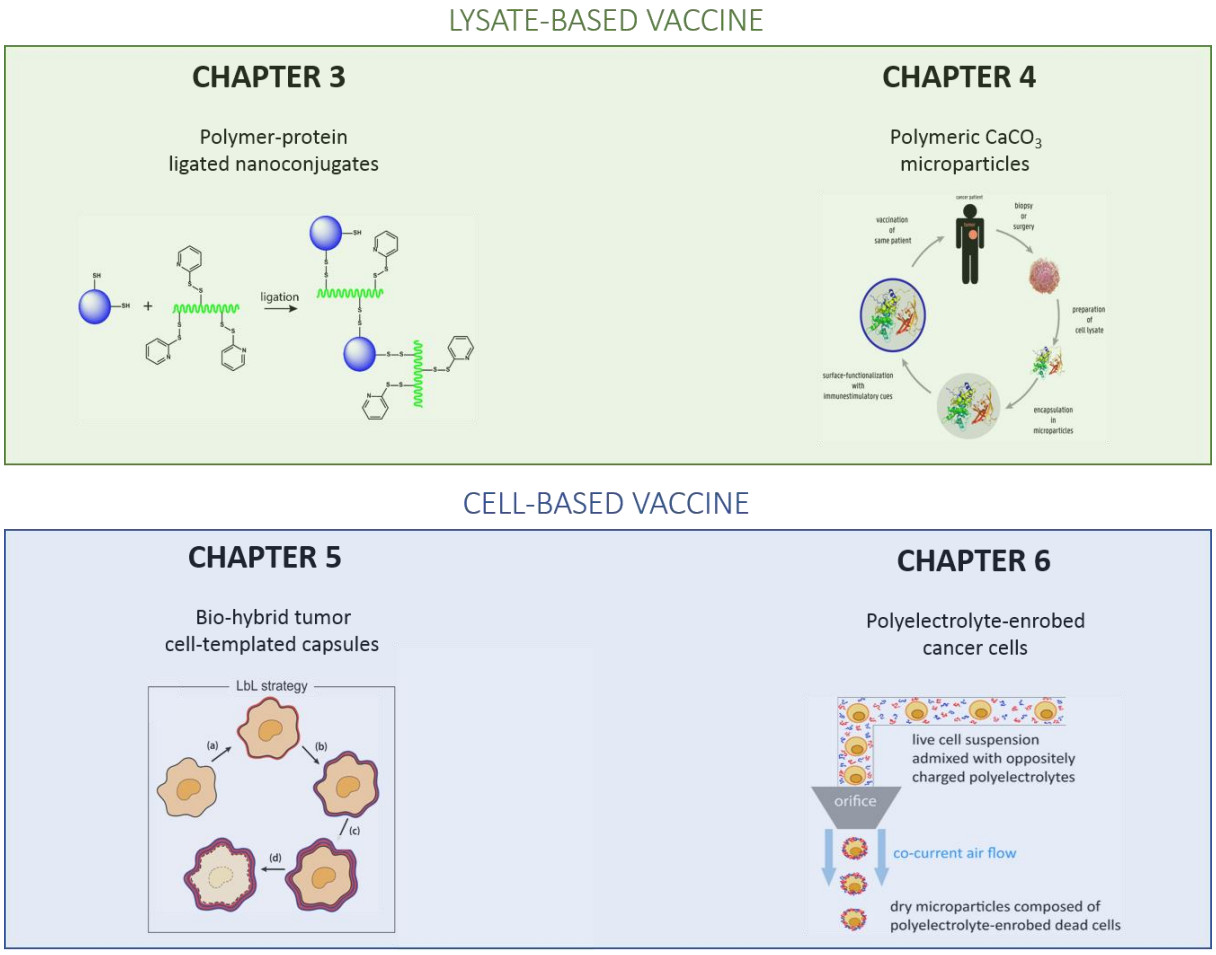


Figure 1. Overview of the formulation strategies that are developed in this thesis, divided into two cell lysate-based vaccines (green) and two intact cell-based vaccines (blue).

In general, extensive focus is devoted in this work to obtain simple, efficient and potent vaccine formulation strategies of cell-derived antigens into pathogen-like particles regarding size and immunogenicity. The latter is either obtained by introduction of TLR-agonists (PAMPs) or immunogenic pre-treatment of the cells considering heat shock protein (DAMPs) expression.

Chapter 3, deals with the design of polymer-protein conjugates formed by disulfide exchange. This concept was grounded on three attractive properties: [1] disulfides can be readily formed with antigens via reaction with free thiols on cysteine residues; [2] disulfides are stable under extracellular conditions but are reduced to free thiols in the cytoplasm of cells; and [3] disulfide exchange. For this purpose, a co-polymer of HPMA and APMA (poly(HPMA-co-APMA)) was synthesized that bears pending pyridyldisulfide moieties (further denoted as poly(HPMA-PDS)) followed by assessment of the conjugation efficiency of the polymer to a model antigen ovalbumin (OVA) whether or not substituted with additional thiols. It was found that poly(HPMA-PDS) is well suited for efficient reversible conjugation of OVA, providing that the protein is modified with protected thiols. *In vitro* analysis revealed that the polymer-protein conjugates show increased cellular uptake, relative to unconjugated protein. This is attributed to disulfide exchange between remaining pyridyldisulfide moieties and exofacial thiols present on the cell surface. Furthermore, the formulation demonstrated to increase antigen presentation by bone-marrow derived DCs (bmDCs) to CD8+ T-cells *in vitro*.

Following characterization of this formulation with OVA, attempts to conjugate cancer cell lysate unfortunately failed. It was not possible to introduce protected thiols to cancer cell lysate due to aggregate formation. Nevertheless, this formulation strategy has potential as a formulation platform for the design of vaccines containing tumor-associated antigens or neo-antigens. Therefore, it would be interesting to conjugate TAAs or neo-antigens to poly(HPMA-PDS) together with further optimization of the formulation regarding the amount of introduced protected thiols. In detail, lowering the percentage of introduced protected thiols on cancer antigens in conjunction with increasing the pending pyridyldisulfide moietes on the polymer is of interest to obtain similar conjugation efficiencies with a minimum risk of epitope loss. Furthermore, assessment of the effect of polymer conjugation on lymphatic antigen transportation as well as conjugation of molecular adjuvants such as TLR-agonists to increase immunogenicity should be performed in future research.

As this work aims to develop, however, cell-derived cancer vaccines, Chapter 4 elaborates on an alternative strategy to encapsulate cancer cell lysate as such, without the need for functionalization of the proteins and thus avoiding epitope loss and aggregation issues. For this purpose, porous calcium carbonate ($CaCO_3$) microparticles obtained by a one-step

precipitation reaction, in the presence of cancer cell lysate, were explored. This approach was chosen based on multiple attractive properties such as its widespread use in protein encapsulation, its high loading capacity for macromolecules, its ease of production and its low cost. In addition, the synthesis can be performed under very mild conditions in aqueous medium without the need of any organic solvents, reactive chemistry or high energy input. Indeed, this approach resulted in the efficient incorporation of cancer cell lysate into non-aggregated spherically shaped $CaCO_3$ microparticles that strongly enhanced uptake efficiency leading to an improvement of cross-presentation by dendritic cells *in vitro* opposed to non-particulate cell lysate. To increase the potency of $CaCO_3$ microparticles as vaccine carriers, immunogenicity was introduced via adsorption of a small molecule toll like receptor 7/8-agonist CL264 conjugated to poly(HPMA-APMA) (further denoted as CL264-poly(HPMA-APMA)) to the microparticle surface. This is essential in order to enable efficient priming of a robust anti-tumor immune response via the induction of DC maturation through TLR-activation. TLR7/8-triggering in particular is attractive in the context of tumor vaccination as this leads to elevated levels of type I IFN and IL-12, which are key cytokines to promote TH1- and cytotoxic T-cell responses required for potent anti-tumor immune responses. The TLR7/8-ligand was conjugated to the polymer backbone as it has been shown previously that lipid-, polymer- and nanoparticle-conjugation of small molecule ligands strongly reduces systemic inflammation and yields potent lymph node localized responses that enhance the adaptive immune response against co-delivered antigens. *In vitro* activation of bmDCs and RAW blue macrophages revealed that the polymer-conjugation of the TLR7/8-agonist did not reduce the activity of the ligand. Moreover, the agonist was more potent when adsorbed onto the microparticle surface which can be explained by the more efficient uptake which leads to enhanced interaction of the TLR-ligand with the receptor upon cell uptake.

Regarding these results, formulation of cancer cell lysate into immunogenic $CaCO_3$ microparticles shows promise as a mild and efficient strategy to encapsulate cancer lysates. Further assessment of the potency of the vaccine can include introduction of one or more additional TLR-agonists alongside follow-up experiments to unravel the induced cytokine spectrum.

The encapsulation of cancer cell lysate, however, does not include cell membrane proteins and stromal proteins, when translated to whole tumor tissue. Thererfore, this work also devoted focus to vaccin formulations involving intact cancer cells. In Chapter 5, live cancer cells were used as templates for layer-by-layer (LbL) assembly of complementary interacting components followed by hypo-osmotic treatment to obtain bio-hybrid capsules loaded with cancer cell lysate within the hollow void of the obtained capsules. The LbL assembly technique, to design a synthetic semi-permeable membrane onto non-planar substrates, is an appealing strategy as it allows easy, all-aqueous and mild encapsulation of a wide variety of species. Initial experiments were performed using the oppositely charged polyelectrolytes, poly-L-arginine (P_LARG) and dextran sulfate (DEXS) based on previous work that showed multilayer capsules composed of these polyelectrolytes are biocompatible, degradable *in vitro* and *in vivo* and induce broad cellular and humoral immune responses against the encapsulated antigen. This led, however, to instantaneous aggregation, cell lysis and cell death upon incubation of the living cells with poly-L-arginine. Less harsh complementary interacting components were therefore chosen to coat the cancer cells. This strategy was based on the use of poly(vinylpyrrolidone) (PVP) and tannic acid (TA) that form hydrogen-bonded complexes. As the aim of our work is to encapsulate whole cancer cells, it is important to preserve cell integrity as much as possible while affecting cell viability as little as possible to retain a maximum amount of cellular proteins within the LbL coating. In this regard, particular focus was devoted to elucidate the optimal coating components and conditions. It was found that deposition of two PVP/TA bilayers followed by hypo-osmotic lysis yielded cell-templated bio-hybrid capsules containing a high amount of encapsulated proteins. Furthermore, it was confirmed that, upon hypo-osmotic lysis, the cancer cells were dead which is of interest as, upon administration, regrowth of new tumors due to residual living cells often occurs in case of whole cell-based lysates. Further, immunogenic properties were engineered into the capsules, in a proof of concept study, by pre-treatment of the cancer cells with heat shock to induce expression of DAMPs, important endogenous immune-activators.

To assess the potential of the bio-hybrid cell-templated capsules, preliminary *in vitro* uptake experiments were performed which revealed only 5% of the capsules to be taken up. We hypothesized that this could be attributed to the large size (above 10 µm) of the cell-templated capsules for efficient uptake by dendritic cells. Several attempts were subsequently made to

decrease the cell-templated capsules in size through exposure to salt or high temperature. This resulted, however, in degradation or aggregation of the particles.

In this regard, an alternative strategy was developed in Chapter 6 to formulate intact cancer cells into vaccine particles by a single-step method which is substantially less labor-intensive and time-consuming and thus avoids unnecessary cell loss opposed to the layer-by-layer coating of cancer cells. A simple, yet efficient single-step method was characterized that encapsulates whole cancer cells in polyelectrolyte microparticles by spray drying. Porous and non-aggregated polyelectrolyte-enrobed microparticles loaded with dead cancer cells were obtained by admixing mannitol and live cancer cells with the oppositely charged polyelectrolytes, DEXS and P_LARG in aqueous medium prior to spray drying. Similar to the cell-templated bio-hybrid capsules (Chapter 5), the polyelectrolyte-enrobed cancer cells were dead which could avoid tumor regrowth upon administration. The polyelectrolyte-enrobed cancer cells, upon redispersion in PBS buffer, were stable as the microparticles did not release cell proteins in the supernatant. *In vitro* evaluation revealed that the microparticles were internalized to a much larger extent by dendritic cells and significantly enhanced antigen cross-presentation, relative to cell lysate. In analogy to the cancer cell lysate-containing $CaCO_3$ microparticles described in Chapter 4, immune-stimulating cues were introduced by co-spray drying of the vaccine components with CL264-poly(HPMA-APMA) yielding immunogenic microparticles that strongly promoted TLR-activation.

These results show the potential of the polyelectrolyte-enrobed cancer cells as immunogenic antigen carriers. Introduction of multiple TLR-ligands and subsequent assessment of the optimal conditions for T-cell priming, as proposed for the lysate-containing $CaCO_3$ microparticles, is thereby also of interest to further increase the potency of this formulation. Furthermore, immunogenic treatment or induction of immunogenic cell death of the cancer cells, prior to spray drying, to induce DAMP-production could be an additional approach to increase the potency of DC activation and CTL-mediated anti-tumor responses.

In conclusion, regarding the cell lysate-based vaccines, the polymeric $CaCO_3$ microparticles appeared to be more promising opposed to the polymer-protein ligated nano-conjugates in terms of applicability. $CaCO_3$ microparticles allowed for efficient encapsulation of cell lysate, without the need of functionalization, into immunogenic particles that efficiently activate

dendritic cells *in vitro*. The cell-based vaccines, on the other, revealed the polyelectrolyte-enrobed cancer cells to be superior compared to the bio-hybrid tumor cell-templated capsules as this method allowed for one-step formulation of cancer cells into polyelectrolyte particles that show promising results *in vitro* in terms of uptake efficiency, MHC-I cross presentation induction and immunogenicity.

For future experiments, it would be of interest to assess the potency of these two promising vaccines *in vivo*, investigating the induction of anti-tumor immunity. Initial *in vivo* experiments could be performed using an immunogenic murine cancer cell line that stably expresses OVA as this enables thorough screening with readily available assays. In addition, it would be particularly interesting to compare the formulation that appears to be the most promising *in vivo* with a neo-antigen containing vaccine.

Overall, this thesis explored four different strategies to efficiently encapsulate cancer cell lysate or cancer cells into immunogenic personalized vaccine particles. This thesis shows the beneficial effect of antigen formulation into pathogen-like particles engineered with immune-stimulating cues, in this case TLR-agonists, with the respect to antigen uptake and activation of dendritic cells.

CHAPTER 9

SAMENVATTING EN ALGEMENE CONCLUSIES

Kanker blijft op dit moment nog steeds één van de grootste doodsoorzaken werelwijd. Daarnaast is het voorspeld dat de incidentie van mannen en vrouwen die worden gediagnosticeerd met kanker in hun leven, zal stijgen tot 40 procent. Gelukkig heeft uitgebreid onderzoek het sterfte percentage drastisch doen dalen door het verbeteren van de huidige therapieën samen met de opkomst van nieuwe therapeutische strategieën in immuno-oncologie. Zoals besproken in hoofdstuk 1, is het immuun systeem in staat om infecties te herkennen en te verwijderen via een slimme en gecombineerde aanval door immuun cellen van het aangeboren en het verworven immuun systeem. Daarnaast blijkt dat het immuun systeem een tegenstrijdige rol heeft in de ontwikkeling van kanker aangezien deze zijn gastheer kan beschermen (immuun-beschermende rol) maar ook kankergroei kan promoten (immuun-ontwijkende rol).

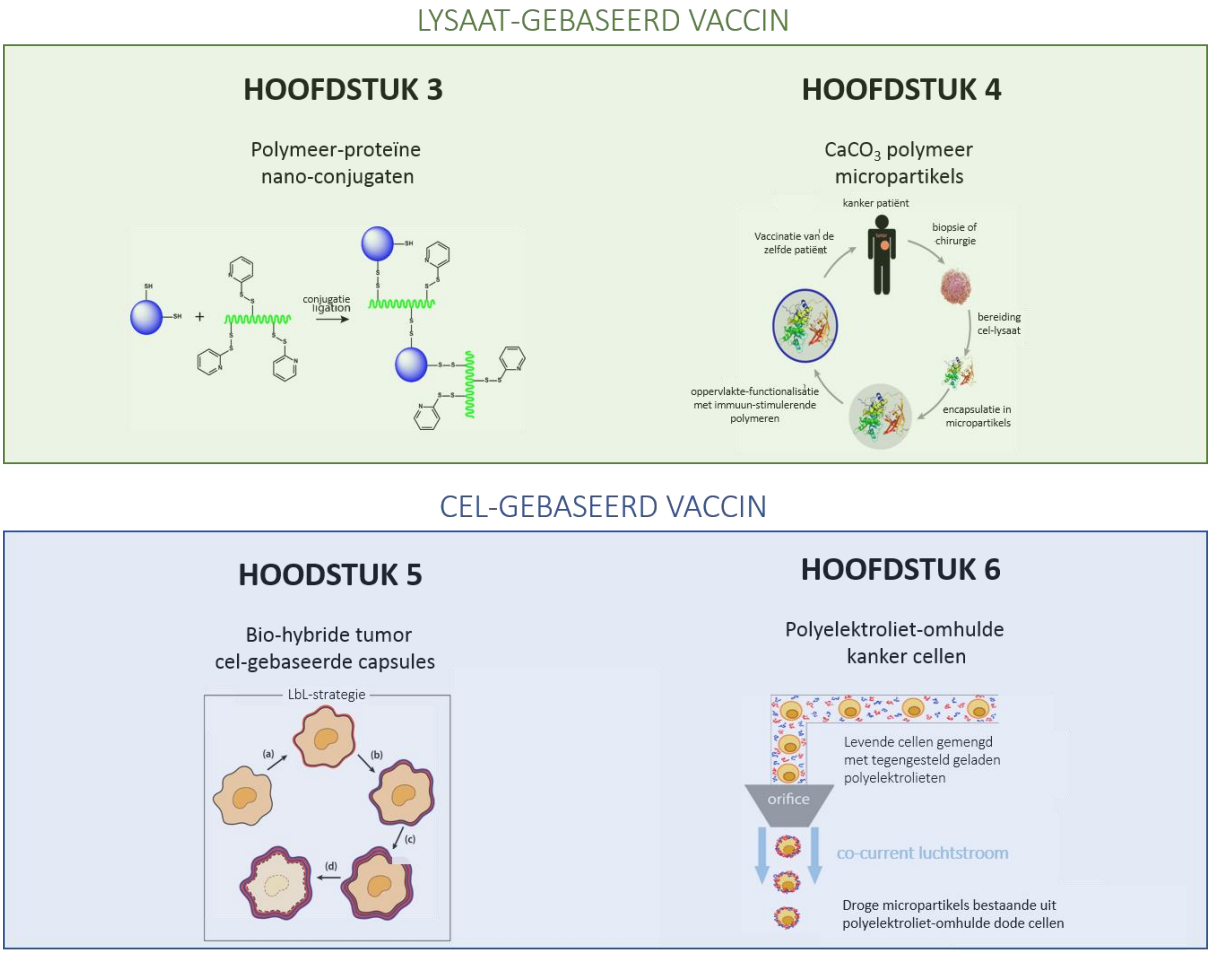
Deze complexe relatie wordt in detail bediscussieerd in hoofdstuk 2. De strategieën van immuun-onderdrukking die door de tumor cellen worden toegepast, om herkenning en eliminatie door het immuun systeem te ontwijken, worden ontrafeld. Daarbij wordt het duidelijk dat subtiele verschillen in immuun celpopulaties de functie van het immuun systeem

drastisch kunnen beïnvloeden van de immuun-beschermende naar de immuun-ontwijkende rol. Dit toont het potentieel aan van kanker immunotherapieën om de balans te verschuiven van een tumor-gunstige naar een tumor-ongunstige omgeving voor kanker cellen via manipulatie van immuun-onderdrukkende cellen en tumor micro-omgeving.

Deze thesis doelt bij te dragen aan de ontwikkeling van anti-kanker vaccins die gebaseerd zijn op cel-lysaat, afgeleid van de tumor van de patient. Het potentieel voordeel hiervan, in vergelijking met de standaard en andere immunotherapeutische strategieën, is het feit dat bij kanker vaccinatie minder sterke bijwerkingen worden gerapporteerd samen met het feit dat immunologisch geheugen wordt opgebouwd die een verlengde bescherming kan bieden tegen uitzaaiingen en herval. Ondanks dat kanker vaccinatie een veelbelovende immunotherapeutische strategie is in het gevecht tegen kanker en ondanks uitvoerig onderzoek, heeft dit nog niet de hoge verwachtingen ingevuld. Er is nog steeds een onvervulde nood aan formulatie strategieën die eenvoudige, milde en efficiënte encapsulatie toelaten van kanker antigenen in immunogene vaccins.

In hoofdstuk 2, worden de voorwaarden voor het efficiënt targeten and activeren van dendritische cellen (DCs) *in vivo* beschreven en wordt de hypothese gesteld dat kanker vaccins moeten lijken op pathogenen om een sterke anti-tumor respons te kunnen vervoorzaken. Dit houdt de formulatie in van kanker antigenen in partikels, om opname efficiëntie te verhogen door DCs, samen met pathogeen- en/of schade-geassocieerde moleculaire patronen (PAMPs en/of DAMPs), om maturatie van DCs te veroorzaken via de stimulatie van receptoren die pathogenen kunnen herkennen. Stimulatie van deze receptoren is essentieel voor het stimuleren van cytotoxische CD8 T-cellen die tumor cellen specifiek kunnen herkennen en vervolgens elimineren. Daarnaast is er nog een belangrijk probleem in het maken van vaccins namelijk het ontbreken van effectiviteit en de lage toepasbaarheid van vele van de huidige strategieën. Dit kan worden toegewezen aan het gebruik van een enkel of meerdere tumor-geassocieerde antigenen die gelimiteerd zijn in gebruik omwille van twee redenen: [1] bij vele types kanker zijn er nog steeds geen antigenen geïdentificeerd; en [2] omdat deze antigenen hogere kans hebben op het mislukken van de behandeling wegens mutatie of het ontbreken van expressie van het antigen door de tumor. Persoonlijke kanker vaccinatie kan deze limitatie omzeilen omdat het immuun systeem niet enkel wordt geactiveerd tegen tumor-geassocieerde

antigenen maar ook tegen tumor-specifieke antigenen van de patiënt zelf waardoor dit potentieel kan leiden tot een potente immuunrespons die uniek is voor de patiënt. Noteer hierbij dat persoonlijke kanker vaccins antigenen bevatten die worden opgezuiverd van kankerweefsel van de patient en er voldoende hoeveelheid kankerweefsel nodig is waardoor dit enkel kan toegepast worden bij vaste tumoren die chirurgisch kunnen worden verwijderd. Enerzijds, houden neo-antigen of nieuw-antigen vaccins individuele tumor-specifieke antigenen in die worden bepaald via genoom-analyse wat bijzonder duur, arbeidsintensief en complex is. Vaccins die anderzijds volwaardige kanker cellen bevatten of kanker cel-lysaat van de patiënt, kunnen hierbij een belangrijk voordeel bieden qua kost en werklast.



Figuur 1. Overzicht van de formulatie strategieën die ontwikkeld zijn in deze thesis, opgedeeld in twee cel-lysaat-gebaseerde vaccins (groen) en twee intacte cel-gebaseerde vaccins (blauw).

Hierop gebaseerd, werden in deze thesis vier verschillende strategieën ontwikkeld die het mogelijk maken om vaccins te maken, afgeleid van de kankercellen van de patient. Een overzicht van deze strategieën wordt geïllustreerd in **Figuur 1**. **Hoofdstuk 3** en **hoofdstuk 4**

behandelen de formulatie van oplosbare kanker cellysaten terwijl hoofdstuk 5 en hoofdstuk 6 focussen op de formulatie van volledige kankercellen in micropartikels. Deze laatste bevatten daardoor additioneel componenten van het membraan van cellen en, in geval van volledig tumorweefsel, proteïnen afkomstig van tumor stroma.

Samengevat, werd in dit werk zeer uitgebreid de focus gelegd op het verkrijgen van eenvoudige, efficiënte en potente vaccin formulatie strategieën, bestaande uit antigenen afgeleid van kankercellen, in partikels die lijken op pathogenen wat betreft grootte en immunogeniciteit. Deze laatste werd verkregen door introductie van TLR-agonisten (PAMPs) of door immunogene voorbehandeling van de cellen met het oog op heat shock proteïne (DAMPs) epxressie.

Hoofdstuk 3 houdt het design in van polymeer-proteïne conjugaten gevormd door uitwisseling van disulfiden. Dit concept werd gebaseerd op drie attractieve eigenschappen: [1] disulfiden kunnen direct worden gevormd met antigenen via reactie met vrije thiolen op de cysteïne-residu's; [2] disulfiden zijn stabiel in extracellulaire condities maar worden gereduceerd tot vrije thiolen in het cytoplasma van cellen; en [3] disulfide-uitwisseling. Hiervoor werd een co-polymeer gesynthetiseerd bestaande uit HPMA en APMA (poly(HPMA-APMA)) die pyridyldisulfide groepen bevat (wordt verder beschreven als poly(HPMA-PDS)) gevolgd door het bepalen van conjugatie-efficiëntie van het polymeer met een voorbeeld-antigen. Het werd bevonden dat poly(HPMA-PDS) geschikt is voor efficiënte reversiebele conjugatie van ovabumine (OVA), op voorwaarde dat het proteïne werd gemodificeerd met beschermde thiol-groepen. *In vitro* analyse toonde aan dat de polymer-proteïne conjugaten verhoogde opname vertoonden, in vergelijking met niet-geconjugeerd proteïnen. Dit kan worden toegewezen aan disulfide uitwisseling van resterende pyridyldisulfide groepen en thiolen die aanwezig zijn op het oppervlak van cellen. Daarbijkomend, werd er aangetoond dat de formulatie leidde tot verhoogde antigen-presentatie door DCs, afgeleid van beenmerg, aan CD8+ T-cellen *in vitro*.

Na karakterisatie van de formulatie met het voorbeeld-antigen OVA werden er verschillende pogingen gedaan om kanker cel-lysaat te conjugeren, die echter faalden. Het was namelijk niet mogelijk om beschermde thiol groepen te introduceren op de kanker cel-lysaat proteïnen wegens problemen met aggregatie. Desondanks heeft deze formulatie strategie potentieel als platform voor het aanmaken van vaccins die tumor-geassocieerde antigenen of neo-antigenen

bevatten. Daarom zou het interessant kunnen zijn om tumor-geassocieerde antigenen of neo-antigenen te conjugeren met poly(HPMA-PDS) samen met verdere optimalisatie van de formulatie aangaande de hoeveelheid geïntroduceerde beschermde thiolen. In detail wordt hiermee het verlagen van het percentage aan geïntroduceerde beschermde thiolen op de kanker antigenen samen met het verhogen van de hoeveelheid pyridyldisulfide groepen op het polymeer beoogd. Dit met het doel om gelijkende conjugatie efficiënties te bekomen met een minimaal risico op verlies van epitopen. Daarnaast, kan het effect van de polymeer-conjugatie op het lymfatisch transport van het antigen worden bestudeerd net als conugatie met moleculaire adjuvantia, zoals TLR-agonisten in de toekomst.

Aangezien deze thesis doelt op de ontwikkeling van kanker vaccins die materiaal van kanker cellen bevatten van de patient, wordt er in hoofdstuk 4 een alternatieve strategie voorgesteld om kanker cel-lysaat te encapsuleren, zonder verdere functionalisatie en dus met vermijden van verlies van epitopen en aggregatie problemen. Hiervoor werden poreuze calcium carbonaat ($CaCO_3$) micropartikels onderzocht d.m.v. een 1-staps precipitatie reactie in de aanwezigheid van kanker cel-lysaat. $CaCO_3$ microparticles werden aangehaald wegens hun interessante eigenschappen zoals het veelzijdig gebruik ervan voor eiwit-encapsulatie, de hoge ladingscapaciteit voor macromoleculen, het gebruiksgemak en de lage kost. Daarnaast, kan de synthese worden uitgevoerd in zeer milde omstandigheden in waterig medium zonder gebruik van organische solventen, reactieve chemie of hoge energie toevoer. Deze aanpak resulteerde in efficiënte encapsulatie van kanker cel-lysaat in niet-geaggregeerde sferische $CaCO_3$ microparticles die sterk verhoogde opname efficiëntie vertoonden samen met verbeterde cross-presentatie door DCs *in vitro*, in tegenstelling tot opgelost kanker cel-lysaat. Om het potentieel te verhogen van de $CaCO_3$ microparticles als vaccins, werd een toll-like receptor-7/8 agonist CL264, geconjugeerd met poly(HPMA-APMA) (verder vermeld als CL264-poly(HPMA-APMA)), geïntroduceerd via adsorptie op het oppervlak van de micropartikels. Het introduceren van immunogeniciteit is essentieel voor het efficiënt induceren van een robuuste anti-tumor immuunrespons via het induceren van DC-maturatie d.m.v. TLR-activatie. TLR7/8 triggering is in het bijzonder interessant in de context van tumor vaccinatie omdat dit leidt tot verhoogde concentraties van type I IFN en IL-12, die TH1- en cytotoxische T-cell responsen promoten die nodig zijn voor potente anti-tumor immuniteit. Het TLR7/8-ligand werd geconjugeerd met een polymeer aangezien het reeds werd aangetoond dat lipide-, polymeer,

en nanopartikel-conjugatie van TLR-liganden zeer sterk de systemische inflammatie reduceert. Dit rendeert in potente responsen gelocaliseerd ter hoogte van de lymfeknopen leidend tot verhoogde adaptieve immuun responsen gericht tegen de antigenen. *In vitro* activatie van DCs, geïsoleerd uit beenmerg, en RAW blue macrofagen toonde aan dat polymeer-conjugatie van de TLR7/8-agonist de activiteit ervan niet benadeelde. Bovendien was de agonist potenter wanneer deze was geadsobeerd op het oppervlak van de micropartikels. Dit kan worden verklaard door de efficiënte opname van micropartikels waardoor de interactie van het TLR-ligand met zijn receptor werd verhoogd.

Aangaande deze resultaten, blijkt de formulatie van kanker cel-lysaat in immunogene $CaCO_3$ micropartikels veelbelovend als milde en efficiënte strategie om kanker cel-lysaten te encapsuleren. Verdere optimilisatie kunnen experimenten inhouden die 1 of meerdere TLR-agonisteren introduceren, gevolgd door het bepalen van het geïnduceerde cytokine spectrum.

Het encapsuleren van kanker cel-lysaat, includeert echter geen celmembraan proteïnen en stroma proteïnen, in geval van compleet tumorweefsel. Daarom werd er in dit werk ook focus gelegd op het formuleren van intacte kanker cellen in vaccins. In hoofdstuk 5, werden levende kanker cellen gebruikt als basis voor layer-by-layer (LbL) coating van complementair interagerende componenten, gevolg door hypo-osmose om bio-hybride capsules te bekomen die kanker cel-lysaat bevatten in de holle ruimte van de partikels. De LbL techniek die wordt gebruikt om een synthetisch halfdoorlatend membraan aan te maken op niet-planaire substraten, is een attractieve techniek omdat het eenvoudige, milde encapsulatie toelaat van een brede waaier aan componenten in waterig medium. Initiële experimenten werden uitgevoerd gebruik makend van tegengesteld geladen polyelektrolieten, poly-L-arginine (P_LARG) en dextraan sulfaat (DEXS). Dit is gebaseerd op eerder onderzoek die resulteerde in biocompatibele, *in vitro* en *in vivo* degradeerbare en meerlagige capsules bestaande uit deze polyelektrolieten die brede cellulaire en humorale immuun responsen induceerden tegen het geëncapsuleerde antigen. Deze aanpak resulteerde echter in onmiddellijke aggregatie, cellyse en celdood van de levende kanker cellen tijdens incubatie met P_LARG. Daarom werden minder sterke complementair interagerende componenten geselecteerd om de kanker cellen te coaten. Deze strategie werd gebaseerd op het gebruik van poly(vinylpyrrolidone) (PVP) en tanninezuur (TA) die waterstofbruggen vormen. Aangezien het doel van deze thesis de

encapsulatie van volledige kanker cellen inhoudt, is het belangrijk om de intergriteit van de cellen zoveel als mogelijk te behouden tijdens de coating. In dit opzicht, werd er bijzondere aandacht geschonken aan het bepalen van de optimale coating componenten en condities. Er werd bevestigd dat, na lyse door hypo-osmose, de kankercellen dood waren wat belangrijk is om hergroei van nieuwe tumoren te vermijden na vaccinatie o.w.v. residuele levende cellen. Dit laatste is vaak het geval in de huidige vaccins van volledige kanker cellysaten. Immunogene eigenschappen werden geïntroduceerd in de capsules, via een proof of concept studie, door het voorbehandelen van de kanker cellen met warmte (heat shock) om expressie van DAMPs, belangrijke endogene immuun activators, te induceren.

Om het potentieel te bepalen van de bio-hybride cel-gebaseerde capsules, werden preliminaire *in vitro* opname experimenten uitgevoerd, waaruit bleek dat slechts 5 % van de partikels worden opgenomen. We wijzen dit toe aan de omvang van de partikels die te groot zijn om efficiënte opname te induceren door dendritische cellen (boven 10 µm). Verschillende pogingen werden uitgevoerd om efficiënte opname door immuun cellen mogelijk te maken. Hiervoor werden de capsules blootgesteld aan zout of hoge temperaturen met het oog op het krimpen van de partikels. Dit resulteerde echter in degradatie of aggregatie van de partikels

Aangezien het niet mogelijk bleek te zijn om efficiënte opname te verkrijgen met de LbL-gecoate kanker cellen, werd een alternatieve strategie ontwikkeld in hoofdstuk 6 voor het formuleren van kanker cellen in vaccin partikels via een één-staps methode. Deze is aanzienlijk minder arbeidsintensief en tijdsrovend en vermijdt bijgevolg onnodig verlies van cellen in tegenstelling tot de layer-by-layer coating van kanker cellen. Dit heeft geleid tot een eenvoudige, maar efficiënte methode om kanker cellen in hun geheel te encapsuleren in polyelektroliet micropartikels via sproeidrogen. Poreuze, niet-geaggregeerde polyelektroliet-omhulde micropartikels, die dode kanker cellen bevatten, werden bekomen via het mengen van mannitol met levende kanker cellen samen met de tegengesteld geladen polyelektrolieten, P_LARG en DEXS in waterig medium net voor het sproeidrogen. Net zoals de bio-hybride capsules (hoofdstuk 5), bevatten de polyelektroliet-omhulde micropartikels enkel dode kanker cellen wat hergroei van tumoren na vaccinatie kan vermijden. De polyelektroliet-omhulde kanker cellen waren stabiel als micropartikels, na resuspensie in PBS buffer, en stelden geen proteïnen vrij in het supernatans. *In vitro* evaluatie toonde aan dat de micropartikels veel meer werden

opgenomen door dendritische cellen en significant de cross-presentatie van antigenen verhoogden, ten op zichte van cel-lysaat. Analoog met de kanker cel-lysaat-bevattende $CaCO_3$ micropartikels, beschreven in hoofdstuk 4, werden immuun-stimulerende eigenschappen geïntrodudeerd via het sproeidrogen van de vaccin componenten in aanwezigheid van CL264-poly(HPMA-APMA). Dit resulteerde in immunogene micropartikels die sterk TLR-activatie induceerden.

Deze resultaten tonen het potentieel aan van de polyelektroliet-omhulde kanker cellen als immunogene antigen vaccins. Introductie van meerdere TLR-liganden gevolgd door het bepaling van de optimale condities voor T-cel activatie, zoals ook voorgesteld voor de lysaat-bevattende $CaCO_3$ micropartikels, zou daarom een interessant vervolg kunnen zijn om het potentieel van deze formulatie te verhogen. Daarnaast, kan immunogene behandeling of inductie van immunogene celdood van de kankercellen, voor het sproeidrogen, ook een meerwaarde betekenen in het verhogen van het potentieel van de formulatie in activatie van DCs en CTL-gedreven anti-tumor responsen.

In conclusie, aangaande de cel-lysaat-gebaseerde vaccins, bleken de $CaCO_3$ polymeer micropartikels meer veelbelovend dan de polymeer-proteïne geligeerde nano-conjugaten qua toepasbaarheid. $CaCO_3$ micropartikels laten namelijk toe om cel-lysaat efficiënt te encapsuleren, zonder functionalisatie ervan, in immunogene partikels die efficiënt dendritische cellen activeren *in vitro*. Voor de cel-gebaseerde vaccins, aan de andere kant, werd aangetoond dat de polyelektroliet-omhulde kanker cellen superieur zijn vergeleken met de bio-hybride cel-gebaseerde partikels. Deze methode maakt het mogelijk om kanker cellen via één stap te formuleren in polyelektroliet partikels die veelbelovende resultaten vertoonden *in vitro* aangaande opname efficiëntie, MHC-I kruispresentatie inductie en immunogeniciteit.

Wat betreft toekomstige experimenten, zou het interessant kunnen zijn om het potentieel te beoordelen van deze twee veelbelovende vaccins *in vivo*, via het bepalen van de inductie van anti-tumor immuniteit. De initiële *in vivo* experimenten kunnen worden uitgevoerd gebruik makende van een immunogene kanker muis cellijn die OVA stabiel tot expressie brengt om grondige screening toe te laten met verkrijgbare assays. Daarnaast, zou het bijzonder interessant zijn om de meest veelbelovende formulatie *in vivo* te vergelijken met het zelfde vaccin dat neo-antigenen bevat.

In het algemeen heeft deze thesis vier verschillende strategieën onderzocht om kanker cel-lysaat of kanker cellen te encapsuleren in immunogene en gepersonaliseerde vaccin partikels. Deze thesis toonde het gunstige effect aan van antigen formulatie in partikels, lijkend op pathogenen, die immuun-stimulerende componenten bevatten (in dit geval TLR-agonisten) met betrekking tot antigen opname en activatie van dendritische cellen.

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Bio-hybrid tumor cell-templated capsules: a generic formulation strategy for tumor-associated antigens in view of immune-therapy
Lybaert, L.; De Vlieghere, E.; De Rycke, R.; Vanparijs, N.; De Wever, O.; De Koker, S.; De Geest, B. G.
European Symposium on Controlled Drug Delivery – Egmond aan Zee, the Netherlands – Apr 16-18 **2014**
FCWO travel grant

Bio-hybrid tumor cell-templated capsules

Lybaert L., De Vlieghere E., De Rycke R., Vanparijs N., De Wever O., De Koker S., De Geest B. G.
Knowledge for growth – Ghent, Belgium – May 8th **2014**

Layer-by-Layer coated cancer cells for cancer vaccination
Lybaert L., De Wever O., De Koker S., De Geest B.G.
Biopharmacy Day – Ghent, Belgium – Dec 18th **2013**

Encapsulation methods for cancer cell lysates into microparticles for cancer vaccination
Lybaert L., De Koker S., Hoogenboom R., De Geest B.G.
Biopharmacy Day – Utrecht, the Netherlands – Nov 9th **2012**

PROFESSIONAL & PERSONAL SKILLS

Presentation skills
Multiple poster and oral presentation at international conferences

Educating skills
Assistant at multiple practical courses of 3rd bachelor and 2nd master students during PhD

Scientific writing
Writing of multiple articles, projects, abstracts
Guidance multiple master and industrial thesis students

Organization skills
Start-up cell lab (supply, protocols, rules, ...)
Teambuilding colleagues/volleyball team

Team player
Volleyball (VOBOG, Ghent)
Teambuilding

Language skills
Dutch – Native language
English – Full professional proficiency
French – Good

Computer skills
Endnote, ChemDraw, CorelDraw, Prism, Specman, CFLow Plus, Image J, Imaris, MS Office