Antibody- and Peptide-based Immunotherapies

Proof-of-concept and safety considerations

ERIKA FLETCHER
The aim of cancer immunotherapy is to eradicate tumours by inducing a tumour-specific immune response. This thesis focuses on how antibodies and peptides can improve antigen presentation and the subsequent tumour-specific T cell response. Tumour recognition by the immune system can be promoted through delivery of antigen in the form of a vaccine. One example is the development of a therapeutic peptide vaccine containing both CD4+ and CD8+ T cell epitopes. So far, peptide vaccinations have shown limited success in clinical trials and further improvements are needed, such as choice of adjuvant and T cell epitopes, as well as targeted delivery of peptides and adjuvants to the same DC.

In paper I, we describe the development of a peptide-peptide conjugate (with a tumour T cell epitope) that, via immune complex formation and FcγR binding, enhance antigen uptake and activation of DCs. The conjugate consists of three tetanus toxin-derived linear B cell epitopes (MTTE) that were identified based on specific IgG antibodies in human serum. Three MTTE peptide sequences were conjugated to a synthetic long peptide (SLP) that consists of a T cell epitope derived from the desired target tumour.

In paper II, the conjugate was evaluated in a modified Chandler loop model containing human blood, mimicking blood in circulation. The conjugate was internalised by human monocytes in an antibody-dependent manner. A conjugate containing the model CMV-derived T cell epitope pp65NLV generated recall T cell responses dependent on MTTE-specific antibodies and the covalent conjugation of the three MTTE with the SLP.

In paper III, a CD40-specific antibody was characterised for local treatment of solid tumours. The antibody eradicated bladder tumours in mice and induced T cell-mediated immunological memory against the tumour.

In paper IV, we characterised the Chandler loop model (used in paper II) for its potential use in predicting cytokine release syndrome (CRS) in response to monoclonal antibodies (mAbs). Superagonistic antibodies (e.g., OKT3) induced rapid cytokine release whereas no cytokine release was induced by antibodies (e.g., cetuximab) associated with low incidence of CRS in the clinic.

In conclusion, this thesis work demonstrates proof-of-concept of improved strategies for antibody- and peptides-based cancer immunotherapies and their potential use in multiple cancer indications.

Keywords: Immune complex, conjugat, vaccine, CD40, whole blood, cytokine release syndrome

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To Joni♥
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I  Mangsbo, SM., Fletcher, E., van Maren, W., Redeker, A., Cordfunke, R., Dinkelaar, J., Ouchaou, K., Codee, J., A. van der Marel, G., Hoogerhout, P., Melief, CJM Ossendorp, F., Drijfhout, JW. Linking T cell epitopes to a common linear B cell epitope; a targeting and adjuvant strategy to improve T cell responses. Submitted manuscript

II  Fletcher, E., van Maren, W., Cordfunke, R., Dinkelaar, J., Codee, J., van der Marel, G., Melief, CJM., Ossendorp, F., Drijfhout, JW., Mangsbo, SM. Formation of immune-complexes by a defined linear tetanus toxin-derived B cell epitope boosts human T cell responses to long peptides. Submitted manuscript


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## Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACT</td>
<td>Adoptive cell transfer</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cell-mediated cytotoxicity</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>Bas</td>
<td>Basophils</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>CAR</td>
<td>Chimeric antigen receptor</td>
</tr>
<tr>
<td>CARPRA</td>
<td>Complement activation-related pseudoallergy</td>
</tr>
<tr>
<td>CCR</td>
<td>Chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>cDC</td>
<td>Conventional DC</td>
</tr>
<tr>
<td>CDC</td>
<td>Complement-dependent cytotoxicity</td>
</tr>
<tr>
<td>CEA</td>
<td>Carcinoembryonic antigen</td>
</tr>
<tr>
<td>CLIP</td>
<td>Class-II associated invariant chain</td>
</tr>
<tr>
<td>CM</td>
<td>Central memory</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CRS</td>
<td>Cytokine release syndrome</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T lymphocyte-associated antigen 4</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage-associated molecular pattern</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribo nucleic acid</td>
</tr>
<tr>
<td>DT</td>
<td>Diphtheria toxin</td>
</tr>
<tr>
<td>DTP</td>
<td>Diphtheria, tetanus and pertussis</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGRF</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EM</td>
<td>Effector memory</td>
</tr>
<tr>
<td>Endo</td>
<td>Endothelial</td>
</tr>
<tr>
<td>Eosi</td>
<td>Eosinophils</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>FcγR</td>
<td>Fcγ receptor</td>
</tr>
<tr>
<td>FcγRn</td>
<td>Fcγ receptor neonatal</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>-----------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GMP</td>
<td>Good manufacturing practice</td>
</tr>
<tr>
<td>gp100</td>
<td>Glycoprotein100</td>
</tr>
<tr>
<td>hCG</td>
<td>Human chorionic gonadotropin</td>
</tr>
<tr>
<td>Her-2</td>
<td>Human epidermal growth factor receptor-2</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papillomavirus</td>
</tr>
<tr>
<td>hTERT</td>
<td>Human telomerase reverse transcriptase</td>
</tr>
<tr>
<td>IC</td>
<td>Immune complex</td>
</tr>
<tr>
<td>IFA</td>
<td>Incomplete Freund’s adjuvant</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>li</td>
<td>Invariant chain</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activating motif</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immunoreceptor tyrosine-based inhibitory motif</td>
</tr>
<tr>
<td>KIR</td>
<td>Killer immunoglobulin like receptor</td>
</tr>
<tr>
<td>KLH</td>
<td>Keyhole limpet hemocyanin</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph node</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAGE</td>
<td>Melanoma antigen E</td>
</tr>
<tr>
<td>Mart-1</td>
<td>Melanoma antigen recognised by T cells-1</td>
</tr>
<tr>
<td>MASP</td>
<td>MBL-associated serine protease</td>
</tr>
<tr>
<td>MBL</td>
<td>Mannose-binding lectin</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIIC</td>
<td>MHC class II compartment</td>
</tr>
<tr>
<td>m-ISA51</td>
<td>Montanide-ISA51</td>
</tr>
<tr>
<td>MoDC</td>
<td>Monocyte-derived dendritic cell</td>
</tr>
<tr>
<td>MR</td>
<td>Mannose receptor</td>
</tr>
<tr>
<td>MTTE</td>
<td>Minimal tetanus-toxoid epitope</td>
</tr>
<tr>
<td>MQ</td>
<td>Macrophage</td>
</tr>
<tr>
<td>Neu</td>
<td>Neutrophils</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PAP</td>
<td>Prostatic acid phosphatase</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PC</td>
<td>Prostate cancer</td>
</tr>
<tr>
<td>PD1</td>
<td>Programmed death 1</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid DC</td>
</tr>
<tr>
<td>pIFNγ</td>
<td>Pegylated interferon gamma</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate specific antigen</td>
</tr>
<tr>
<td>SlanDC</td>
<td>6-sulfo LacNAc+ dendritic cell</td>
</tr>
<tr>
<td>SLP</td>
<td>Synthetic long peptide</td>
</tr>
<tr>
<td>STn</td>
<td>Sialyl-Tn</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>Syn</td>
<td>Syncytiotrophoblasts</td>
</tr>
<tr>
<td>TAA</td>
<td>Tumour-associated antigen</td>
</tr>
<tr>
<td>TAP</td>
<td>Transporter associated with antigen processing</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>Tumour growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TIL</td>
<td>Tumour-infiltrating leukocyte</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>Treg</td>
<td>T regulatory</td>
</tr>
<tr>
<td>TCC</td>
<td>Terminal complement complex</td>
</tr>
</tbody>
</table>
Populärvetenskaplig sammanfattning


I delarbete IV har vi karakteriserat blodloopsystemet (samma som i paper II) för dess potential att detektera farliga immunrespons som vissa antikroppar kan orsaka. För att förutse dessa responsor genomgår antikroppar tester i mus- och humant blod innan de får ges till människa. Alla dessa metoder har för- och nackdelar. Vi karakteriserade ett system som historiskt använts för att analysera interaktionen mellan blod och materialytor som avses användas som implantat. I loopsystemet genererade antikropparna samma immunologiska effekter som när de har getts till patienter i kliniken, vilket validerar systemets noggrannhet för att vara förutseende för farliga responser.
Cancer is a disease that involves transformation of normal cells. Many inherited and environmental factors, such as smoking and sunlight exposure, are known to be involved in the pathogenesis of cancer. These factors alter the DNA of normal cells that make them multiply out of control and grow into malignant tumour lesions. In most cases, accumulation of multiple mutations in several genes is the key feature of malignant tumour development. This is one of the reasons why many cancer forms affect the elderly. Cancer is a heterogeneous disease, which means that tumours can have very diverse genetic mutations and can develop in many different locations in the body. Tumours can arise in various parts of the body; for example, lung, breast, prostate, liver, skin, stomach and bladder are common sites for malignancy. Cancer is a worldwide healthcare problem affecting one out of three. The standard treatments involve surgery, chemotherapy and radiation, which in general target rapidly growing cells but lack the specificity for tumour cells. This means that normal cells are also affected by these treatment modalities, leading to adverse events/toxicity.

Cancer cells evade recognition by the immune system via multiple mechanisms, eventually growing into a tumour mass. The aim of cancer immunotherapy is to eliminate cancer by inducing a tumour-specific immune response, in a fashion similar to how our immune system naturally recognises and eliminates invading microorganisms. Cancer immunotherapy is more specific than conventional therapies and has the potential of being less toxic, if the proper selective immune response is induced. Immunotherapy of cancer is attractive since in theory, only a primary local anti-tumour response is needed to induce systemic anti-tumour responses. Thus, it may not be the drug dose and exposure time that determines the outcome, but rather the potential of activated immune cells to home to metastatic lesions and target the spreading disease. This uniqueness in that a local immune response can become effective against metastatic diseases, is a key difference compared to both chemotherapy and targeted therapies. The first famous case of immunotherapy of cancer patients was a vaccine consisting of inactivated S. pyrogenes and Serratia marcescens bacteria, which the surgeon William Coley successfully treated sarcoma patients with in the 1890s [1]. Furthermore, the Nobel Prize awarded to Köhler and Milstein in 1975 for the development of the hybridoma technology was the first step towards the generation of human monoclonal antibodies. The therapeutic success of metastatic disease with
the human monoclonal antibodies ipilimumab [2] and nivolumab, together with genetically engineered T cells (known as CAR T cells), resulted in the announcement of cancer immunotherapy as the “Breakthrough of the year” in 2013 by the leading scientific journal Science.

The promise and potential of earlier established cancer therapies and their effect on the immune response is becoming more and more widely recognised. Timing and dose of cytostatic drugs, as well as radiation, may influence certain types of immune cells and can work in synergy with immunotherapies to reduce tumour burden. The future will most likely focus on how to combine various therapies to treat a specific cancer patient based on the molecular signature of this or her tumour to be able to realise the goal of individualised cancer therapy.

This thesis focuses on characterising new antibody and peptide drug candidates in model systems, with the aim of developing novel cancer therapies to be used in the clinic.
Overview of the immune system

Our body’s immune system is composed of a great variety of cells and proteins that cooperatively defend us against infectious microorganisms such as bacteria and viruses. The immune system is also essential for clearing the body of abnormal (e.g., tumour cells) and dead cells. Immune cells are derived from the bone marrow and originate from haematopoietic stem cells that differentiate into either a lymphoid or myeloid progenitor. Lymphoid progenitors differentiate into natural killer (NK) cells, T cells and B cells, whereas myeloid progenitors differentiate into granulocytes, monocytes, erythrocytes and platelets. The immune system is divided into the innate and adaptive immune response. The innate immune response is in general quick to respond and gives the same response during the first and the second infection with the same microorganism. In contrast, the adaptive immune response acts faster upon re-exposure, since the first infection generates an immunological memory to the specific microorganism.

The innate response

The innate arm of the immune system is the first line of defence that consists of physical barriers (skin and mucosa), immune cells including NK cells, granulocytes and monocytes, and soluble plasma proteins that make up the cascade systems. The cells and proteins of the innate immune system recognise damage-associated molecular patterns (DAMPs) that are released by damaged cells and pathogen-associated molecular patterns (PAMPs) on microorganisms such as microbial nucleic acids and surface glycoproteins. The recognition of DAMPs and PAMPs induce the elimination of non-self-intruders, as well as stimulate the release of signals that attract and activate cells of the adaptive immune system, which is the second line of defence.

The adaptive response

The adaptive arm of the immune system is executed by B and T cells, derived from the bone marrow. B cells recognise macromolecular antigens directly with their B cell receptor (BCR) whereas the T cell receptor (TCR) on T cells recognise antigens presented by major histocompatibility complex (MHC) molecules on the cell surface. After antigen recognition and activation, B cells and T cells undergo clonal expansion and affinity maturation of their antigen-specific receptor, which promotes the immune response against the antigen. B cells produce antibodies that coat the antigen to prevent entry into host cells and label it for destruction by other immune cells (i.e., humoral immunity). Activated T cells can help the B cell response (helper CD4+ T cells) or migrate to the periphery and directly kill the recognised pathogen (executed by CD8+ T cells via cell-mediated immunity). When the target
antigen is cleared, a small fraction of B and T cells remain as memory cells that can respond more quickly the second time to the same pathogen. Memory, specificity and recognition diversity are the major characteristics that distinguish the adaptive from the innate immune response. However, the interplay between the two is great, and the dendritic cell (DC) plays a central role as a major cellular link. DCs are an essential part of this thesis and will be discussed in more detail below, with a special focus on antigen presentation capacities.

Antigen presentation

Antigen presenting cells

All nucleated cells can present intracellular-derived antigens such as viral and self-proteins on their external surface. Additionally, professional antigen presenting cells (APCs), including dendritic cells (DCs) and B cells, can present both intracellular- and extracellular-derived antigens to T cells. APCs can present extracellular antigens to CD4+ T cells, and through a mechanism called cross-presentation, to CD8+ T cells. The focus of this section is on DCs and how they present antigens to T cells.

The discovery of DCs by Steinman and Cohn [3] in 1973 was awarded the Nobel Prize in Physiology or Medicine in 2011. DCs are a heterogeneous cell population that can differentiate from both myeloid and lymphoid progenitors [4]. The heterogeneity of DCs means that they do not express only one lineage surface marker like T cells, which are defined by the expression of CD3. Adding further complexity, the surface markers defining different DC populations are not the same in mouse and human, making it hard to generalise and apply findings derived from one species to the other. There are two major subpopulations of DCs called conventional (cDCs) and plasmacytoid DCs (pDCs). cDCs are found in blood and lymphoid tissues, and can be further divided into CD141+ and CD1c+ DCs. These human DC subsets are functionally equivalent to mouse CD8α+ and CD8α- cDCs [5, 6]. Mouse CD8α+ are superior cross-presenters [7, 8]; however, whether or not the human counterpart CD141+ are superior over the other human DC subsets is unclear [9]. pDCs promote anti-viral responses by migrating to inflamed lymph nodes (LNs) and secreting type I IFN [10]. In the skin, there are resident Langerhans cells (DCs of skin and mucosa) and dermal DCs (CD1a+ or CD14+) that migrate to skin-draining LNs for antigen presentation. Monocytes can differentiate into DCs [11] and a more recently discovered blood DC expressing surface CD16 (SlanDCs) [12, 13].

DCs are scavenger cells that, in an immature state, search and internalise antigens in the periphery (e.g., skin, tissues and blood) with the help of multiple surface receptors including CD91, DEC205, CD36, integrins and Fcγ
receptors (FcγRs) [14-16]. In the presence of danger signals such as DAMPs and PAMPs, or immune stimulatory signals such as cytokines, DCs down-regulate and upregulate receptors, improving their antigen presentation capacity and making them less efficient in antigen uptake. DAMPs and PAMPs are recognised by toll-like receptors (TLRs) expressed on DCs [17]. Additionally, when DCs are activated, they express the chemokine receptor CCR-7 which promotes their migration to secondary lymph nodes where they present antigens to T cells [18].

The presentation of antigens to T cells requires three types of signals for optimal T cell activation (Figure 1; reviewed in [19]). Signal 1 is the engagement of the peptide-loaded MHC molecule on the DC with the TCR on the T cell. Signal 2 is the engagement of costimulatory molecules (CD70, CD80/86, and CD40) with the respective ligands/receptors on T cells (CD27, CD28 and CD40L). Signal 3 is the release of immune stimulatory cytokines by DCs including IL-12, TNFα and IL-6. Furthermore, DCs can produce immunosuppressive cytokines (e.g., IL-10 and TGF-β) which can induce T cells to become anergic, further described in the section T cells.

![Figure 1. Antigen presentation of APC to T cells requires three signals for optimal T cell activation: (1) TCR recognition of the presented peptide in MHC class I/II, (2), costimulatory signals such as CD28 interaction with CD80/86, and (3) IL-12 secreted by DCs promoting T cell activation.](image)

**CD40 and DC licensing**

The costimulatory receptor CD40 is a member of the TNF receptor superfamily and is expressed on APCs (e.g., DCs and B cells), as well as on tumours such as bladder, breast and ovary [20]. CD40 binds CD40L, expressed on activated CD4+ T cells, B cells, epithelial cell, endothelial and
platelets [21-24]. CD40-CD40L engagement results in the trimerisation of CD40 resulting in intracellular signalling of the CD40-expressing cell, apoptosis of CD40+ tumours, activation of CD40+ DCs, and stimulation of CD40+ B cells to become IgG-producing plasma cells (see section B cells). Activated CD4+ T cells licence DCs, via CD40-CD40L engagement, to express MHCII, CD80 and CD86, and secrete IL-12. Licensed DCs promote antigen-specific CD8+ responses and tilt the T cell response towards a Th1 response (see section T cell), which is associated with a strong anti-tumour response [25]. The CD40-CD40L interaction is essential for generating fully mature DCs; therefore, CD40 is a potential target in tumour immunotherapy (discussed in the section CD40-specific mAbs).

MHC class I presentation
The antigens presented to T cells are protein antigens that require processing into shorter peptides before they fit optimally in the MHC molecule. There are two major MHC subtypes referred to as MHC class I (MHCI) and MHC class II (MHCII) that present peptides to CD8+ and CD4+ T cells, respectively (reviewed in [26]). MHCI molecules are encoded by three allelic polymorphic genes (HLA-A, HLA-B and HLA-C in human) and consist of a transmembrane heavy chain and a supporting light chain (β2-microglobulin). Intracellular proteins are processed by the proteasome and peptides are subsequently translocated to the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP). In the ER, the stability of MHCI molecules are supported by chaperones that are released when a peptide fits into the MHC groove. Peptide-loaded MHCI molecules are subsequently transported to the surface for antigen presentation to CD8+ T cells. The presentation of intracellular antigen to CD8+ T cells can result in destruction of the presenting cell or peripheral tolerance, further described in the section T cells.

MHC class II presentation
In contrast to MHCI, the expression of MHCII is mainly found on professional APCs that present extracellular peptides to CD4+ T cells. However, MHCII expression can be induced on endothelial cells by IFNγ stimulation [27]. MHC class II molecules are encoded by three allelic polymorphic genes (HLA-DR, HLA-DQ and HLA-DP in humans) and consist of two transmembrane domains, one α- and one β-chain. The two chains are assembled and stabilised by the invariant chain (Ii) in the ER. The assembled MHCII is transported to the late endosomal compartment (MIIC) where Ii is digested, leaving only a small peptide fragment (CLIP) in the peptide-binding groove. In the MIIC, the MHCII encounters exogenous antigens that are degraded by proteases and replace CLIP to form a peptide-loaded MHCII
complex which is transported to the surface for presentation to CD4+ T cells. Antigen presentation to CD4+ T cells generates T cells that orchestrate a broad range of immune responses, including CD8+ T cell responses.

Cross-presentation

Exogenous antigens can also be presented by MHCI on DCs through a mechanism called cross-presentation (Figure 2) [28]. Cross-presentation of exogenous antigens has two proposed intracellular pathways, the cytosolic and the vacuolar pathway. In the cytosolic pathway, phagocytosed antigens enter the cytosol, are processed by the proteasome and are either loaded on MHCI in the ER via transport through TAP, or transported back into the phagosome for MHCI loading [29, 30]. In the vacuolar pathway, antigens are degraded by proteases and loaded on MHCI in the phagosome [31]. Although the mechanisms of cross-presentation are poorly understood, the importance of cross-presentation for tumour and viral destruction is well-recognised from many studies [32-36]. Cross-presentation of antibody-coated antigens is further discussed in the section FcγRs.

![Figure 2. Schematic representation of antigen presentation by DCs. The figure is reprinted with permission from [37]. See text above for pathway description.](image-url)
T cells

T cells are the effector cells of the adaptive immune response that, after encountering proper antigen presentation by DCs, orchestrate the elimination of microorganisms and/or cells. Precursor T cells migrate from the bone marrow to the thymus where they start to express the α- and β-chain of the TCR and the dual expression of two CD4 and CD8 co-receptors. The TCR associates with CD3 that upon antigen recognition sends intracellular signals via immunoreceptor tyrosine-based activating motifs (ITAMs). Genetic re-arrangements of the antigen binding portions of the TCR generate a large pool of T cells with different specificities. T cells are then educated in the thymus by positive and negative selection, which ensures that no self-reactive T cells enter circulation (a selection process called central tolerance) [38]. In the thymus, thymic epithelial cells present self-antigens and the T cells that bind the peptide-loaded MHC complex weakly are promoted to survive by receiving survival signals (positive selection); however, cells that do not bind are eliminated by apoptosis. The recognition of either MHCI or MHCII induces the loss of either CD4 or CD8 expression. In negative selection, T cells that bind strongly either die through apoptosis or are induced to differentiate into regulatory T cells (Tregs). The education of T cells in the thymus results in the release of naïve T cells into the circulation with low TCR avidity for MHC molecules presenting self-antigens.

Naïve T cells are activated when recognising antigens presented by APCs, and in the presence of sufficient co-stimulation, they undergo clonal expansion (see section Antigen presenting cells and Figure 1). In the absence of co-stimulation, antigen recognition can render T cells anergic and unresponsive to antigenic stimuli (peripheral tolerance). Activated T cells also express inhibitory molecules, including cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed death 1 (PD1) receptor. CTLA-4 is upregulated early in T cell priming and inhibits immune responses by either sending inhibitory intracellular signals that block T cell activation, or by binding CD80/86 with higher affinity and therefore competing out CD28 co-stimulation [39]. PD-1 bind its ligand PD-L1/PD-L2 on APCs or target cells, and is important for peripheral tolerance [40].

T cells are grouped into CD8+ cytotoxic T lymphocytes (CTLs) and CD4+ helper T cells (Th). CTLs are effector T cells that, upon antigen recognition, kill target cells through the release of perforin and granzymes. Additionally, CTLs express death receptors such as FasL on their surface, which induces apoptosis of the target cell when engaging Fas on the cell surface [41]. Th cells are broadly divided into Th1, Th2, Th17 and Tregs based on their cytokine release profile and function; there are also more subtypes that will not be further described herein. Th1 cells are associated with the production of IFNγ, IL-2 and TNFα, and are important for CTL activation and anti-tumour responses. Th2 cells are associated with the production...
of IL-4, IL-5 and IL-10, and function to eliminate extracellular pathogens such as parasitic worms (helminths). Th17 cells produce IL-17, induce inflammation and are linked to autoimmunity, and it is currently being debated whether they have pro- or anti-tumour effects (reviewed in [42]).

Antigen recognition and T cell activation generate, in addition to the effector T cells described above, a small number of antigen-specific memory cells that are either located in lymph nodes (central memory [CM] T cells) or in the periphery (effector memory [EM] T cells). CM cells express, like naïve T cells, CD62L and CCR7, while EM cells have low surface expression of these markers [43]. Upon antigen recognition splice variants of the CD45 gene are generated making it possible to distinguish naïve T cells (CD45RA) from antigen experienced T cells (CD45RO) [44]. Upon re-challenge with the same pathogen, memory T cells can differentiate to effector T cells responding much faster than during the first encounter with the same pathogen.

B cells
Similarly to T cells, B cells generate memory cells allowing a rapid immune response upon re-infection with the same pathogen. Immature B cells migrate from the bone marrow to secondary lymphoid organs for maturation. Immature B cells are permitted to leave the bone marrow after rearrangement of the immunoglobulin (Ig) genes that generate a heavy and light chain that, together as a heterodimer, form the antigen-specific BCR [45]. The complete BCR additionally contains one α- and one β-chain with the intracellular signalling domain ITAM that upon antigen-recognition stimulates downstream signalling that promotes B cell activation.

In contrast to T cells, B cells directly bind their antigen without MHC presentation and can therefore recognise a wide range of epitopes such as proteins, macromolecules, carbohydrates and nucleic acids. When B cells interact with an antigen they migrate to germinal centres in secondary lymph nodes and undergo a series of changes resulting in a highly antigen-specific immune response. The changes include: clonal expansion (generating Ig-producing plasma cells and memory cells), somatic hypermutation (enhancing specificity to the same antigen in a mechanism called affinity maturation), and gene recombination of the heavy chain (class-switch) [46]. The B cell response against many antigens requires help from CD4+ T cells. Therefore, activated B cells internalise the antigen and present the antigen on MHCII molecules to activate CD4+ T cells, which in turn help to enhance the capacity of B cells to become Ig-producing plasma cells [47].

There are five different Ig classes including IgM, IgD, IgG, IgA and IgE (referred to as the Ig isotype). When activated, the surface-bound BCR is exchanged for secreted Igs (also known as antibodies) and in a primary in-
Infection these mainly consist of IgM antibodies; whereas during a secondary infection with the same pathogen, IgG antibodies are mainly produced [46]. The structure of an antibody is divided into an antigen-binding part called Fab and an effector fragment called Fc. Secreted IgM are decavalent consisting of five heterodimers that create 10 antigen binding sites, whereas IgG are bivalent consisting of two heterodimers that together create two antigen-binding sites (Figure 3) [48]. As a result of this, IgMs bind their target with high avidity; however, often with lower affinity than IgGs which are products of an affinity maturation process. IgG antibodies are further divided into IgG1-4 isotypes (in human) which is further described in the section Fcγ Receptors.

Released antibodies circulate in the bloodstream and coat the target antigen to for example, prevent microorganisms from entering and infecting host cells (also known as neutralisation). In addition, the coating mechanism also functions as a way of recruiting phagocytes (see section Fcγ Receptors). Additionally, both IgM and IgG antibodies are recognised by the complement component C1q (described in the section The complement system).

![Figure 3. Schematic drawing of the structure of a pentameric IgM and a monomeric IgG antibody. IgM is viewed from above and IgG from the side. A pentameric IgM antibody is approx. 970 kDa and an IgG antibody is approx. 150kDa. The antigen binding sites on IgM/IgG (and FcγRs on IgG) are roughly indicated with arrows.](image)

**Fcγ Receptors**

IgG antibodies are divided into four subclasses (IgG1-IgG4) that are induced by different immunological stimuli. In general, protein antigens stimulate
IgG1 and IgG3, polysaccharide antigens stimulate IgG2, and repeated antigen exposure stimulates IgG4 production [49].

The Fc-part of IgG antibodies are ligands of a receptor family called FcγRs, which are expressed on a wide variety of haematopoietic cells, including DCs [50]. There are two major groups of human FcγRs, the activating (FcγRI, FcγRIIA, FcγRIIC, FcγRIIIA and FcγRIIIB) and the inhibitory (FcγRIIB) receptors (Figure 4). FcγRs are also known as CD64 (FcγRI), CD32 (FcγRII) and CD16 (FcγRIII). FcγRn is an intracellular receptor involved in recycling and transport of IgGs [51]. FcγRI is a high affinity IgG receptor that can bind monomeric IgGs, whereas the majority of the other FcγRs require multimeric IgGs (immune complexes [ICs]) for binding [52]. The binding of FcγRs to complexed IgGs results in crosslinking of multiple receptors which promote intracellular signalling via ITAMs on activating FcγRs and ITIM on the inhibitory FcγR. NK cells express almost exclusively the activating FcγRIIIA and when binding complexed IgGs, the target cell expressing the antigen is killed through a process called antibody-dependent cellular cytotoxicity (ADCC) [53]. The activating and inhibitory FcγRs are co-expressed on many cell types (monocytes, DCs and neutrophils) and mediate opposing functions where the balance between the two determines the outcome of the immune response (immune activation or tolerance) [54, 55]. The different isotypes (IgG1, IgG2, IgG3 and IgG4) bind FcγRs with different affinities. All FcγRs bind complexed IgG1 and IgG3, FcγRIIA and FcγRIIIA bind complexed IgG2, and complexed IgG4 was recently shown to bind several FcγRs (FcγRI, FcγRIIA, FcγRIIB, FcγRIIC and FcγRIIIA) [50, 52]. Furthermore, the different isotypes can induce different biological responses depending on what FcγR they bind, which is an important consideration in monoclonal antibody therapy (see section Therapeutic monoclonal antibodies).

**Immune complexes**

Antibodies bind antigens on the cell surface, but can also bind soluble antigens and thereby form immune complexes (ICs). ICs can be recognised by FcγRs promoting antigen uptake and APC activation through crosslinking of activating FcγRs. In fact, antibodies enhance antigen uptake up to a factor of 100 compared to antigen alone [56, 57]. Furthermore, complexed antigens promote DC maturation and are more efficiently cross-presented by DCs both in vitro and in vivo compared to soluble antigen alone [56, 57]. Complexed antigens are proposed protected against degradation in an antigen storage compartment facilitating long-term CTL priming [58]. The uptake of ICs by human moDCs stimulates DC activation through FcγRIIA crosslinking [59, 60]. Therapeutic IgG1 antibodies can induce ADCC via FcγRIIIA and have been proposed by DiLillo et. al. [61] to secondarily induce tumour-specific memory T cells (i.e., an anti-tumour vaccinal effect). In theory, this
occurs by release of IC of the therapeutic antibody bound to tumour material that is targeted to DCs in an FcRIIA-dependent manner. A similar Fc-mediated vaccination effect has been documented previously [62].

![Diagram of FcγR structure and binding affinity](image)

**Figure 4.** Human FcγR structure, binding affinity for IgG subclasses when in complexed form and their cellular expression patterns. The figure was adapted from [50] and the binding IgG binding affinities were obtained from [63]. The expression pattern distribution is derived from [50]. There is also a high affinity receptor FcγRn that binds monomeric IgGs and is involved in antibody transportation/recycling (not included in the figure). * Two polymorphic variants of FcγRIIA (H131 / R131) ** Two polymorphic variants of FcγRIIIA (V158 / F158). Mo=monocytes, MQ=microphages, DC=dendritic cells, Neu=neutrophils, MC=mast cells, Bas=basophils, Eos=Eosinophils, Endo=endothelial cells and syn=syncytiotrophoblasts. Neu+ and MC+ indicate inducible expression on these cell types. (Neu) and (Mo/MQ) indicates receptor expression on a low percentage of cells or certain subsets. The “+” indicates binding and the number of “+” indicates the magnitude with a scale from “+” to “+++”. “–” means no binding.

A brief focus on selected innate immune cells

**Monocytes and Macrophages**

Monocytes are myeloid-derived cells of the innate immune system. Monocytes circulate in the bloodstream and are mainly characterised by their expression of CD14. Monocytes can give rise to multiple types of mature cell types. Monocytes migrate into tissues, differentiate into various forms of macrophages (or DCs), and release pro-inflammatory cytokines such as TNFα, IL-1β and IL-6. The differentiation into macrophages is dependent on
the local environment where IFNγ induces pro-inflammatory macrophages (referred to as M1), and IL-4 and IL-10 induce a more immunosuppressive macrophage subtype (referred to as M2) [64]. The pro-inflammatory M1 macrophage secretes TNFα, IL-6 and IL-12 and thereby promotes Th1 responses, which are important for anti-tumour responses and clearing intracellular pathogens. In contrast, M2 macrophages are immunosuppressive, secreting TGF-β and thereby promoting Th2 responses.

NK cells
Natural killer cells (NK cells) are lymphocytes of the innate immune system that are identified by their surface expression of CD56 and lack of CD3 (in humans). NK cells are derived from the bone marrow and mature in secondary lymphoid organs. NK cells can kill target cells directly by secreting perforin or cytokines (IFNγ) that promote Th1 responses. The activation of NK cells is tightly controlled by inhibitory and activating receptors. For example, human NK cells express the inhibitory receptor KIR (Killer cell Ig-like receptors) which recognises MHCI on the cell surface of neighbouring cells. MHCI is downregulated on virus infected cells and tumour cells [65], removing the inhibition generated by KIR and enabling the NK cell to kill the infected cells or tumour cells, while leaving MHCI expressing uninfected/non-tumour self-cells intact [66]. However, for NK activation to occur, stimulation via activating receptors such as FcγRIIIA and NKG2D is required [67, 68]. FcγRIIIA recognises IgG-coated cells and kills by ADCC. The induction of ADCC stimulates NK release of cytotoxic granule content (e.g., perforin and granzymes) and IFNγ secretion, promoting adaptive responses such as antigen presentation [69].

The complement system
Definition
In addition to cells, the innate immune system consists of more than 30 plasma proteins and glycoproteins that together make up the complement system. Some of the complement proteins are proteases that after an initial activation generate cleavage products in a sequential cascade. The cleavage products either attach to the surface and tag the target cells for elimination, or are released as soluble molecules that attract immune cells to the site of complement activation. The complement components are mainly produced by the liver; however, upon stimulation by IL-6, TNFα and IFNγ macrophages can produce complement components in tissues [70]. The complement system has many important functions including defence against intrud-
ing microorganisms, removal of dead/modified cells, clearing circulating immune complexes (ICs), tissue regeneration and lipid metabolism [71].

Activation pathways and the TCC

The complement system can be activated through three main pathways: the alternative, the classical and the lectin pathway (outlined in Figure 5). The alternative pathway is spontaneously activated by hydrolysis of the complement component C3 [72] creating an initial soluble C3 convertase that cleaves C3 into C3a and C3b. C3b molecules attach to the target surface and form C3 convertase with factor B (C3bBb).

The classical pathway is activated by the complement component C1q that recognises complexed IgG, complexed IgM [73] or pentraxins (e.g. C-reactive protein; CRP) [74]. The binding of C1q to its ligand causes a conformational change that activates the proteases C1r and C1s, which together with C1q form the C1 complex (C1qr2s2). The protease C1s cleaves C4 and C2 into their cleavage products C4a, C4b, C2a and C2b. C4b attaches to the surface and together with 2b creates C3 convertase (C4b2b). The same C3 convertase is generated by the lectin pathway where the mannose binding lectin (MBL) recognises carbohydrate patterns, and together with MBL-associated serine proteases (MASPs), form a complex that cleaves C4 and C2 [75].

Independent of the initial activation pathway, further C3 cleavage and build-up of C3b on the target cell results in the formation of C5 convertases (C3bBb3b or C4b2b3b) that cleave C5 into C5a and C5b. The C5b fragment associates with the hydrophilic glycoproteins C6, C7, C8 and C9, which together form the cytolytic terminal complement complex (TCC) [76]. The TCC forms a lethal pore in the membrane of the target cell.
Figure 5. Schematic representation of the three activation pathways of the proteolytic complement cascade. The classical pathway is activated by C1q recognising IgM or IgG coated antigens and the lectin pathway by MBL recognising carbohydrates on bacteria. The alternative pathway is activated by spontaneous hydrolysis of C3 which can in its hydrolysed form associate with Factor B. Factor D can then cleave Factor B forming Factor Ba and the C3 convertase C3bBb. Reprinted with permission from [77].

Anaphylatoxins and regulation

Complement activation, in addition to the formation of the TTC, result in release of the soluble anaphylatoxins (C3a and C5a) that recruit neutrophils, monocytes and macrophages to the site of complement activation [78]. The phagocytes recognise C3b, C4b and C1q on the target cell via their complement receptors CR1-4 and eliminate the target cell through phagocytosis [79].

The destructive nature of the complement system requires strict regulation to prevent tissue damage and the development of autoimmune diseases. Apoptotic cells are recognised by C-reactive protein (CRP) that together with C1q and factor H inhibit C5 convertase and TCC formation, thereby limiting complement activation to phagocytosis of the apoptotic cell without inducing inflammation [80]. Additionally, the classical and lectin pathways are inhibited by the C1 inhibitor (C1INH) which inhibits the proteases C1r, C1s and MASP [81]. The alternative pathway is inhibited by the membrane
bound regulators CD46 and CD55 that promote C3 degradation [82]. Both soluble (CFHR1) and surface bound (CD59) regulators inhibit C5 convertase and assembly of the TCC [83, 84].

C1q on monocytes

The C1q molecule not only activates the classical pathway, but it is also an important component in a great number of mechanisms; for example, autoimmunity, wound repair, as well as regulation of B cells, T cells and DCs (reviewed in [85]). Complement proteins are mainly synthesised by the liver, whereas C1q is additionally synthesised by monocytes [86], macrophages, DCs [87], epithelial and endothelial cells [64]. In response to IL-6 and TNF, macrophages can produce early complement components including C1, C3 and C4 [88]. C1q of the classical pathway binds IgG-coated antigens, promoting IC clearance from the circulation [89] and enhancing IC phagocytosis by human monocytes [90]. The recent discovery of C1q on the cell surface of monocytes is thought to be important for the regulation of the monocyte to DC transition [91]. Ghebrehiwet et al. [92] speculates that unoccupied C1q have a regulatory role in maintaining immature monocytes (CD14^high and CD11c^high) in a steady state, and when C1q binds ICs, maturation is induced leading to expression of HLA-DR^{high} and CD86^{high}. In agreement, monocyte-derived DCs (MoDCs) maintained their immature status when co-cultured with soluble C1q [91] whereas they mature (expressing CD83, CD86 and MHCII) when cultured on surface immobilised C1q [93], which mimics C1q crosslinking on the cell surface after binding soluble ICs.

Tumour immunology

The existence of immunity against tumours is supported by: the existence of tumour-infiltrating T cells (TILs) [94, 95] and the good overall survival that has been associated with tumour-specific TILs in the tumour [96, 97]. In 1957, Burnet described the immune surveillance theory which propose that immune cells search our body for abnormal cells and eliminate them [98]. This theory has over the years been re-evaluated to the immunoediting theory which consists of three phases: elimination, equilibrium and escape (reviewed in [99]). During the elimination phase, abnormal cells are recognised and removed by the immune system presumably by both innate and adaptive responses. During equilibrium, the immune system controls tumour growth without eliminating all tumour cells. The genetic instability of tumours and the selective pressure provided by the immune system may result in tumour cells that can escape recognition and therefore grow into tumour lesions, which is the final phase referred to as the escape phase. Tumours avoid recognition by several mechanisms such as secreting immunosuppressive
cytokines (e.g., TGF-β and IL-10) [100] or surface receptors (e.g., PD-L1) [101, 102], down-regulating MHC expression (or hampering with the antigen-presentation machinery) to reduce presentation and recognition [103, 104], and increasing the expression of complement inhibitors [105].

Tumour associated antigens

Tumour cells represent transformed normal cells that have undergone genetic mutations causing them to multiply out of control. Tumour cells are caused by both inherited and environmentally acquired genetic mutations. The genetic instability of cancer cells results in high mutation frequency and production of proteins that are different from endogenous proteins, also known as tumour-associated antigens (TAAs). TAAs can be divided into self-proteins that are overexpressed or abnormally expressed (e.g., expression of lineage-specific genes or developmental genes expressed in adult) and non-self proteins (e.g., generated through mutations; also known as neo-antigens or virally acquired) [106] (see Table 1 for examples of TAAs).

The immunogenicity of neoantigens is presumed to be greater than abnormally expressed proteins as the T cells that recognise neoantigens have not undergone central tolerance [107] (the same applies to virally acquired antigens). Another type of proposed TAAs is phosphopeptides that are generated by abnormal phosphorylation during malignant transformation. Mohammed et. al. [108] show that deregulated phosphorylation by malignant cells can enhance the affinity for MHC molecules or alter the repertoire of T cells that can recognise the presented peptide, thereby creating neoantigens in the form of phosphopeptides. Similarly, aberrant posttranslational modifications can create neoantigens in the form of glycopeptides [109].

For presentation of TAAs by MHCI, antigen processing by the proteasome is required. Antigen processing is also performed by an alternative proteasome referred to as the immunoproteasome, further described in the next section.
<table>
<thead>
<tr>
<th>Origin</th>
<th>Type of TAA</th>
<th>TAA</th>
<th>Cancer type</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutated self-antigens</td>
<td>Neoantigen/Unique tumour antigen</td>
<td>Source can vary e.g., Ras and p53</td>
<td>Often high presence in cancer forms induced by known mutagens</td>
<td>[110]</td>
</tr>
<tr>
<td>Non-self (Viral)</td>
<td>Virally-derived</td>
<td>HPV E6/E7</td>
<td>Cervical cancer, H&amp;N</td>
<td>[111]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EBV</td>
<td>Burkitts lymphoma</td>
<td>[112]</td>
</tr>
<tr>
<td>Self-antigens</td>
<td>Lineage specific</td>
<td>NY-ESO-1</td>
<td>Bladder, melanoma among multiple other tumour types</td>
<td>[113]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MAGE</td>
<td>Multiple tumour types</td>
<td>[114]</td>
</tr>
<tr>
<td>Tissue differentiation</td>
<td>gp100</td>
<td>Malignant melanoma</td>
<td></td>
<td>[115]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Melan-A/Mart-1</td>
<td>Malignant melanoma</td>
<td>[116]</td>
</tr>
<tr>
<td></td>
<td>PSA and PAP</td>
<td>Prostate cancer</td>
<td></td>
<td>[117]</td>
</tr>
<tr>
<td>Overexpressed</td>
<td>Her-2/Neu</td>
<td>Breast cancer among multiple other tumour types</td>
<td>[118]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>hTERT</td>
<td>Multiple tumour types</td>
<td>[119]</td>
</tr>
<tr>
<td>Oncofetal</td>
<td>CEA</td>
<td>Colorectal carcinoma</td>
<td></td>
<td>[120]</td>
</tr>
<tr>
<td>Posttranslationally altered</td>
<td>Glyco- and phospho-peptides</td>
<td>Leukaemia</td>
<td></td>
<td>[108, 109, 121]</td>
</tr>
</tbody>
</table>

Abbreviations: HPV = human papillomavirus, H&N = Head and neck, EBV = Epstein-Barr virus, MAGE = melanoma antigen E, gp100 = glycoprotein100, Mart-1 = melanoma antigen recognised by T cells-1, PSA = prostate specific antigen, PAP = prostatic acid phosphatase, Her-2/Neu = human epidermal growth factor receptor-2, hTERT = human telomerase reverse transcriptase and CEA carcinoembryonic antigen.

### The immunoproteasome

The proteasome is a large protein complex that cleaves intracellular antigens into peptides for MHCI presentation. The core of the proteasome consists of β-subunits (β1, β2 and β5) with proteolytic capacity [122]. A set of three alternative forms of the β-subunits (β1i, β2i and β5i) are constitutively expressed in DCs and lymphocytes; however, they can be induced in other cells by IFNγ, and together form the immunoproteasome [123, 124]. The immunoproteasome cleaves peptides preferentially different from the constitutively expressed proteasome and thereby generate a different set of peptide products [125]. The alternative repertoire of peptides results in the presentation of many unique peptides, though at the expense of others [126]. Proteasomes containing only one or two of the alternative β-subunits generate different peptides and are referred to as intermediate proteasomes. Guillaume et. al. [127] showed that two tumour-associated antigens (TAAs) were exclusively cleaved by the intermediate proteasomes, but were destroyed by the immunoproteasome. Although the intermediate proteasome was expressed at only a low percentage, the antigens generated were sufficient for inducing CTL responses. This highlights that tumour expression of multiple proteasomes generate a diverse repertoire of antigens which may be
essential for successful cancer immunotherapies that are dependent on tumour MHCI presentation of TAAs.

Cancer immunotherapy

In the last few decades the field of cancer immunotherapy has expanded enormously, with drugs and candidate drugs targeting both the innate and adaptive branches of the immune system. In addition, tumour-targeting drugs can induce long-lived immune responses through a combination of innate and adaptive responses following the cytolysis induced through ADCC/CDC or via antibody-drug conjugates. The aim of cancer immunotherapy is to eliminate tumours by inducing a tumour-specific immune response. Tumours are endogenously-derived and their recognition by T cells is therefore limited by central and peripheral tolerance to prevent autoimmunity. Breaking immune tolerance is therefore essential for successful immunotherapy. Important immunotherapies that boost tumour-specific T cells include adoptive T cell transfer (ACT), genetically engineered CAR T cells, activating/blocking antibodies (e.g., anti-CD40, anti-CTLA-4 and anti-PD-1) and therapeutic vaccines (e.g., tumour cells, viruses, proteins and peptides) [128].

Therapeutic cancer vaccination

Therapeutic cancer vaccination induces cellular immune responses against an existing disease, which differs from classical prophylactic vaccination that mainly induces the production of neutralising antibodies thereby preventing primary infection/tumour induction. Cancer vaccine strategies aim to deliver tumour-related material (e.g., irradiated tumour, cell line, proteins or peptides) and adjuvants to patients to generate sufficient tumour-specific responses (reviewed in [129]). Tumour-cell based vaccines can be in the form of autologous tumour cells or cell lines. The advantage of using the autologous vaccination strategy is that all TAAs of the specific tumour is used; however, the disadvantage is that the treatment is dependent upon the tumour providing sufficient tumour material and therefore may limit the types of tumours/patients that can be treated. On the contrary, by using cell lines as the tumour material it is possible to create a cost effective off-the-shelf vaccine, although with the disadvantage of not including sufficient TAAs required for certain tumours.

Another type of cell-based vaccine is the autologous administration of DCs that are loaded with antigen and activated ex vivo. The advantage is that the loading and activation of the DCs is controlled, however, the procedure is laborious and expensive. Another strategy is to deliver TAAs in vivo
through DNA/RNA- and virus-based strategies that provide at least some adjuvant properties by themselves; however, so far limited efficacy has been seen in the clinic. A more cost-effective vaccination strategy for in vivo delivery is via peptide/protein vaccines, which require additional adjuvants and where the disadvantage is that prior knowledge of TAAs is required.

Peptide vaccination
Therapeutic short peptide vaccines originally consisted of TAA-derived peptides of approximately 8-10 amino acids that directly bind MHCI for presentation to CD8+ T cells [130, 131]. However, short peptide vaccines can have limited use as they are designed for a specific HLA allele. Additionally, short peptides are presented by cells other than professional APCs and this may, in the absence of co-stimulation, result in immunological tolerance instead of immunity against the immunised TAA peptides/tumour [132]. Vaccines with longer peptides require internalisation, processing and presentation on MHCI by DCs and can thereby induce greater CD8+ T cell responses than short peptides [133, 134], and promote the eradication of established tumours in mice [132]. Longer peptides can also include multiple HLA epitopes, enabling treatment of a less selected patient population (depending on what epitopes are present in the peptide stretch).

Strict HLA allele dependence is avoided by using long overlapping peptides (spanning a whole protein) or mixtures of synthetic long peptides (SLPs), spanning several TAAs, and thereby expanding the number of patients that can be treated. Although the selection of SLP sequences for a peptide vaccine requires prior knowledge of the MHC epitopes of a TAA compared to whole protein vaccines (which contain all MHC epitopes), SLPs have been suggested as preferable to whole protein vaccines since DCs are superior in processing and presenting SLP-derived epitopes compared to whole protein-derived epitopes [135]. The limitation of peptide vaccines is their poor half-life in vivo along with the possible need for multiple synthetic peptides to induce proper immune activation, thereby challenging GMP production as compared to producing one defined molecule (e.g., a protein).

One other perspective is that longer peptide stretches, as well as proteins, can harbour CD4+ Th epitopes that are known to greatly enhance the induction of protective CD8+ T cell immunity [136] through licensing of DCs via CD40-CD40L signalling [137, 138]. In clinical trials, SLP vaccines induced low toxicity in cervical cancer patients [139] and have shown promising results when treating HPV-induced pre-cancerous lesions [139, 140]. However, there is clearly room for improvements in peptide vaccination; for example by enhancing DC maturation [141] as well as the delivery method, and possibly ensuring that the vaccine contains sufficient numbers of both Th and CTL epitopes. Successful development of alternative adjuvants is essential for the future use of therapeutic vaccination, which is the topic of the next section.
Peptide vaccine formulations

The ability of vaccines to generate potent antigen-specific T cell responses is highly dependent on the type of adjuvant that is used. Peptide vaccines are most commonly administrated in oil-based adjuvants like incomplete Freund’s adjuvant (IFA; also known as Montanide-ISA 51) that protect peptides from degradation and allow for a slow release formulation, while inducing an inflammatory response [142]. Despite induction of tumour-specific CD8+ T cells, the lack of therapeutic effect of Montanide-based vaccines is possibly due to sequestering of T cells at the vaccination site where they become dysfunctional [143]. To improve peptide vaccination, attempts have been made to combine or replace Montanide ISA-51 with one or several adjuvants (see Table 2) such as cytokines (GM-CSF, IL-2 and IFNα) [144-146], TLR agonists (Picibanil® and Hiltonol®) [147, 148] and CD40 agonists [149-151].

Table 2. Examples of peptide vaccine formulations that have entered clinical trials

<table>
<thead>
<tr>
<th>Peptide Formulation</th>
<th>Study</th>
<th>Indication</th>
<th>TAAs</th>
<th>Notes</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-ISA-51 GM-CSF</td>
<td>Phase II</td>
<td>Metastatic melanoma</td>
<td>MART-1 Gp100 Tyrosinase</td>
<td>No enhanced immunogenicity with low dose GM-CSF</td>
<td>[152]</td>
</tr>
<tr>
<td>DepoVax</td>
<td>Phase I</td>
<td>Advanced breast, ovarian and prostate cancer</td>
<td>e.g., TOPO2A and JUP</td>
<td>Ag-specific T cell responses</td>
<td>[153, 154]</td>
</tr>
<tr>
<td>M-ISA-51</td>
<td>Phase I</td>
<td>Cervical cancer patients</td>
<td>HPV16 E6 E7</td>
<td>Ag-specific CD4+ and CD8+ responses</td>
<td>[139, 140]</td>
</tr>
<tr>
<td>M-ISA-51 PolyICLC</td>
<td>Phase I</td>
<td>Advanced ovarian cancer</td>
<td>NY-ESO-1</td>
<td>Ag-specific Th1 responses</td>
<td>[148, 155]</td>
</tr>
<tr>
<td>M-ISA-51 OK-432</td>
<td>Phase I</td>
<td>Advanced cancer patients</td>
<td>NY-ESO-1</td>
<td>Ag-specific cellular and humoral responses</td>
<td>[147]</td>
</tr>
<tr>
<td>PolyICLC Resiquimod</td>
<td>Phase I/II</td>
<td>Melanoma</td>
<td>LPV7</td>
<td>NCT02126579 (Clinicaltrials.gov)</td>
<td>-</td>
</tr>
<tr>
<td>PolyICLC</td>
<td>Phase I</td>
<td>Newly diagnosed glioblastoma</td>
<td>Personalised neo-antigen</td>
<td>NCT02510950 (Clinicaltrials.gov)</td>
<td>-</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Phase I/Ia</td>
<td>Metastatic hormone-naive prostate cancer</td>
<td>hTERT</td>
<td>Few adverse events, Ag-specific T cell responses</td>
<td>[156]</td>
</tr>
<tr>
<td>M-ISA-51 plIFNα</td>
<td>Phase I/II</td>
<td>Colorectal cancer</td>
<td>p53</td>
<td>Ag-specific T cell responses</td>
<td>[157]</td>
</tr>
</tbody>
</table>

Abbreviations: TAAs= tumour-associated antigens, m-ISA-51 = Montanide-ISA-51, GM-CSF= granulocyte macrophage colony-stimulating factor, Mart-1= melanoma-associated antigen recognised by T cells, pg100= glycoprotein100, TOPO2A= topoisomerase 2a, Ag= antigen, HPV= human papillomavirus, PolyICLC= Hiltonol®, OK-432= Picibanil®, LPV= long peptide vaccine, plIFNα = PEGylated IFNα.

GM-CSF is a DC maturing cytokine that failed to provide additional effects of Montanide ISA-51 in a clinical trial with metastatic melanoma patients
However, with a higher dose GM-CSF, and as the only adjuvant, minor responses could be seen in prostate cancer patients [156]. IFNα is an interesting cytokine to combine with peptide vaccines as it stimulates DC maturation, cross-presentation to CTLs, and is proposed to induce the third signal required (such as IL-12) for CTLs to clonally expand and produce IFNγ [158]. In mice, IFNα promotes proliferation and accumulation of antigen-specific T cells, which subsequently inhibit tumour growth [159]. The efficacy of a peptide vaccine with p53-derived epitopes to activate antigen-specific T cells was greatly enhanced in combination with IFNα in a Phase I/II clinical trial with colorectal cancer patients [157].

An alternative way to enhance DC maturation is through TLR stimulation, mimicking the danger signal present during an infection. Peptide vaccines with TLR agonists generate anti-tumour responses [160] and can have a synergistic effect when several TLR agonists are combined [161]. Synergistic effects have also been seen by combining TLR agonists with a CD40 agonist that provide the licensing signal required to generate fully mature DCs, which subsequently induce tumour-specific CTL responses [150, 151].

Although preclinical studies with peptide vaccines are promising, the level of clinical responses is disappointing so far [162, 163]. The peptide vaccines described above are all mixtures of peptides and adjuvant co-injected at the same site which does not fully ensure targeting of the peptide and adjuvant to the same cell. Co-targeting of peptide and adjuvants to the same cell is possible through covalent conjugations, further described in the next section.

Conjugate vaccines

A DC should take up antigen and be induced to become activated/mature for being able to trigger a potent antigen-specific T cell responses. To assure co-targeting of antigen and adjuvant to the same DC, antigen-adjuvant conjugates have been developed such as TLR agonist-peptide conjugates [164] (see Table 3 for examples of conjugate vaccines that have reached clinical trials). TLR agonist-peptide conjugates promote DC maturation and efficient anti-tumour responses in mice [164-166]. The responses induced by these conjugates are more efficient than when adjuvant and peptide are administered as a mixture [164], which may be due to both receptor-mediated uptake and the adjuvant signal to the cell that receives the material. Furthermore, Zom et. al. [167] describes efficient DC maturation and subsequent T cell activation of cervical cancer patient-derived cells ex vivo, in response to a TLR agonist-peptide conjugate that is currently in a Phase I clinical trial (NCT02821494).

Another strategy to specifically target peptides to DCs has been to conjugate the peptides to a monoclonal antibody (mAb) specific for receptors expressed on the cell surface of DCs, such as DEC205 and the mannose re-
ceptor (MR) (amongst many others) [168]. DEC205 is an endocytic receptor that can be targeted with antibody-protein conjugates resulting in cross-presentation [169]; however, additional adjuvants are required to avoid peripheral tolerance and generate anti-tumour responses [170, 171]. In clinical trials, these strategies have generated both antigen-specific cellular and humoral responses [172, 173].

Table 3. Examples of clinical trials with conjugate vaccines

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Conjugate</th>
<th>Study</th>
<th>Indication</th>
<th>TAAs</th>
<th>Notes</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>long</td>
<td>Peptide-Amplivant (TLR agonist)</td>
<td>Phase I</td>
<td>HPV16 positive tumours</td>
<td>HPV16 E6</td>
<td>NCT02821494 (Clinicaltrials.gov)</td>
<td>-</td>
</tr>
<tr>
<td>long</td>
<td>PEP-3-KLH</td>
<td>Phase I</td>
<td>Glioblastoma multiforme</td>
<td>EGFRvIII</td>
<td>NCT00626015 (Clinicaltrials.gov)</td>
<td>-</td>
</tr>
<tr>
<td>full-length protein</td>
<td>Protein-DEC205-mAb Resiquimod polyICLC</td>
<td>Phase I</td>
<td>Advanced malignancies</td>
<td>NY-ESO-1</td>
<td>Ag-specific cellular and humoral responses</td>
<td>[173]</td>
</tr>
<tr>
<td>full-length protein</td>
<td>Protein-MR-mAb GM-CSF Resiquimod polyICLC</td>
<td>Phase I</td>
<td>Advanced epithelial malignancies</td>
<td>hCG-β</td>
<td>Ag-specific cellular and humoral responses</td>
<td>[172]</td>
</tr>
<tr>
<td>long</td>
<td>Oil-emulsion hCG-DT</td>
<td>Phase II</td>
<td>Metastatic colorectal cancer</td>
<td>hCG-β</td>
<td>hCG and DT-specific antibody responses</td>
<td>[174]</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>STn-KLH</td>
<td>Phase III</td>
<td>Metastatic breast cancer</td>
<td>STn (carbohydrate)</td>
<td>Safe, humoral responses, no overall benefit</td>
<td>[175]</td>
</tr>
</tbody>
</table>

Abbreviations: MR= mannose receptor, hCG-β= beta-human chorionic gonadotropin, STn-KLH= Sialyl-Tn-keyhole limpet hemocyanin, mAb = monoclonal antibody, Ag = antigen, GM-CSF= granulocyte-macrophage colony-stimulating factor, DT= diphtheria toxin, HPV= human papillomavirus, TLR= Toll-like receptor, EGFR= epidermal growth factor

The inclusion of a helper epitope in peptide vaccines is essential for inducing potent anti-tumour CTL responses [136]. Helper epitopes can be in the form of tumour epitopes, but also as non-specific helper sequences such as the tetanus toxoid helper sequence [176, 177] and keyhole limpet hemocyanin (KLH) [178-180]. KLH is a known carrier protein used to induce both humoral and cellular immune responses against a conjugated molecule, such as peptides, proteins or carbohydrates [181]. KLH conjugated to the carbohydrate STn (Theratope®) generated promising results in phase I and II clinical trials, however, was disappointing in a large Phase III trial with metastatic breast cancer patients [175]. This vaccine contained a carbohydrate antigen, so only humoral responses were induced; it is possible that additional T cell responses could give a better outcome in future vaccine designs. Another weakness of this trial was the lack of selecting patients based on the antigen expression on the tumour (i.e., patient stratification). Accurate patient stratification has given clinical success when applied to small molecules, for example for epidermal growth factor receptor (EGFR) targeted therapies that
are effective in lung cancer patients with a mutated EGFR [182]. Indeed, immunotherapies, along with tumour vaccines, may benefit from improved patient selection for improved clinical success rates. This is because the therapeutic effect of peptide vaccines requires not only induction of strong cellular responses against tumour antigens, but it also requires that the target tumour presents the tumour antigen on MHC molecules for recognition and killing by CTLs.

Therapeutic monoclonal antibodies

Monoclonal antibodies (mAbs) represent another promising strategy of how the immune system can be modulated to treat human disease. Murine mAbs were initially generated in 1975 by Köhler and Milstein when they developed hybridoma technology [183]. In 1992, muromonab (OKT3) was the first mAb approved by the FDA to suppress renal allograft rejection [184] and since then more than 40 mAbs are FDA approved and over 300 are currently under development [185]. The first therapeutic mAbs, including muromonab, were mouse antibodies that are greatly limited by the generation of human anti-mouse antibodies which reduce their half-life, thereby reducing therapeutic efficacy [186]. Therefore, enormous efforts to improve recombinant DNA technology were made to generate less immunogenic chimeric [187] and humanised antibodies with longer half-life and improved efficacy [188, 189]. Today, fully human antibodies can be made through phage display libraries [188].

The therapeutic effect of monoclonal antibodies can be generated directly by the Fab fragment binding its target, inducing an agonistic effect (e.g., anti-CD40 and TGN1412) or antagonistically by blocking the binding of natural ligands (e.g., natalizumab) (Figure 6). Additionally, mAbs can act indirectly by inducing Fc-mediated effector functions including antibody-mediated-cytotoxicity (ADCC) and complement-dependent-cytotoxicity (CDC). In addition, the high target specificity of mAb make them useful as vehicles for targeting other immunotherapeutics to the tumour; for example, liposomes loaded with a radiolabelled derivative of daunorubicin [190] and T cells engineered with a tumour-specific chimeric antigen receptor (CAR) [191].
The choice of the Fc part (isotype) of an IgG antibody requires careful consideration depending on the therapeutic application, as this can either enhance or limit effector functions such as ADCC and complement-dependent cytotoxicity (CDC), but also downstream signalling induced by the antibody/target interaction. Different IgG isotypes bind FcγRs and complement components with different affinity. IgG1 and IgG3 have high affinity for FcγRs and C1q, making them suitable candidates when ADCC and CDC effector functions are desired [53]. The direct killing via ADCC and CDC is of great interest when targeting cancer cells, which is the reason that many anti-cancer mAbs are generated as an IgG1 antibody (e.g., rituximab [53] and alemtuzumab). IgG1 is often preferred over IgG3 since the latter binds weakly to FcRn and therefore has a shorter serum half-life. IgG2 and IgG4 have less ability to induce ADCC and CDC due to their generally low or lack of affinity for FcγRs and C1q [50, 193], and they are therefore often chosen when treating inflammatory diseases where the induction of ADCC and CDC is not desired (as with natalizumab). IgG4 was, however, recently discovered to bind FcγRI, FcγRIIA, FcγRIIB, FcγRIIC and FcγRIIIA [50, 52] which should be kept in mind when designing new mAbs. Fc-mediated effects other than ADCC and CDC potentiate the therapeutic effect of CD40-
specific mAbs and the superagonistic effect of TGN1412, and this is described in the next few sections below.

CD40-specific mAbs

The central role of CD40 to mediate DC licensing makes the receptor an interesting target for cancer immunotherapy. In preclinical studies, CD40-specific agonists induce anti-tumour CTL responses with promising therapeutic results [194-196]. The main goal of CD40-specific agonists is to override the need for T helper cell-induced licensing of DCs, thereby allowing for a drug-induced optimisation of antigen presentation to stimulate tumour-specific CTLs. This is also dependent on the presence of TAAs that DCs can process and present to T cells. Therefore, treatment with CD40 agonists in preclinical studies has been combined with tumour vaccines [197] as a source of TAAs, or chemotherapeutics that induce tumour cell death and thereby cause the release of TAAs [36, 198]. In addition, murine models have shown that CD40 agonistic antibodies work well in close proximity to the tumour/tumour-draining lymph node, and depend on antigen release for proper induction of CTLs [199-201]. CD40 agonists have also been combined with IL-2 and TLR agonists, where other cell types have been proposed to exert an anti-tumour effect such as B cells [202] and macrophages [203].

CD40 is expressed on many cell types and adverse events caused by systemically delivered CD40 agonists in mice include liver damage [204]. Via local administration of CD40 agonists it is possible to reduce the dose, and thereby toxicity, without compromising efficient anti-tumour responses of both local and disseminated tumours [199, 200]. In humans, systemic delivery of CD40 agonists is dose-limited by adverse events such as liver toxicity as well as CRS [205, 206]. Johnson et al. [207] reported that CRS-induced by treatment with a CD40 agonist could be managed with corticosteroids without hampering the therapeutic effect.

The first CD40 agonist in a clinical trial was a fully human IgG2 antibody given to patients with advanced solid malignancies [206]. The results were promising, showing a safe profile and observed clinical activity with a 14% response rate. The potency of CD40 agonists in transgenic mice was later found to be greatly enhanced by modifying their Fc part from IgG2 to IgG1 [208-210]. IgG1 antibodies have greater affinity for FcγRIIB than IgG2 [63], which ensure crosslinking of the antibody and therefore the CD40 receptor. However, whether crosslinking of CD40 agonists is required for therapeutic effect in humans is being debated [211, 212].

Most clinical trials are with anti-CD40 IgG1 antibodies, but there is also one with an IgG2 antibody. These CD40-specific antibodies are assessed in combination with anti-PD-1 antibody (NCT2706353), chemotherapeutics (NCT02588443), or alone (NCT02482168). In paper III of this thesis, we
describe the development and preclinical proof-of-concept of a CD40-specific antibody (ADC-1013) specifically created for local administration. The first clinical trial of ADC-1013 was recently completed. This trial included patients diagnosed with advanced solid tumours (NCT02379741).

Cytokine release syndrome (CRS)

Therapeutic mAbs are most commonly administrated intravenously and can induce several different infusion reactions such as anaphylaxis, anaphylactoid reactions, complement activation-related pseudoallergy (CARPRA), and cytokine release syndrome (CRS) (reviewed in [213]). The cause of a rapid anaphylaxis reaction can be pre-existing IgE antibodies that result in the release of vasoactive mediators from mast cells. Cetuximab is an example of where IgE antibodies against a carbohydrate on the antibody cause an anaphylactic reaction in sensitised individuals [214]. Anaphylactoid reactions are also caused by mast cells but in an IgE-independent manner. CARPRA is caused by complement activation which causes the release of anaphylatoxins and subsequent release of vasoactive mediators from phagocytes such as mast cells and basophils (e.g., rituximab) [215].

CRS is generally characterised by the induction of TNFα and IFNγ after 1-2 hours, followed by IL-6 and other cytokines, such as IL-2 and IL-8, depending on the target cell and Fc-mediated effects. CRS is graded 1-5 with the following criteria [213]:

1. Mild reactions where the infusion continues
2. CRS symptoms, where the infusion is stopped and the patient responds to symptomatic treatments
3. Prolonged CRS symptoms where hospitalization is required
4. Life-threatening symptoms that require ventilator support
5. Death

The first report of CRS caused by an mAb was the CD3-specific mAb muromonab (OKT3) [184], and more recently, the focus on improving prediction and management of CRS induced by mAbs has exploded since the incidence with TGN1412 in 2006 [216].

TGN1412

The humanized IgG4 mAb TGN1412 targets the costimulatory molecule CD28 expressed on T cells. TGN1412 is a superagonistic antibody, meaning that it can activate T cells without engaging the T cell receptor [217]. It was initially postulated to only activate and expand T regulatory cells [218] for the treatment of autoimmune disorders [219]. After extensive pre-clinical
safety tests, TGN1412 was approved for a Phase I clinical trial in healthy volunteers by both the German and UK regulatory agencies [220].

In March 2006 the trial was initiated with 6 volunteers, who after 90 minutes post TGN1412 infusion experienced severe headache, lumbar myalgia, hypotension, fever and tachycardia amongst many other side effects over the following few hours. The volunteers eventually experienced multi-organ failure and despite the severity all individuals survived [216]. The cause of the severity of TGN1412 infusion in these volunteers was the induction of an immediate CRS with high levels of IL-2, TNFα, IFNγ, IL-6, IL-8, IL-1β and IL-10 [216]. Additionally, the severe CRS was not predictable by the standard pre-clinical safety tests performed prior to the clinical trial, emphasising that a more optimal strategy was required for in vitro and in vivo toxicology prior to a first-in-human clinical trial.

Why did the safety tests fail?

The failure of the safety tests for mAbs may depend on several reasons including: an unexpected effect of binding its target, cross-reaction with another target, target expression by unknown cells/tissues, lack of homology in structure/function of target molecule in the animal model [221] and unexpected Fc-mediated effects (ADCC or CDC). Historically, many therapeutic mAbs targeting tumour cells were generally considered safe in human, however, the range of different targets has rapidly increased and new toxicity problems need careful consideration when, for example, the mAb is an immunomodulating antibody. In vitro safety tests of mAbs for the prediction of immediate cytokine release in humans are commonly performed with human peripheral blood mononuclear cells (PBMCs) or whole blood, as this biological material is more accessible than tissue samples. The problem with the in vitro safety tests performed with TGN1412 was later revealed to be connected to the aqueous display of the antibody, which means that there is an absence of cytokine production in the previously standard whole blood and PBMC assays used. Afterwards, when TGN1412 was coated by air-drying, to allow the solvent to evaporate, TGN1412 was found capable of stimulating PBMCs to produce IL-2, TNFα, IFNγ and IL-6 with similarities to the cytokine profile of the volunteers in the clinical trial [222]. Later, aqueous TGN1412 was shown to induce cytokine release by PBMCs in high-density cultures [223] in an FcγRIIB-dependent manner [224, 225].

The choice of in vivo model depends strongly on the structure and functional homology of the target molecule with its human counterpart. The amino acid sequence of human CD28 is 100% homologous with the CD28 of cynomolgus monkey and was therefore chosen as the in vivo animal model for toxicity studies [226]. In agreement, in vitro studies showed that TGN1412 had similar affinity for the human and cynomolgus monkey CD28 molecule [226]. The cytokine release in cynomolgus money was very low in
response to 500x higher TGN1412 dose than the dose given to the volunteers (0.1 mg/kg) in the clinical trial [220, 227]. This species difference was initially postulated to depend on differences in function and/or intracellular signaling pathways [222]. However, Eastwood et al [228] proposed a likely explanation for this species difference by the difference in expression pattern of CD28 by CD4+ effector memory T cells. It was shown that expression of CD28 was absent on effector memory T helper cells in cynomolgus monkey but present in humans. Therefore, the importance of biological knowledge of a mAb and its target molecule is essential when choosing the right \textit{in vitro} and \textit{in vivo} tests for safety prediction.
Aims of the current investigation

Paper I
Since immune complexes can be used to deliver material into antigen presenting cells and at the same time induce their activation, we investigated the potential of synthetic peptide vaccines via an immune complex-delivery approach. The aim was initially to identify a linear B cell epitope that could be conjugated to synthetic peptides harbouring T cell epitopes and to study the effect of this conjugate on DC and T cell activation. Our prerequisite for this peptide was that most donors should have antibodies to this sequence and be of an IgG isotype. Additionally, we aimed to identify a method by which peptides could be linked to each other with a chemistry allowing for GMP production.

Paper II
The aim of this paper was to study if human T cell responses could be improved by the conjugation of the MTTE-sequence (identified in paper I) to a peptide containing the model CMV-derived T cell epitope (pp65NLV). Initially, we set out to study the uptake of the conjugate in human cells along with T cell activation. Ultimately, we aimed to identify a possible mechanism of action of uptake/T cell activation and to determine if antibody titres would affect T cell activation.

Paper III
Several anti-CD40 agonistic antibodies have been developed over the years with the intention for clinical use. Herein we set out to modify an existing anti-CD40 antibody to make it suitable for local tumour immunotherapy and to validate this antibody in a preclinical model system.

Paper IV
PBMC and whole blood assays are used to investigate cytokine release induced by mAbs. The limitations in these systems are lack of immunoglobulins as well as functional cascade systems. Modified Chandler loop models have historically been used to study the interaction of foreign surfaces with
blood and later autologous cell/blood interactions. However, the system has great potential in evaluating cytokine release in response to mAbs. Here we aimed to assess a modified Chandler loop model for its potential in predicting cytokine release and mechanism-of-action of immunotherapeutics.
Methods

Therapeutic vaccination strategy

Our vaccination strategy presented herein aims to improve therapeutic SLP vaccination with the help of endogenous circulating antibodies [229]. A model peptide conjugate vaccine, referred to as [MTTE]3-SLP, was developed and validated in murine model systems (Paper I). The model conjugate used herein consists of a linear SLP designed according to the target of interest (with one or more T cell epitopes, albeit the final aim is to build a vaccine containing a mixture of multiple SLPs [i.e., multiple T cell epitopes]). The SLP is linked to a B cell epitope consisting of three identical linear minimal tetanus toxoid-epitope (MTTE) peptides. The MTTE peptide was chosen based on the majority of humans having MTTE-specific circulating IgG antibodies as a result of standard tetanus toxoid vaccination programs. On a linker molecule, the SLP and three MTTE peptides are conjugated (Figure 7) by copper-free click chemistry to limit copper-induced toxicity. The peptide-peptide conjugate can form soluble ICs with endogenous circulating antibodies, thereby enhancing peptide uptake by DCs via FcγR and/or complement receptors (Figure 7 [1]). The ICs stimulate DCs to secrete IL-12 and express costimulatory molecules (e.g., CD86 and MHCII) on the cell surface (Figure 7 [2]). The SLPs are processed and presented to both CD4+ and CD8+ T cells (Figure 7 [3]). CD4+ T cells promote CD8+ T cell killing ability via CD40L-CD40 ligation, enhancing DC maturation. As a result, activated CD8+ T cells migrate to and kill the target cells (e.g., tumour cells) (Figure 7 [4]).
Figure 7. Schematic illustration of the vaccination strategy. The SLP conjugate, with the help of endogenous circulating antibodies, is delivered to (1) and activates DCs (2) that promote T cell-mediated killing of tumour cells (3 and 4). The figure is taken from Figure 6 in paper I). MTTE= minimal tetanus toxoid-epitope, SLP= synthetic long peptide, Ag= antigen.

The circulating whole blood loop assay

In the blood loop assay, whole blood from a healthy individual is collected in an open system where the donor blood flow rate and gravity determine the acquisition rate, in contrast to the classically used closed vacutainer blood acquisition method where the vacuum sucks the blood into the tube. The open system of blood acquisition that exerts less pressure on the blood, compared to the closed system, minimises the required amount of anti-coagulant and therefore preserves complement cascade proteins. The blood is immediately mixed with the thrombin-specific inhibitor hirudin (Paper II) or low concentration of heparin (Paper IV), at doses that ensure a preserved complement system [230] when blood circulation is maintained. The blood circu-
lates within loops made of PVC-tubing that are connected with hollow metal connectors. The loops rotate on a wheel maintained within a 37°C incubator to mimic human blood circulation (Figure 8). All materials in direct contact with the blood are surface heparinised to prevent coagulation and complement activation. By adding molecules of interest (e.g., peptides and antibodies), their interaction with complement intact whole blood is feasible over time by sampling blood at different time points. By immediately mixing blood aliquots with the chelating agent EDTA, which terminates coagulation and complement activation, the desired analyses of both blood cells and plasma, (e.g., complement activation, cytokine release, immune cell activation and cellular distribution of labelled molecules) can be performed.

**Figure 8.** Schematic drawing of the circulating blood loop assay. Whole blood circulates in loops that rotate on a wheel at 37°C to mimic human blood circulation [231, 232].
Summary of papers

Paper I

A tetanus toxin-derived linear peptide (MTTE) was identified by screening serum from 17 donors for antibodies specific for tetanus toxin-derived overlapping peptides. The majority of donors had IgG but not IgM MTTE-specific antibodies. To identify the optimal peptide for antibody binding, different peptide versions (single amino acid changes and free C- or N-terminus) of the originally identified peptide were made, and screened for antibody binding with ELISA using serum from high-titre donors. None of the amino acid changes improved antibody binding; thus, the original sequence was deemed optimal candidate peptide and a free N-terminus was found required for antibody binding. The C-terminus was therefore used to create peptide-peptide conjugates. The formation of conjugates was possible by the development of a chemical protocol with the possibility to link four peptides (three MTTE sequences and one peptide containing T cell epitopes) to a core linker molecule. Conjugates with three MTTE sequences and an SLP (containing a surrogate T cell epitope [SIINFEKL]) activated mouse DCs (surface expression of MHCII and CD40, and secretion of IL-12p40) and enhanced T cell priming in the presence of MTTE-specific antibodies in vitro. Conjugates with one MTTE sequence failed to activate DCs and prime T cells, whereas conjugates with two or three MTTE sequences per T cell epitope performed equally well in inducing DC activation and T cell priming.

Paper II

In the modified Chandler loop, a conjugate with three MTTE sequences labelled with an Alexa Fluor488® was taken up by human blood monocytes and CD1c+ DCs in an antibody-dependent manner; this was not detected when studying cells that do not express Fc receptors (e.g., T cells and erythrocytes). The classical complement component C1q was partly found to be involved in the uptake mechanisms rather than FcγRs, and in one instance, a C1q block abolished T cell activation. A surrogate conjugate with three MTTE sequences and an SLP containing the CMV-derived model T cell epitope (pp65NLV) resulted in improved CD8+ T cell recall responses compared to conjugates with one and two MTTE sequences. It was required to have the SLP linked to the MTTE sequences in order to induce recall T cell responses, as no T cell responses were induced when three MTTE were attached to the core molecule and the SLP were administered in an unconjugated state. The low anti-MTTE titres in some donors could be boosted with a DTP vaccination mainly generating MTTE-specific IgG1 antibodies; this was followed by an increase in CD8+ T cell recall responses.
Paper III

The ADC-1013 antibody was developed for local cancer immunotherapy, and was therefore optimized with a high affinity for its target CD40 at low pH to mimic the tumour milieu. ADC-1013 stimulated human DCs in vitro to express the costimulatory molecules CD80 and CD86, and to secrete IL-12. The effect of ADC1013 on tumour growth was evaluated using two mouse models; first with immunodeficient NSG mice that were transplanted with a human bladder cancer cell line, and secondly, with immunocompetent human CD40 transgenic (hCD40tg) mice transplanted with a mouse bladder cancer cell line (hCD40 negative). In the NSG model, ADC1013 induced killing of CD40-positive tumour cells, and in the hCD40tg model, ADC1013 induced killing of CD40-negative tumour cells. This demonstrates a dual mechanism of action of killing CD40 expressing tumour cells directly or by licensing APCs to cross-present tumour antigen to T cells. Furthermore, long-term tumour-specific T cell-mediated immunological memory was induced by ADC-1013 in the immunocompetent hCD40tg model.

In conclusion, local administration of ADC1013 eradicates bladder cancer in mice models and induces long-term immunological memory.

Paper IV

We evaluated a modified Chandler loop model for its potential use in predicting cytokine release syndrome (CRS) induced by therapeutic monoclonal antibodies (mAbs). The agonistic antibodies anti-CD3 (OKT3) and anti-CD28 (ANC28.1) induced cytokine release in the loop assay after only 4 hours of incubation. In contrast, non-agonistic antibodies such as cetuximab and natalizumab were unable to induce cytokine release in the loop assay, in line with the low incidence of CRS in patients. A TGN1412-like antibody induced CD4+ memory cells to produce IL-2, consistent with the healthy controls in the clinical trial in London [216]. By blocking different components of the CDC and ADCC pathways, we could identify that alemtuzumab kills CD3+ cells via CDC and B cells via ADCC in human whole blood.

Future perspective

Paper I and II

We are in the revision process of these submitted papers. Currently, we are incorporating more donors into the analysis of the one, two and three MTTE comparison to further investigate how many MTTEs are needed to form optimal immune complexes and thereby improve antigen uptake and antigen presentation. The current human data in paper II include recall responses with one antigen derived from the CMV pp65 protein and we are currently in
the process of amending another antigen by generating conjugates with T cell epitopes derived from the influenza virus and Epstein-Barr virus. We also aim to perform further in vivo studies in animal models.

The peptide conjugate vaccine project was awarded funding from the BIO-X programme (Uppsala BIO) and the funded project was initiated in 2014 through collaboration between our academic group and our collaborators at the LUMC, Leiden, The Netherlands. This project also gave the impetus to establish Immuneed AB, founded to enable the project to work towards a clinical trial. The funded project aimed to characterise the concept further in model systems, to prepare a GMP batch of [MTTE]3-SLPs from prostate cancer (PC)-specific TAAs, and to test the toxicity of the drug candidate. With an ethical permit, we have been able to obtain blood samples from prostate cancer patients before and after they have received a DTP booster vaccination. For proof-of-concept, we ran blood loop experiments to look at recall responses generated by the vaccine in a similar fashion to the surrogate conjugate in Paper II. The data is under analysis (at the time of printing this thesis) and the conjugate vaccine has generated a recall response in blood from some patients, which we are currently correlating with the patients’ level/isotype of MTTE-specific IgG antibodies, HLA-type and clinical data.

For this peptide conjugate strategy and other therapeutic vaccination strategies to be successful in clinical trials, three important factors need to be carefully considered; (1) patient stratification (i.e., selecting patients whose tumour presents the TAAs on their MHC), (2) whether additional adjuvants are required or if Fc-mediated DC activation is sufficient, and (3) whether there is a need to combine with checkpoint blockers or other agents to maintain activated T cells in the immunosuppressive environment in the tumour milieu. Lessons from other DC-targeting conjugates include, for example, the DEC205-specific antibody conjugated to hCG-β where additional adjuvants are required to avoid tolerance [170, 171]. However, the difference is that DCs are activated by FcγR-targeting of immune complex but not by DEC205-targeting of specific antibodies.

Paper III
The first clinical trial with locally injected ADC-1013 was completed in March 2017. Prior to completion, the trial had been amended with an intravenous administration arm. Results are expected to be reported late 2017. CD40 agonistic antibodies are a focus for many companies and the great interest in Fc receptor cross-linking for optimal efficacy of anti-CD40 antibodies has spurred new interest in the development of this field. Biotechnology advances have allowed for innovative designs of antibodies that can enable improved CD40 cross-linking and thereby enhanced efficacy. An example is the development of hexavalent single-chain receptor-binding
domains (e.g., anti-TNFR drug candidates by Apogenix). However, it still remains to be understood how to handle the administration as increased efficacy through these innovative approaches may lead to both liver toxicity and cytokine release. As anti-CD40 antibodies are mediating improved antigen presentation, the co-evolution of CD40 agonistic therapies with tumour vaccine development is natural and may lead to new drug candidates over time. Furthermore, the interesting synergistic effect of CD40 agonistic antibodies and TLR agonists to boost therapeutic vaccination and thereby anti-tumour responses in mice is an interesting concept for future clinical trials.

Paper IV

The in-depth characterisation of the whole blood loop system as a cytokine release assessment tool allows the assay to be used for preclinical safety studies. Companies have also used the system for this purpose and we foresee that a publication will allow expansion of its use. The opportunity to study cellular biodistribution (paper II) is also of interest in the preclinical and clinical development phase of monoclonal antibodies and we foresee that this system can be useful to understand the interplay between on-target (antigen-specific) and off-target (Fc-mediated effects) interactions that monoclonal antibodies have, along with how bispecific antibodies behave in circulation. In addition, the system has been useful for studying the vaccine candidate in patient blood, see section Paper I and Paper II above.
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References


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