Lentivector-based cancer immunotherapy silencing PD-L1 and modulating cytokine priming; Development of *ex vivo* myeloid-derived suppressor cells to assess therapeutic efficacy.

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Thesis submitted to the University College London for the degree of Doctor of Philosophy

2015
DECLARATION

I, Therese Maria Liechtenstein, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

[Signature]

Therese Liechtenstein
ABSTRACT

Cancer immunotherapy strategies based on lentiviral vector (lentivector) transduction have recently been shown to be safe and effective in the clinic. The objective of cancer immunotherapy is to induce anti-cancer immune responses. Cytotoxic T cell (CTL) responses are particularly effective at recognizing and eradicating cancer cells and their activation is the main objective of most cancer immunotherapy treatments. Effective CTL responses depend on activation by antigen presenting cells (APCs), presenting tumour-associated antigens (TAAs) in the context of appropriate co-stimulatory and cytokine signals. In general, cancer immunotherapy has largely been ineffective due to tumour-induced immune suppression. One immunosuppressive mechanism employed by tumours relies on the accumulation of tumour-infiltrating myeloid-derived suppressor cells (MDSCs). Effective cancer immunotherapy treatments therefore need to stimulate tumour-specific CTLs as well as counteract the activity of tumour-infiltrating immunosuppressive cells.

The first part of this thesis is based on the construction and evaluation of lentivector vaccines. The lentivector vaccines simultaneously expressed TAAs and cytokines, combined with silencing of the co-inhibitory molecule programmed death 1 ligand 1 (PD-L1). A collection of lentivector vaccines expressing an array of different T cell-polarising cytokines was generated and their T cell stimulatory and anti-tumour efficacies were assessed in vitro and in vivo.

In the second part of this thesis a highly efficient and rapid method to produce large numbers of melanoma-infiltrating MDSCs ex vivo was developed, without inducing tumours in mice. Ex vivo MDSC phenotype, differentiation, and immunosuppressive activities were extensively studied. The novel ex vivo melanoma MDSCs were further used to evaluate the lentivector vaccines generated in the first part of this thesis. Simultaneous delivery of IL12 and a PD-L1-silencing microRNA was the only combination that could counteract ex vivo MDSC suppressive activities, correlating with therapeutically relevant anti-melanoma activities in a syngeneic B16 melanoma mouse model.
ACKNOWLEDGEMENTS

First and foremost I thank my supervisor Dr. David Escors for his support and guidance. His infinite enthusiasm for the field of cancer immunology never let me forget how important and exciting this research is. David’s ability to cheer you up and look at the world from an unconventional angle was indispensable for the completion of this thesis. He was an inspiration not only because of his expertise in immunology but also as a great example of how to lead and take care of people.

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One of the most important lessons in writing a PhD is to develop a critical mind and keep going during difficult circumstances. I thank my family and friends for pushing me further. My mother deserves a special thank you as an eternal defender of the importance of science and my father for inspiring me to always strive for success. Especially important was the distraction caused by my sister, cousins, and friends, which let me take my mind of things from time to time.
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ABBREVIATIONS:

ACOX3  Acyl-CoA oxidase
ACTN4  Alpha actinin 4
AKT    Protein kinase B
ALDH3  Aldehyde dehydrogenase
APC    Antigen presenting cell
ApoB   Apolipoprotein B
ARG-1  Arginase 1
ATRA   All-trans-retinoic acid
BM     Bone marrow
BM-DC  Bone marrow-derived dendritic cell
C/EBPβ CCAAT/enhancer-binding protein beta
CD     Cluster of Differentiation
cDNA   complementary DNA
CFSE   Carboxyfluorescein succinimidyl ester
CKB    Creatine kinase B
CLP    Common lymphoid progenitor
CLTA   Clathrin light chain A
CM     Conditioning Medium
cMoP   Common monocyte progenitor
CMP    Common myeloid progenitor
CMV    Cytomegalovirus
COX-2  Cyclooxygenase-2
cPPT    Central polypurine tract
CPT1A   Carnitine O-palmitoyltransferase
CTL    Cytotoxic T lymphocyte
CTLA-4  Cytotoxic T lymphocyte-associated protein 4
DC     Dendritic cell
DC-SIGN Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DMEM   Dulbecco’s Modified Eagle’s Medium
DNA    Deoxyribonucleic Acid
dNTP   deoxynucleotide triphosphate
dsDNA  double-stranded DNA
DTT    Dithiothreitol
ECL    Enhanced chemiluminescence
EDTA   Ethylenediaminetetraacetic acid
ELISA  Enzyme-linked immunosorbent assay
ELIspot Enzyme-linked immunospot assay
EMH    Extramedullary haematopoiesis
EP-4   Prostaglandin E receptor 4
EPHX-1 Epoxide hydrolase 1
ER     Endoplasmic reticulum
ERK    Extracellularly regulated protein kinase
FACS   Fluorescence-activated cell sorting
FAS    Fas cell surface death receptor
<table>
<thead>
<tr>
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<tr>
<td>FASL</td>
<td>Fas cell surface death receptor ligand</td>
</tr>
<tr>
<td>FBS</td>
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</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FBL</td>
<td>Fibrillarin</td>
</tr>
<tr>
<td>FDR</td>
<td>False discovery rate</td>
</tr>
<tr>
<td>FLT3</td>
<td>Fms-like tyrosine kinase 3</td>
</tr>
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<tr>
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<td>Granulocyte colony-stimulating factor</td>
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<td>Green fluorescent protein</td>
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<tr>
<td>GITR</td>
<td>Glucocorticoid-induced TNFR-related protein</td>
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<td>Interferon</td>
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</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove’s Modified Dulbecco’s Medium</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>iMC</td>
<td>immature myeloid cell</td>
</tr>
<tr>
<td>IRG-1</td>
<td>Immuneresponsive gene 1 protein</td>
</tr>
<tr>
<td>iTRAQ</td>
<td>Isobaric tag for relative and absolute quantitation</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus family of tyrosine kinases</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun protein kinase</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani medium/Lennox broth</td>
</tr>
<tr>
<td>LMNB2</td>
<td>Lamin B2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTR</td>
<td>Long Terminal Repeat</td>
</tr>
<tr>
<td>LV</td>
<td>Lentiviral vector</td>
</tr>
<tr>
<td>M1</td>
<td>Classically activated macrophages</td>
</tr>
<tr>
<td>M2</td>
<td>Alternatively activated macrophages</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony stimulating factor</td>
</tr>
<tr>
<td>MCSF-R</td>
<td>Macrophage colony stimulating factor receptor</td>
</tr>
<tr>
<td>MAGE</td>
<td>Melanoma antigen gene</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MART-1</td>
<td>Melanoma-associated antigen recognized by T cells</td>
</tr>
<tr>
<td>MCA</td>
<td>methyl-cholanthrene</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein 1</td>
</tr>
<tr>
<td>MDP</td>
<td>Macrophage/DC progenitor</td>
</tr>
<tr>
<td>MDSC</td>
<td>Myeloid-derived suppressor cell</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIF</td>
<td>Macrophage migration inhibitory factor</td>
</tr>
<tr>
<td>M-MDSC</td>
<td>Monocytic Myeloid-derived suppressor cell</td>
</tr>
<tr>
<td>MLR</td>
<td>Mixed lymphocyte reaction</td>
</tr>
<tr>
<td>MLV</td>
<td>Mouse leukemia virus</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem mass spectrometry</td>
</tr>
<tr>
<td>MSC</td>
<td>Myeloid suppressor cell</td>
</tr>
<tr>
<td>MTHFD2</td>
<td>Methylenetetrahydrofolate dehydrogenase</td>
</tr>
<tr>
<td>MYD88</td>
<td>Myeloid differentiation factor 88</td>
</tr>
<tr>
<td>N1</td>
<td>Anti-tumourigenic tumour-associated neutrophil</td>
</tr>
<tr>
<td>N2</td>
<td>Pro-tumourigenic tumour-associated neutrophil</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NDUFV2</td>
<td>NADH dehydrogenase flavoprotein 2</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer (cell)</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NOS2</td>
<td>Nitric oxide synthase 2</td>
</tr>
<tr>
<td>Nox2</td>
<td>NADPH Oxidase</td>
</tr>
<tr>
<td>NP</td>
<td>Nucleoprotein</td>
</tr>
<tr>
<td>NS</td>
<td>Natural suppressor (cell)</td>
</tr>
<tr>
<td>NSF</td>
<td>Vesicle fusing ATPase</td>
</tr>
<tr>
<td>NY-ESO-1</td>
<td>Cancer testis antigen</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>Superoxide Anion</td>
</tr>
<tr>
<td>OAT</td>
<td>Ornithine aminotransferase</td>
</tr>
<tr>
<td>OH</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>ONOO$^-$</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>P450R</td>
<td>Cytochrome p450 reductase</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood monocyte</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>TRP1</td>
<td>Tyrosinase-related protein 1</td>
</tr>
<tr>
<td>TRP2</td>
<td>Tyrosinase-related protein 2</td>
</tr>
<tr>
<td>VAPA</td>
<td>Vesicle-associated membrane protein-associated protein A</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VSV-G</td>
<td>Vesicular stomatitis virus G protein</td>
</tr>
<tr>
<td>WPRE</td>
<td>Woodchuck hepatitis virus posttranscriptional regulatory element</td>
</tr>
<tr>
<td>X-SCID</td>
<td>X-linked severe immunodeficiency</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION

Ever since immunotherapy and vaccination were successfully applied against infectious diseases have immunologists been dreaming of applying the same strategies to the fight against cancer. In this way, the immune system could effectively and specifically target cancer cells, leading to long-term immunological memory and prevention of tumour re-establishment. These hopes have largely been unfulfilled as cancer immunotherapy strategies and vaccinations have been rather unsuccessful in the clinic, especially concerning non-virally induced cancers. Cancer cells derive from the host’s own cells and thus cancer immunotherapy strategies have to break immunological tolerance to self-antigens to raise cancer-specific immune responses. This is a rather difficult undertaking as the immune system regulates itself to prevent auto-immunity, thus representing a major barrier for successful cancer immunotherapy strategies. Nevertheless, anti-cancer immune responses have been successfully raised in pre-clinical and clinical studies (Bronte, Apolloni et al. 2000; Germeau, Ma et al. 2005; Chapatte, Colombetti et al. 2006). But as early clinical studies showed, this is not enough to effectively eliminate tumours (Rosenberg, Sherry et al. 2005; Boon, Coulie et al. 2006). Once cancer-specific effector cells have been raised, they are frequently inactivated by the suppressive tumour environment, diminishing the efficacy of the treatment. The immunosuppressive tumour micro- and macro-environment represents the second major barrier that cancer immunotherapeutic treatments need to overcome. It could be argued that tumour cells have developed an independent life, with purposes distinct and harmful to the organism they belong to. This view is emphasized by such statements as the following: “In the last 10 years, it has become evident that a tumor becomes aware of its susceptibility to immune attack and elaborates many defences against it” (Cavallo, De Giovanni et al. 2011). Thus, raising an effective immune response against cancer cells is (in most cases) a much more challenging undertaking than against foreign pathogens.

The complex interactions between cancer and the immune system are described in this chapter. In addition, current cancer immunotherapeutic treatments and the use of lentiviral vectors (lentivectors) as gene transfer vectors are described and discussed.
1.1. CANCER AND THE IMMUNE SYSTEM

Due to the complexity of cancer and the difficulty of treating and curing these malignancies there exists a high, unmet medical demand to find more targeted and effective therapies. Current treatments are accompanied by serious adverse events and more targeted therapies are needed to circumvent collateral damage to healthy tissues. Cancer is such a difficult disease to treat because cancer cells develop from normal tissues and retain a high similarity to the tissue they develop from. The difficulty lies in finding treatments that can distinguish such similar non-transformed cells. Our immune system remains the most effective and sophisticated system to distinguish and fight disease-causing processes. However, in the fight against cancer our immune system needs some extra help.

Cancer Immunology can be viewed as one of the most dynamic fields of biological research. At the same time it remains one of the most controversial areas of biology, in which even basic concepts are still under debate. The exact role of the immune system in cancer remains controversial as the relationship between immune cells and cancer cells are highly complex and can lead to various opposing effects, including anti-tumour protection, tumour promotion, or no visible effects at all (Ichim 2005; Weinberg 2006). The immune system is in a precarious situation because under physiological conditions it is crucial to maintain peripheral tolerance to prevent auto-immunity and initiate mechanisms terminating immune responses after pathogen clearance. However, peripheral tolerance and the presence of immunosuppressive cell types in turn facilitate tumour immune evasion. There is a thin line separating over-reactivity to self-tissues and tumour tolerance, which explains the duality of the immune system in respect to cancer responses.

Nevertheless, it is now widely accepted that the immune system can recognize and eliminate cancer cells, supporting the theories of cancer immunosurveillance and elimination. To complicate things more, the various activities of several immune cells can change the course of cancer progression and contribute to variability in responses to different tumour types and within different individuals.
1.1.1. CANCER AND THE TUMOUR MICROENVIRONMENT

Traditionally, the hallmarks of cancer were viewed as the accumulation of genetic and epigenetic mutations in genes regulating cell cycle processes, including proliferation, replication, and apoptosis. Transformed cells could go on to acquire further mutations leading to tumour invasion, angiogenesis, and metastasis. The accumulation of mutations in proto-oncogenes or tumour-suppressor genes is a gradual process. Thus, tumours consist of a heterogeneous group of cancer cells at various stages of transformation. Cancer cells can be subcategorized into various types, including cancer stem cells, cancer cells, and invasive cancer cells. Deregulated growth of cells and metastasis to other organs interfere with the physiological function of the invaded organs, leading to disease symptoms (Hanahan and Weinberg 2000; Hanahan and Weinberg 2011).

Importantly, it has become increasingly clear that tumours do not only consist of transformed neoplastic cancer cells. Tumour cells form complex tissues with multiple cell types that have been recruited to the nascent tumour site. Non-transformed cells within tumour tissues are generally termed the tumour stroma. Cells of the tumour stroma interact with each other and with cancer cells, leading to the development of the tumour microenvironment. These interactions further enhance tumour development by enhancing tumour progression, vascularization, immune evasion, and eventually metastasis. Pericytes, for example, play a role in maintaining tumour vasculature while myeloid cells aid in tumour angiogenesis and immunosuppression. The tumour microenvironment hence contains several distinct cell types, including cancer-associated fibroblasts, endothelial cells, pericytes, and cancer cells at various stages of transformation. The tumour microenvironment is further infiltrated by various types of immune cells, exhibiting both anti-tumour effector and tumour-promoting functions (Hanahan and Weinberg 2011).

As tumour cells disrupt normal tissue architecture tumours trigger a “chronic” inflammatory environment. In addition, tumour cells secrete cytokines and growth factors abnormally, leading to further inflammation and recruitment of leukocytes to the microenvironment. These tumour-secreted factors suppress anti-tumour effector cells and skew infiltrating immune cells to a suppressive phenotype, as we will discuss
Inflammation plays a crucial role in tumour progression, and chronic inflammation is strongly associated with tumour initiation. Chronic inflammation can be described as a “wound that never heals”, leading to enhanced renewal of cells. This in turn increases the likelihood of acquiring neoplastic transformations. The constant activities of recruited phagocytic cells lead to an accumulation of reactive oxygen and nitrogen species (ROS and RNS) that can damage DNA, further enhancing the acquisition of mutations (Fernandes, DE Medeiros Fernandes et al. 2015). Thus it is generally accepted that the tumour microenvironment plays a key role in promoting tumour progression. Chromosomal instability of cancer cells is a major factor contributing to tumour progression, leading to transformation of cells through accumulation of genetic and epigenetic mutations. However, of equal importance is the environment, using chronic inflammation and tolerogenic immune responses for tumour initiation, progression and immune evasion.

1.1.2. CANCER IMMUNOSURVEILLANCE

Transformed neoplastic cells exhibit altered genomes and unstable transcriptomes, leading to presentation of altered antigens or altered levels of antigen density (Pardoll 2003). These mutated or aberrantly expressed forms of self-proteins are termed tumour-associated antigens (TAAs). Natural TAA-specific CD4 and CD8 responses take place in cancer patients but these TAA-specific T cells are mostly anergic or tolerogenic due to thymic deletion of strongly auto-reactive T cells and their inactivation by several peripheral tolerogenic mechanisms (Bakker, Schreurs et al. 1994; Boon, Coulie et al. 2006). Nonetheless, the existence of TAA-specific responses indicates the ability of the immune system to recognize and potentially fight cancer cells, paralleling its role in infectious disease.

The theory of cancer immunosurveillance was first proposed by Paul Ehrlich in 1909, applying his knowledge of immune responses in infectious diseases to the field of cancer. He proposed that the immune system could recognize and eliminate nascent tumour cells before they could establish a clinically evident tumour (Ehrlich 1909). This theory has been controversial ever since. The theory of cancer immunosurveillance was refined by Burnet in the mid 1900s (Burnet 1970) but went through a phase of rejection
following experiments in CBA/H nude mice in the 1970s. These experiments suggested that mice without an immune system developed spontaneous and methylcholantherene (MCA)-induced tumours in the same manner as immunocompetent mice (Stutman 1974). It was not known then that nude mice lacking a thymus are not entirely immunodeficient. Experiments in the 1990s and early 2000s revived the field of cancer immunosurveillance, showing that IFNγ (Interferon gamma) and lymphocytes are crucial for anti-tumour responses in mice (Shankaran, Ikeda et al. 2001; Teng, Swann et al. 2008). Further studies using mice deficient in various immune effector cell populations including NK (Natural killer), Th1 (T helper 1) or CTL (Cytotoxic T Lymphocyte) cells, demonstrated increased tumour incidence. Tumour incidence was even higher when combining T and NK cell deficiencies, suggesting that all these effector cell populations are involved in anti-tumour immune responses (Kim, Emi et al. 2007; Teng, Swann et al. 2008; Hanahan and Weinberg 2011).

The theory of immunosurveillance is further supported by an increased incidence of cancers in immunocompromised individuals. Many of the cancers that arise in immunocompromised patients are virally-induced. However, it is becoming clear now that the incidence of non-viral cancers such as melanoma also increases upon immunosuppression (Vajdic and van Leeuwen 2009; Vajdic, van Leeuwen et al. 2009; Hanahan and Weinberg 2011). Similar to the results observed in nude mice, patients with chronically suppressed immune systems do not exhibit a higher incidence of non-viral cancers. Again, these individuals may be deficient in T and B cell populations but still exhibit functioning NK cells and other innate immune cell populations. All of these observations have augmented the controversy of the role of the immune system in cancer. These arguments may be over-simplified when discussing a topic as wide and complex as cancer immunology (Hanahan and Weinberg 2011). In addition, due to the lack of clinical efficacy of tumour vaccines and other classical cancer immunotherapy treatments, the theory of immunosurveillance remained controversial for a long time. Recent clinical efficacy of new blocking antibodies such as immune checkpoint modulators (Brahmer, Drake et al. 2010; Topalian, Hodi et al. 2012; Trinh and Hwu 2012) and other novel immunotherapeutic approaches (Porter, Levine et al. 2011) have revived the field of cancer immunology and immunotherapy. As the interactions between the immune system and cancer are highly complex and the immune system
plays a dual role in tumour progression, the field of cancer immunology will remain a dynamic one for some time to come (Schreiber, Old et al. 2011; Escors 2014). As I will discuss later, the induction of tolerogenic T cells and myeloid cells play an equally important role in tumour etymology.

1.1.3. CANCER IMMUNOEDITING AND ESCAPE

Due to recent advances in cancer immunology showing that innate and adaptive immune effector cells can recognize and eliminate tumour cells (Girardi, Oppenheim et al. 2001; Pardoll 2001; Shankaran, Ikeda et al. 2001; Takeda, Smyth et al. 2001; Peggs, Quezada et al. 2009; Soong, Song et al. 2014) the concept of cancer immunosurveillance is now widely accepted (Dunn, Old et al. 2004; Zitvogel, Tesniere et al. 2006). As tumours arise nonetheless, it is evident that in many instances tumours avoid immune detection or develop mechanisms to escape and limit detection. The anti-cancer immune response thus leads to the selection of cancer cells that have adapted to survive in an immunocompetent host (Shankaran, Ikeda et al. 2001). This process has been termed cancer immunoediting and consists of three phases; elimination, equilibrium, and eventually escape (Dunn, Old et al. 2004). The initial phase of recognition and elimination consists of innate and adaptive immune responses, correlating to immune system functions in pathogen-related diseases. As the targets are transformed cells the main adaptive immune responses employed to eliminate them are T cell-mediated cytotoxic responses. The following paragraphs summarize the anti-tumour immune responses (Fig. 1.1), leading to recognition, elimination, cancer immunoediting, and eventually escape.

Chronic inflammation caused by tissue remodelling and disruption attracts immune cells to the growing tumour site (Fig. 1.1.A). The initiation of an anti-tumour immune response starts with the attraction and activation of macrophages, NK cells, NK T cells, and γδ T cells. These innate immune cells are attracted through secretion of pro-inflammatory cytokines and chemokines (Matzinger 1994; Kim, Emi et al. 2007; Hanahan and Weinberg 2011). Innate immune cells in turn produce chemokines and cytokines, including IFNγ and TNFα (tumour necrosis factor alpha), leading to the recruitment of additional leukocytes to the tumour microenvironment. NK cells are a primary source of
IFNγ, which can activate NK cells and macrophages to become cytotoxic. IFNγ has also direct and indirect anti-cancer effects. Direct effects include cancer cell cytotoxicity and inhibition of tumour growth by regulating proliferation, apoptosis, and angiogenesis. In addition, IFNγ affects cancer immunogenicity and promotes cancer cell recognition and elimination (Kim, Emi et al. 2007; Miller, Maher et al. 2009; Zaidi and Merlino 2011). Activated NK cells are capable of recognizing and eliminating cancer cells, as shown by several studies in mice and humans. NK cell-mediated cytotoxicity is initiated by granule-dependent perforin and granzyme secretion (Ichim 2005; Cullen and Martin 2008; Woo, Corrales et al. 2015). NK cell-mediated and IFNγ-mediated control of tumour growth can also be initiated by TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) binding (Takeda, Smyth et al. 2001). IFNγ secreted by NK cells polarizes macrophages to the classically activated M1 phenotype, which secretes large amounts of TNFα, IL12, and IL23, and exhibits tumouricidal activities (Galli, Borregaard et al. 2011).

The initial anti-tumour attack of the innate immune system releases TAAs from necrotic cancer cells, which is important for the initiation of adaptive immune responses (Fig. 1.1.B). Effector cells of the innate immune response (such as NK cells) do not need to be activated by professional antigen presenting cells (APCs). On the other hand, effector cells of the adaptive immune response acquire effector activities upon activation in the context of appropriate signals delivered by APCs. Dendritic cells (DCs) are nowadays considered to be the main professional APCs regulating adaptive immunity. DCs are also attracted to the tumour microenvironment through inflammatory signals (Matzinger 1994). Upon arrival DCs take up necrotic cancer cells, process TAAs, mature, and migrate to tumour-draining lymph nodes (Fig. 1.1.B). In tumour-draining lymph nodes DCs activate CD4⁺ T cells by presenting TAA peptides complexed to major histocompatibility complex (MHC) II molecules. DCs also cross-present TAA peptides complexed to MHC I molecules to CD8⁺ T cells (Huang, Golumbek et al. 1994; Lipscomb and Masten 2002; Goold, Escors et al. 2011)(Fig. 1.1.C). While CD8⁺ T cells are viewed as the main anti-cancer effector cells, activation of CD4⁺ T cells is needed to efficiently stimulate CD8⁺ T cells to obtain CTL activities. CD4⁺ T cells acquire different phenotypes and functions, depending on the cytokines that TAA-presenting DCs secrete. For anti-cancer immunity the desired CD4⁺ T cell phenotype is Th1 (T helper 1), as they aid in the activation of CTL responses. Secretion of IFNγ is necessary for Th1
differentiation, activation, and homeostasis and inhibits CD4+ T cell polarization to Th2 (T helper 2). Type M1 macrophages secrete TNFα, IL12, and IL23, and thus help in driving Th1 and Th17 responses (another pro-inflammatory T cell type) (Galli, Borregaard et al. 2011). Of note, IFNγ also has a role in regulatory T cell (Treg) differentiation, activation, expansion, and survival, reflecting the self-regulatory mechanisms of the immune system. Th1 cells in turn secrete mainly IFNγ, but also IL2 and IL12, which are important for CTL differentiation, expansion and survival. Stimulation by Th1 aids in the clonal expansion of activated effector CD8+ T cells. In addition, Th1 cells are needed for differentiation of CD8+ memory T cells and their reactivation upon encounter with the same TAAs (Macatonia, Hosken et al. 1995; Schmidt 2002; Curtsinger, Johnson et al. 2003; Curtsinger, Lins et al. 2003; Gerloni and Zanetti 2005; Knutson and Disis 2005; Zaidi and Merlino 2011; Liechtenstein, Dufait et al. 2012).

Activated CD4+ and CD8+ T cells infiltrate tumours and kill cancer cells in an antigen-specific manner (Fig. 1.1.D). Th1 and CTL cells can kill directly by IFNγ (and TNFα) secretion, which activates death receptors on tumour cell surface membranes and has anti-proliferative and anti-angiogenic effects (Knutson and Disis 2005; Kim, Emi et al. 2007; Tyciakova, Matsukova et al. 2015). IFNγ further leads to the up-regulation of MHC and co-stimulatory molecules on APCs, enhancing antigen presentation, activation of cell-mediated immunity, and cancer cell recognition and elimination. Secretion of IFNγ by T cells causes up-regulation of MHC I molecules also on tumour cells, enhancing their immunogenicity (Miller, Maher et al. 2009; Zaidi and Merlino 2011). While these CD4+ IFNγ+ cells exhibit tumour cytotoxicity, CD8+ CTLs remain the main anti-tumour effector cell type. Upon antigen recognition CTLs synthesize and exocytose granules containing perforin and granzymes within the immunological synapse. Once the content of these granules is released in the cytoplasm of target cells, caspases (a family of death-inducing proteases) are activated, killing the target cells. Alternatively, cell death can be induced by granzyme-mediated proteolysis. CTLs can use another mechanism to induce cytotoxicity in target cells as they express ligands of the TNF superfamily (such as FasL) on their cell surface. Ligation of death receptors induces target cell death (Cullen and Martin 2008).

Once CD8+ T cells are fully activated and acquire CTL functions they can effectively eliminate TAA-presenting cancer cells (Pardoll 2001; Soong, Song et al. 2014).
Interestingly, this process selects cancer cells for less immunogenic cancer cell variants, which survive the attack of the immune system. Due to the heterogeneity and chromosomal instability of transformed cells, nascent tumours contain a wide variety of cancer cells with rapid mutagenic potential. As immunogenic cancer variants are attacked and eliminated, cancer cells resistant or invisible to the immune system escape and expand. These escaping cells exhibit reduced immunogenicity and accumulate mutations that increase their resistance to immune responses. This immunoediting phenomenon is supported by experiments transferring tumour cells from immunocompromised mice to immunocompetent mice. In these experiments it was shown that tumour cells from immunocompromised mice were more immunogenic due to reduced selection pressure, leading to higher tumour rejection rates once transferred to immunocompetent hosts (Svane, Engel et al. 1996; Engel, Svane et al. 1997). Recent studies further assessed the mechanisms of cancer immunoediting, using highly immunogenic MCA-induced sarcoma cell lines from immunodeficient \textit{Rag2}^{-/-} mice. Unedited sarcoma cells from immunodeficient mice resembled nascent primary tumour cells and could be rejected by immunocompetent mice. Using cancer exome sequencing and \textit{in silico} epitope prediction algorithms the authors identified highly immunogenic, tumour-specific mutational antigens (neoantigens) in unedited sarcoma cells. These neoantigens conferred high immunogenicity to unedited sarcoma cells and served as targets for the elimination phase of cancer immunoediting. T cells mediated immunoselection of sarcoma cells lacking strong rejection antigens, leading to tumour outgrowth (Matsushita, Vesely et al. 2012). Importantly, T cell reactivity against patient-specific neoantigens has also been shown in melanoma patients and thus neoantigens can remain targets in edited and established tumours (Linnemann, van Buuren et al. 2014).

Thus, there are two classes of cancer rejection antigens. Neoantigens form the first class and are peptides that are not present in the normal human genome. These novel protein sequences are formed by tumour-specific DNA alterations. Neoantigens may be particularly interesting for cancer immunotherapy as they are not subjected to central T cell tolerance. The second class of cancer rejection antigens is represented by nonmutated proteins, usually referred to as TAAs. T cell tolerance to TAAs exists but is limited, for example due to restricted tissue expression pattern (Schumacher and
Schreiber 2015). TAA-specific CD4\(^+\) and CD8\(^+\) T cells are mainly anergic or exhibit low affinity T cell receptors (TCRs), further aiding tumour escape from the equilibrium phase. These cell-mediated responses are thus not potent enough to eradicate the tumour completely but rather exert a gradual immune selective pressure. A recently proposed theory from our laboratory proposes that there is a requirement for a critical mass of T cells for effective tumour eradication (Bricogne, Laranga et al. 2012; Karwacz, Arce et al. 2012; Escors 2014).

Immune selective pressure thus leads to the survival of poorly immunogenic cancer cell variants. These escaping cancer cells exhibit various genetic and epigenetic mutations, leading amongst others to reduced expression of MHC I or TAA molecules on the cell surface (Spiotto, Yu et al. 2002; Dunn, Old et al. 2004; Zitvogel, Tesniere et al. 2006). Increasing antigen density can overcome immunological ignorance and lead to rejection of tumours, which is mostly dependent on APCs (Spiotto, Yu et al. 2002). For effective cross-presentation and CTL activation to take place, fully functional and mature DCs need to be present. This is not the case in the tumour microenvironment, in which mainly immature or tolerogenic DCs can be found. DCs are not the only deficient immune cell type present within the tumour microenvironment, and the same applies to other tumour-infiltrating myeloid and lymphoid cells. Cancer cells do not only escape by becoming less immunogenic. Tumour cells actively subvert the immune system by secreting tumour-derived growth factors and cytokines, skewing the immune response to a tolerogenic path (Zitvogel, Tesniere et al. 2006). The equilibrium phase between the immune system and escaping cancer cells is viewed as the longest phase of immunooediting (Dunn, Old et al. 2004). At the end, cancer cells with reduced immunogenicity and higher resistance to immune attack eventually lead to further tumour growth. These “super” cancer cells then subvert the immune system and use it for their own purposes, manipulating all levels of the immune system response to favour angiogenesis, tumour progression, and metastasis.
Fig. 1.1. Innate and adaptive immune responses to nascent tumours. (A) Tissue remodelling-induced inflammation attracts innate immune cells, such as NK cells and macrophages. NK cells induce tumour cell death through IFNγ and secretion of granules containing perforin (Perf) and granzymes (Grz). Secretion of cytokines and chemokines by innate immune cells induce further infiltration of other immune cells, such as DCs. (B) Attack of the innate immune system leads to release of TAAs by necrotic cancer cells. DCs phagocytose TAAs and present them on their surface, complexed to MHC molecules. Recognition of danger signals lead to the maturation of DCs, up-regulating co-stimulatory molecules. (C) Matured DCs home to the tumour-draining lymph nodes where they present TAAs to T cells in a positive co-stimulatory context. Cytokine secretion within the immunological synapse leads to T cell polarization and acquisition of effector functions. (D) Effector T cells infiltrate tumours, where they recognize and eliminate cancer cells expressing cognate TAAs.
1.1.4. ALTERED MYELOPOIESIS

Depending on the cancer type, tumour cells secrete various factors, including VEGF (vascular endothelial growth factor) (Gabrilovich, Chen et al. 1996; Young, Kolesiak et al. 1999; Ohm, Gabrilovich et al. 2003), GM-CSF (granulocyte macrophage colony-stimulating factor) (Morales, Kmieciak et al. 2010), G-CSF (granulocyte colony-stimulating factor) (Marigo, Bosio et al. 2010), PGE$_2$ (Prostaglandin E2) (Sharma, Yang et al. 2005), IL6 (Marigo, Bosio et al. 2010), IL10, TGFβ (Fridlender, Sun et al. 2009; Inman 2011), and soluble Fas (Fas cell surface death receptor) and FasL (Fas ligand) (Erdogan, Uzaslan et al. 2005) amongst others. These tumour-derived factors act locally to promote tumour progression or skew immune cell polarization. In addition, many of these factors extend their suppressive effects to lymph nodes and spleen and enhance the recruitment of bone marrow (BM)-derived cells to the tumour. Tumour-secreted factors that reach the BM significantly alter myelopoiesis, resulting in the release of high numbers of undifferentiated and immature myeloid cells to the circulation. These immature myeloid cells get recruited into the tumour, where they suppress rather than stimulate anti-tumour immune responses (Rabinovich, Gabrilovich et al. 2007).

Haematopoiesis is tightly regulated under physiological conditions but this system is unhinged under the assault of a strong influx of tumour-derived growth factors. Haematopoiesis originates from a pluripotent haematopoietic stem cell (HSC). Successive differentiation steps give rise to progeny in two lineage branches, the myeloid and lymphoid lineages (Fig. 1.2.A). Each differentiation step restricts the cells closer to one lineage and is accompanied by a loss of self-renewal capacities. The HSC can differentiate into two different progenitor cells, the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP). CLPs give rise to NK, T, and B cells, while CMP progeny are divided into two further branches. The megakaryocyte/erythrocyte progenitor and the granulocyte/macrophage progenitors give rise to these two branches, leading to the generation of megakaryocytes and erythrocytes, monocytes, macrophages, granulocytes, DCs, and mast cells (Akashi, Traver et al. 2000). Fig. 1.2.A summarizes the text book view of haematopoiesis. This process is much more complicated though. Haematopoiesis should not be considered as a strictly hierarchical system but should rather be viewed as a highly interconnected
system (Fig. 1.2.B)(Gabrilovich, Ostrand-Rosenberg et al. 2012). Other progenitor stages have been identified, such as the common macrophage/DC progenitor (MDP) or the common monocyte progenitor (cMoP) (Hettinger, Richards et al. 2013), adding further plasticity to the system.

During physiological steady-state haematopoiesis myeloid proliferation and mobilization from BM and spleen is regulated by GM-CSF and G-CSF (Demetri and Griffin 1991; Summers, Rankin et al. 2010). G-CSF regulates granulopoiesis. Thus, G-CSF induces proliferation of granulocytic precursors and their release from BM. As G-CSF is also a frequent tumour-secreted factor it may not be surprising that its enhanced secretion leads to the accumulation of immature granulocytes in tumour-bearing hosts (Fig. 1.2.C). In addition, IL6, IL13, and GM-CSF also stimulate granulopoiesis (Summers, Rankin et al. 2010). Tumour-secreted or exogeneous GM-CSF enhances accumulation of immature myeloid cells with immunosuppressive properties in murine tumour models and cancer patients (Young, Young et al. 1990; Pak, Wright et al. 1995; Bronte, Chappell et al. 1999; Serafini, Carbley et al. 2004). GM-CSF has been used in several clinical trials to induce DC responses but high-dose GM-CSF instead mobilizes suppressive myeloid cells, thus explaining the limited efficacy of these treatments (Serafini, Carbley et al. 2004). Transferring tumour cells engineered to secrete high levels of GM-CSF leads to splenomegaly in mice and increases myeloid cell infiltration in tumour tissue (Dufait, Schwarze et al. 2015). Interestingly, a study showed that there is a correlation between the number of splenic immunosuppressive myeloid cells from tumour-bearing mice and enhanced transcription of splenic G-CSF. Tumour-infiltrating immunosuppressive myeloid cell numbers on the other hand directly correlated with enhanced GM-CSF transcription in spleen. In addition, tumour-infiltrating immature myeloid numbers correlated directly with tumour cell number and tumour burden (Abe, Dafferner et al. 2010). Thus, tumours disturb the tightly regulated haematopoietic system and reverse the quiescence of HSCs by secreting high levels of haematopoietic growth factors. As a result HSCs proliferate and thereby increase the level of myelopoiesis. Further, tumour-secreted growth factors and chemokines impair myeloid differentiation and lead to the mobilization of myeloid progenitors before they can fully differentiate. In this way, immature myeloid cells disseminate to blood and peripheral organs and finally infiltrate and accumulate in spleen and tumour tissues (Gabrilovich, Ostrand-Rosenberg et al.
2012; Younos, Dafferter et al. 2012; Talmadge and Gabrilovich 2013). Premature mobilization of immature myeloid cells leads to a decrease of functional DCs and other APCs at the tumour site and lymphoid organs, unable to respond appropriately to danger signals or present antigen effectively (Gabrilovich, Ciernik et al. 1996; Gabrilovich 2004).

As different tumours secrete distinct types or levels of growth factors, cytokines, and chemokines, each tumour is characterized by variations of predominant immature myeloid cell populations, accumulation sites, and tumour infiltration. In summary, tumour-secreted factors affect the macroenvironment by altering myelopoiesis, leading to immature myeloid cell mobilization, accumulation, differentiation, and function (Gabrilovich and Nagaraj 2009; Ostrand-Rosenberg and Sinha 2009; Gabrilovich, Ostrand-Rosenberg et al. 2012; Talmadge and Gabrilovich 2013) (Fig. 1.2.C).
**Fig. 1.2. Haematopoiesis under physiological and high tumour burden conditions.**

(A) Scheme represents haematopoiesis in a simplified, text book view. (B) Scheme represents fluidity and interconnectivity of haematopoiesis. Black frames highlight the three main terminally differentiated myeloid cell types; DCs, granulocytes, and macrophages. Figure B was designed according to information from Gabrilovich et al., Nat Rev Immunol, Vol. 12(4), 253-68, 2012. (C) Tumour-derived factors alter myelopoiesis, leading to the mobilization of large numbers of immature myeloid cells. Red frames highlight tumour-promoting myeloid and lymphoid cells.
1.1.5. IMMUNOSUPPRESSIVE LEUKOCYTES

Tumour immune subversion can act systemically to flood the host with dysfunctional myeloid cells. Tumour-infiltrating immature myeloid cells are further subjected to tumour-secreted factors in the tumour environment, skewing their polarization and function further. Tolerogenic and immunosuppressive myeloid cells inhibit T cell and NK cell-mediated anti-tumour activities and contribute to vascularization, tumour progression, and metastasis (Gabrilovich and Nagaraj 2009; Ostrand-Rosenberg and Sinha 2009; Gabrilovich, Ostrand-Rosenberg et al. 2012; Talmadge and Gabrilovich 2013). Tumours not only alter the myelopoietic compartment but also subvert adaptive immune responses by polarizing T cells to tolerogenic types. Immunosuppression is mediated by various immune cell types, including regulatory T cells (Treg), M2 tumour-associated macrophages (TAMs), tumour-associated neutrophils (TANs), myeloid-derived suppressor cells (MDSCs), and tolerogenic DCs.

Regulatory T cells are CD4⁺ T cells that can be identified at least in mice through the high expression of CD25 and the transcription factor FoxP3. Natural Tregs derive from auto-reactive T cells that escape clonal deletion in the thymus. These cells are crucial for central tolerance and keep a balance between immune responses to foreign pathogens and inhibition of self-directed immune responses. On the other hand, inducible Tregs differentiate from CD4⁺ T cells in the periphery (Sakaguchi, Yamaguchi et al. 2008). When naïve CD4⁺ T cells encounter antigen presented by tolerogenic DCs, they differentiate to Tregs. Negative co-stimulation and TGFβ secretion during antigen presentation are involved in Treg induction by enhancing Foxp3 expression (Akbari, Freeman et al. 2002; Mahnke, Qian et al. 2003; You, Leforban et al. 2007; Chappert, Leboeuf et al. 2008; Wang, Pino-Lagos et al. 2008; Arce, Breckpot et al. 2011). Tregs secrete immunosuppressive cytokines IL10 and TGFβ and inhibit TAA-specific Th1 and CTL responses, rendering anti-tumour cell-mediated responses ineffective (Beyer and Schultze 2006; Kabelitz, Wesch et al. 2006; Zhou, Drake et al. 2006; Gallimore and Godkin 2008; Whiteside, Schuler et al. 2012). Importantly, circulating and tumour-infiltrating Treg numbers are increased in cancer patients (Wolf, Wolf et al. 2003; Wang, Lee et al. 2004) and regulatory T cell infiltration is generally associated with poor prognosis. High tumour-infiltrating regulatory T cells are associated with tumour
invasiveness and the intratumoural balance of Tregs and CTLs can be used as a prognostic marker in several human cancers, including amongst many others hepatocellular carcinoma, gastric cancer, endometrial, and ovarian cancer (Gao, Qiu et al. 2007; de Jong, Leffers et al. 2009; Leffers, Gooden et al. 2009; Shen, Zhou et al. 2010). In these studies, intratumoural high FoxP3+/CD8⁺ T lymphocyte ratios could predict worse overall survival (Shen, Zhou et al. 2010) while high ratios of tumour-infiltrating CD8⁺/FoxP3⁺ T lymphocytes were associated with improved prognosis (de Jong 2009, Leffers 2009).

There are three terminally differentiated myeloid cell types; DCs, granulocytes, and macrophages (Fig. 1.2). These are all important for an effective innate and adaptive immune response (Gabrilovich, Ostrand-Rosenberg et al. 2012). Although the involvement of myeloid cells in tumour progression has been noticed for several decades, it has become increasingly clear over the last decade that myeloid cell functions are altered by tumours for their growth and progression. DCs are generally viewed as the master regulators of adaptive immune responses. Under physiological conditions, DCs are crucial for antigen processing and presentation, initiating antigen-specific immune responses. However, these functions can only be performed by fully functional DCs (Liechtenstein, Dufait et al. 2012). Various tumour-derived factors and hypoxia have been shown to prevent DC differentiation, maturation, and activation (Mancino, Schioppa et al. 2008; Gabrilovich, Ostrand-Rosenberg et al. 2012). VEGF-mediated inhibition of DC maturation is caused by NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) blockade in HSCs (Gabrilovich, Ishida et al. 1998; Oyama, Ran et al. 1998) while tumour-derived IL6 prevents DC maturation through STAT3 (Signal transducer and activator of transcription 3) activation (Park, Nakagawa et al. 2004). IL10, TGFβ, and GM-CSF all modulate DC maturation and additionally polarize DCs to acquire tolerogenic functions (Rutella, Danese et al. 2006). Immature/tolerogenic DCs are resistant to maturation stimuli, express co-inhibitory molecules, and secrete TGFβ and IDO (indoleamine 2,3-dioxygenase), thus suppressing T effector responses by augmenting Treg levels and causing effector T cell apoptosis (Li, Zhang et al. 2008; Wang, Pino-Lagos et al. 2008; Arce, Breckpot et al. 2011).

DCs within the tumour environment initiate Treg responses rather than Th1 and CTL responses, in this way suppressing TAA-specific immune responses. Like DCs,
macrophages are also professional APCs, capable of phagocytosing pathogens and presenting antigen on MHC I and II molecules. Macrophages are tissue-resident cells that have differentiated from monocytes and are normally involved in the elimination of infectious agents, wound healing, and regulation of adaptive immunity (Mosser and Edwards 2008; Gabrilovich, Ostrand-Rosenberg et al. 2012). Macrophages can be further categorized into M1 and M2 phenotypes, depending on the signals they receive from their environment. When macrophages are activated by bacterial products or IFNγ they acquire M1 properties and secrete high levels of IL12. M1 macrophages are also associated with anti-tumour activities. Macrophages that get activated by cytokines IL4, IL10, or IL13, on the other hand, acquire an M2 phenotype. The M2 phenotype is associated with several tumour-promoting properties such as T cell suppression and apoptosis, Treg attraction and activation, tumour growth, and angiogenesis. Macrophages within the tumour are frequently called tumour-associated macrophages (TAMs) and acquire an M2 phenotype due to tumour-secreted factors and the chronically inflamed environment. TAMs secrete high amounts of IL10, TGFβ, and PGE2 and thus attract Treg cells to the tumour. As TAMs have lost IL12 expression and cytotoxic capabilities, they are unable to effectively stimulate adaptive immune responses or engage in direct tumour cytotoxic activities. TAMs further induce T cell suppression and initiation of T cell apoptosis by arginase-1, ROS, peroxynitrite, and PD-L1-dependent mechanisms (Biswas, Gangi et al. 2006; Kusmartsev and Gabrilovich 2006; Mantovani, Sica et al. 2009; Gabrilovich, Ostrand-Rosenberg et al. 2012). Similarly, TAN tumour-infiltration is also observed. Neutrophils are myeloid cells that contain cytoplasmic granules with high histotoxic potential. Due to their cytotoxicity, the processes of granulocyte formation, mobilization, and activation are normally tightly regulated. Under physiological conditions they are rapidly recruited to inflammatory sites, where they kill invading pathogens thorough phagocytosis, release of granular enzymes and proteins, and ROS-mediated mechanisms (Summers, Rankin et al. 2010). Neutrophils can also be subcategorized into N1 and N2 phenotypes. N2 neutrophils are associated with suppressive properties and their polarization seems to depend on TGFβ. N1 neutrophils on the other hand express immunostimulatory cytokines, less arginase, and exhibit anti-tumour activities (Fridlender, Sun et al. 2009). Importantly, myeloid cell
types such as TAMs and TANs differ in frequency, gene expression, and function, depending on tumour type (Elpkek, Cremasco et al. 2014).

1.1.6. MYELOID-DERIVED SUPPRESSOR CELLS

All three terminally differentiated myeloid cell types, DCs, granulocytes, and macrophages, can be polarized to acquire immunosuppressive activities. Tumours extend their activities to the BM and alter myelopoiesis, leading to the mobilization of high numbers of immature myeloid cell types. Already in the early 20th century it was described that tumour progression was associated with an increase in extra-medullary haematopoiesis (EMH) and neutrophilia (Sonnenfeld 1929). In the 1960s it was further described that EMH was associated with increased haematopoietic colony-stimulating factors and abnormal myeloid cell differentiation. These abnormal myeloid cells induced a leukaemoid reaction, which was associated with tumour growth and myeloid cell infiltration (Lappat and Cawein 1964; Robinson 1965; Talmadge and Gabrilovich 2013). Cancer-related NS (natural suppressor) cells were first described in 1989 in tumour-bearing mice. These NS cells suppressed immune responses and were unresponsive to mitogens (Subiza, Viñuela et al. 1989). Later it was appreciated that the accumulation of immature myeloid cells leads to vascularization and immune evasion of tumours (Talmadge and Gabrilovich 2013).

Nowadays, these cancer-induced heterogeneous populations of immature and immunosuppressive myeloid cells are called myeloid-derived suppressor cells (MDSCs) by most immunologists, although some continue to call them immature myeloid cells (iMCs) or myeloid suppressor cells (MSCs) (Talmadge and Gabrilovich 2013). MDSCs are comprised of myeloid progenitors and precursors of macrophages, DCs, and granulocytes at various stages of differentiation (Condamine and Gabrilovich 2011). Their phenotypic characterization remains rather controversial due to cell heterogeneity, investigator-dependent phenotypic marker profiles, and tumour-dependent variability. Tumour-dependent variability stems from the fact that different tumours secrete different growth factors and cytokines (Peranzoni, Zilio et al. 2010; Talmadge and Gabrilovich 2013). Thus, the most important characteristic of MDSCs remains their immunosuppressive activity. Although they are mostly described in
cancer-related studies, they also exist under physiological conditions, where their function is to limit damage to tissues by the immune system (Bronte, Wang et al. 1998) (Strober, Okada et al. 1984; Youn and Gabrilovich 2010). For treatment of autoimmunity-related diseases it is of interest to raise MDSC responses while they obstruct anti-tumour immune responses in patients with high tumour burden (Poschke and Kiessling 2012).

In tumour-bearing mice they are defined as CD11b+ GR-1+ cells with T cell inhibitory activities. GR-1 consists of two epitopes, Ly6C and Ly6G (Bronte, Apolloni et al. 2000; Youn, Nagaraj et al. 2008). In humans, the MDSC phenotype has remained rather controversial and less defined but can be summarized as expressing either CD33 or CD11b and low or no HLA-DR. These markers reflect their myeloid lineage and low antigen presenting capabilities (Almand, Clark et al. 2001; Poschke and Kiessling 2012). Murine and human MDSCs can be further divided into two subsets according to morphology and surface marker expression. MDSCs exhibiting a monocytic-like morphology are called monocytic MDSCs (M-MDSCs). In mice M-MDSCs are CD11b+ Ly6Glow Ly-6C hi while in humans CD33+ HLA-DRflow CD14low (Filipazzi, Valentì et al. 2007; Youn, Nagaraj et al. 2008). MDSCs exhibiting a granulocytic-like morphology are called granulocytic MDSCs (G-MDSCs) or polymorphonuclear MDSCs (PMN-MDSCs). G-MDSCs in mice are CD11b+ Ly6Ghi Ly-6Cinterm/c and in humans CD33+ HLA-DRflow CD15+ (Youn, Nagaraj et al. 2008; Ko, Zea et al. 2009; Poschke and Kiessling 2012). To this date there remains controversy on the recognition of MDSCs as distinct cell lineages. MDSCs express high phenotypical and functional plasticity in mice and humans, further complicating the issue. MDSC differentiation and function depends on the environment they are in. Many factors, including growth factors, cytokines, chemokines, hypoxia, and glucose levels influence their differentiation, accumulation, and function (Gabrilovich, Ostrand-Rosenberg et al. 2012). This reflects the plasticity of the myeloid cell system (Fig. 1.2). It should not be forgotten that immature myeloid cells with the same phenotype exist in the BM of naïve mice, albeit without suppressive functions. It has therefore been suggested that these immature myeloid cells need to be activated following their expansion, to unfold their suppressive activity (Talmadge 2007). The exact combination of signals and mechanisms influencing MDSC accumulation, function,
and activation remain a subject of current studies (Gabrilovich, Ostrand-Rosenberg et al. 2012; Talmadge and Gabrilovich 2013).

Nonetheless, it is now widely accepted that accumulation of MDSCs strongly correlates with cancer progression (Almand, Resser et al. 2000; Diaz-Montero, Salem et al. 2009; Solito, Falisi et al. 2011; Christiansson, Söderlund et al. 2013; Markowitz, Wesolowski et al. 2013; Jiang, Guo et al. 2014) and metastasis (Diaz-Montero, Salem et al. 2009) in patients. Murine tumour models also show a direct relationship between MDSC numbers and tumour burden and progression (Donkor, Lahue et al. 2009; Abe, Dafferner et al. 2010; Steding, Wu et al. 2011; Younos, Dafferner et al. 2012) as well as metastasis (Abe, Dafferner et al. 2010). In addition, there is an inverse correlation between MDSC numbers and T cell numbers in tumour-bearing mice (Donkor, Lahue et al. 2009; Abe, Dafferner et al. 2010). This is supported by studies showing that tumour removal decreases MDSC numbers and increases T cell numbers (Rashid, Nagahashi et al. 2013). Further, depletion of MDSCs can restore NK and T cell function and numbers in mouse models and patients (Mirza, Fishman et al. 2006; Kusmartsev, Su et al. 2008; Pan, Wang et al. 2008; Li, Han et al. 2009; Porembka, Mitchem et al. 2012).

MDSCs negatively impact NK cell function and induce NK cell anergy, an effect mediated by membrane-bound TGFβ (Li, Han et al. 2009). Effector T cells can be inhibited by MDSCs in antigen-specific as well as unspecific ways. Antigen-unspecific inhibition relies on several mechanisms including secretion of immunosuppressive cytokines IL10 and TGFβ, depletion of L-arginine by arginase-1 and/or iNOS activity (Gabrilovich and Nagaraj 2009), and secretion of reactive oxygen and nitrogen species (ROS and RNS) (Nagaraj, Gupta et al. 2007; Corzo, Cotter et al. 2009). Antigen-specific T cell suppression is initiated by secreting immunosuppressive cytokines and signalling through negative co-stimulatory molecules during antigen presentation. Antigen presentation in this context leads to the expansion of T cells with no or only limited effector capabilities. If these T cells encounter the same antigen they become unresponsive (anergic). These mechanisms also lead to the induction of Treg responses, further suppressing effector T cell responses (Nagaraj, Gupta et al. 2007; Gabrilovich and Nagaraj 2009; Escors 2014). Tumour-promoting activities of MDSCs are not restricted to inhibition of anti-tumour immune effector cells. MDSCs also aid in promoting angiogenesis (Yang, DeBusk et al. 2004; Murdoch, Muthana et al. 2008) and
metastasis (Diaz-Montero, Salem et al. 2009; Abe, Dafferner et al. 2010; Condamine, Ramachandran et al. 2015) by secreting cytokines, chemokines, and matrix metalloproteinases (MMPs) (Yang, DeBusk et al. 2004; Gabrilovich and Nagaraj 2009; Gabrilovich, Ostrand-Rosenberg et al. 2012; Liu, Lai et al. 2012). For instance, MMPs aid in the modification of the extracellular matrix, thus aiding in tissue remodelling, invasion, angiogenesis, and metastasis (Yang, DeBusk et al. 2004; Gutschalk, Yanamandra et al. 2012; Liu, Lai et al. 2012). The tumour microenvironment is a highly complex site and tumour-infiltrating myeloid populations engage in cross-talk with other cell types. Thus, cross-talk between MDSCs and TAMs exacerbates myeloid-mediated immune suppression. IL10 secreted by MDSCs decreases IL12 and increases IL10 secretion by TAMs, thus potentiating suppressive mechanisms (Sinha, Clements et al. 2007). Close interactions also exists between MDSCs and tumour cells. MDSCs help tumour cells escape the immune system and aid in progression, angiogenesis, and metastasis. Tumour cells on the other hand are largely causing MDSC development and accumulation.
1.2. CANCER IMMUNOTHERAPY

As our knowledge of cancer has developed, including the identification of cancer-causing mutations, implementation of cancer-screening techniques, and the study of cancer immunology over the past decades, the possibility for more effective and targeted therapies has arisen. Traditional cancer therapies such as chemotherapy are characterized by high collateral damage and limited efficacy in the long-term. A promising approach relies on boosting the immune system’s natural defences against tumours, termed cancer immunotherapy. The aim of cancer immunotherapy is to stimulate the immune system to recognize and eradicate tumour cells without harming healthy tissue. Especially desired is the activation of adaptive immune responses, as they act in a targeted way and can induce immunological memory. As CTLs are the main anti-tumour effector cells, boosting TAA-specific CTL responses is the objective of most cancer immunotherapy treatments. Nevertheless, there are still several major problems associated with the induction of anti-tumour immune responses. The first one is the natural tolerance of T lymphocytes against TAAs. A cancer immunotherapy treatment must therefore overcome this natural tolerance. Once a treatment has resulted in generating anti-tumour CTL responses it needs to ensure that these effector cells are not inactivated by the immunosuppressive tumour microenvironment. This represents the second major barrier to efficient induction of anti-tumour immune responses.

Cancer immunotherapy has classically focused on two immune cell types for manipulation, as described in previous sections. The first are DCs and the second are the CTLs themselves. Researchers are still discovering more TAAs. Before experimental evidence on the existence of TAAs started to appear in the 1960s, it was widely believed that most cancer cells are invisible to the immune system (Haughton and Amos 1968). Due to the genomic instability of transformed cells they acquire genetic and epigenetic mutations that are reflected in the range of altered antigens they present on their surface. These TAAs make cancer cells susceptible to the attack of the immune system and can be exploited by cancer immunologists (Zitvogel, Tesniere et al. 2006). Nowadays there is a large collection of known TAAs and it remains a very active area of research (Brichard, Van Pel et al. 1993; Bakker, Schreurs et al. 1994) (Castelli, Tarsini et al. 1999; Cheever, Allison et al. 2009).
Summarising, there are six major therapeutic strategies for cancer immunotherapy: Cytokine treatment, tumour vaccines, adoptive transfer of TAA-specific CTLs, adoptive transfer of TAA-presenting DCs, immune checkpoint modulators, and depletion of MDSCs and Tregs. Combination of several of these immunotherapeutic strategies often has synergistic effects, boosting the immune response at several levels.

1.2.1. CYTOKINE TREATMENTS

Cytokines are small signalling proteins, and major modulators in immune processes and can be divided into interleukins (ILs), chemokines, interferons (IFNs), tumour necrosis factors (TNFs), mesenchymal growth factors, and adipokines. Cytokines are characterized by their pleiotropicity, as they possess multiple and various biological functions. Their specific activities over target cells are determined by the differential expression of receptors on target cell types (Dinarello 2007). Cytokines can be administered to either activate the immune system or to induce direct anti-tumour effects. IL6, TNFα, and IFNγ exhibit direct anti-tumour cytotoxicity in vitro but they show strong toxicity in vivo and even promote tumour growth or exert immunosuppressive effects through the activation of negative feedback mechanisms to dampen inflammation (Disis 2014). IL12 also has direct anti-cancer cytotoxic effects but its systemic administration is highly toxic (Cohen 1995; Leonard, Sherman et al. 1997; Car, Eng et al. 1999; Colombo and Trinchieri 2002). Thus, although several cytokines exhibit anti-tumour activity, systemic administration frequently leads to highly undesired side effects.

Cytokines that activate and expand NK and T cells such as IL2 and IFNα, have been used in patients more or less successfully (Atkins, Lotze et al. 1999; Flaherty, Othus et al. 2014). IL2 is approved for treatment in cancer patients but its pro-inflammatory effects can be problematic as these are not easily tolerated by many patients. In addition, low efficacy has been observed in metastatic melanoma and renal cell carcinoma (Fehniger, Cooper et al. 2002; Dinarello 2007; Disis 2014). IFNα is approved for malignant melanoma, leukaemia, and lymphoma but its clinical efficacy is limited by some immunosuppressive activities within cancer patients (Tarhini, Gogas et al. 2012; Disis 2014).
GM-CSF has been used and approved in humans for haematopoietic reconstitution, recruitment of mature DCs, and DC maturation and activation. GM-CSF administration in clinical trials has led to promising results, for example in melanoma patients (Daud, Mirza et al. 2008). On the other hand, GM-CSF has recently been shown to favour tumour invasion in mice, by elevating MMP (matrix metalloproteinase) expression levels in the tumour environment (Gutschalk, Yanamandra et al. 2012). As discussed in the previous section, high levels of GM-CSF do not recruit mature DCs but rather alter myelopoiesis, resulting in accumulation of dysfunctional DCs and other immature myeloid cells (Serafini, Carbley et al. 2004). IL7, IL11, IL12, IL15, IL21, IFNβ, and IFNγ are being evaluated for cancer immunotherapy (Disis 2014) but their pleiotropicity makes it difficult to use cytokines in cancer patients, especially when administered systemically. Local delivery and combination with other immunotherapeutic treatments may be able to circumvent their undesired side effects, at least for certain cytokines.

1.2.2. TUMOUR VACCINES

Natural TAA-specific CD4 and CD8 T cell responses take place in patients. The objective of tumour vaccines is to enhance TAA presentation to immune cells, thus inducing their activation and differentiation to anti-TAA effector T cells. There are at least three different types of tumour vaccines; protein/peptide vaccines, cell-based vaccines, and genetic vaccines.

Protein/peptide vaccines consist of either entire TAAs or various peptides derived from them. As TAAs are poorly immunogenic, protein/peptide vaccines are usually co-administered with immunostimulatory adjuvants. Most peptide-based vaccines in clinical trials target cancer-testis antigens, differentiation-associated antigens, or oncofoetal antigens, mostly in combination with adjuvants and immune modulators. Peptide vaccines induce TAA-specific T cells but clinical efficacy remains low (Guo, Manjili et al. 2013). Nevertheless, recent successes have validated this approach. A recent Phase III trial for advanced melanoma combined the administration of gp100 peptide with IL2 and Monanide ISA-51 adjuvant, which increased median overall survival (Schwartzentruber, Lawson et al. 2011). Peptide vaccines have one clear disadvantage,
which is that only a limited number of epitopes from TAAs are presented to T cells, and this may not sufficiently stimulate different cohorts of CD4 and CD8 T cells that may cooperate in cytotoxic activities. Stimulation of CD4 T cell responses is important to effectively raise CD8 T cell responses (Guo, Manjili et al. 2013).

Tumour cell-based vaccines are more costly than peptide-based vaccines but an advantage is that APCs process the TAAs themselves into peptides, ensuring that CD4 and CD8 T cell responses are stimulated. In addition, the whole range of TAAs is supplied to the patient’s immune system rather than a selected few. Tumour cell-based vaccines are either autologous (from patient) or allogeneic (from allogeneic tumour or human tumour cell lines). Autologous tumour cell vaccines are produced by irradiating tumour cells from the patient. Immunostimulatory agents are added to irradiated tumour cells and the tumour vaccine is then administered to the patient (Guo, Manjili et al. 2013). Autologous tumour cell vaccines genetically modified to secrete GM-CSF (Gvax) have shown certain efficacy in the clinic (O’Rourke, Schmidt et al. 1997; Nemunaitis 2003; Hodi and Dranoff 2006). This procedure is rather costly and can only be applied to certain tumours, as it is dependent on the supply of sufficient tumour sample for vaccine generation (Guo, Manjili et al. 2013). Allogeneic tumour cell vaccines are less costly as they are not patient-specific. While several allogeneic cancer vaccines showed promising results in Phase II clinical studies, they were discontinued due to disappointing results in Phase III studies. Combination therapies using allogeneic cancer vaccines with immune checkpoint modulators are now being pursued (Garcia and Dreicer 2011; Guo, Manjili et al. 2013).

For peptide- and cell-based tumour vaccines the selection of the right adjuvant is highly important as using the wrong adjuvant can obstruct vaccine efficacy. IFA (incomplete Freund’s adjuvant) analogues have frequently been used as adjuvants for vaccination. TAA delivery in IFA has recently been shown to cause sequestration and deletion of TAA-specific CTLs at the vaccination site, rendering the tumour vaccine ineffective. Thus, delivery of TAAs alone did not cause antigen deposits but did not raise strong CTL responses (Hailemichael, Dai et al. 2013). Hence, genetic vaccines and especially viral-based vaccination may represent a more effective option. For instance, lentiviral vector transduction does not cause antigen deposits at the injection site but leads to long-term transgene expression in cells at the injection site, leading to induction
of anti-tumour CTL responses (Dullaers, Van Meirvenne et al. 2006; Breckpot and Escors 2009; Liechtenstein, Perez-Janices et al. 2013).

Genetic tumour vaccines are based on TAA DNA transfer to patients, either encoding TAAs as full proteins or as peptides. APCs are the main target of TAA DNA, so that they can present TAAs and initiate anti-TAA immune responses. TAA DNA can be transferred through several methods; through bacterial plasmids, as RNA, or supplied by viral vector transduction. DNA vaccines have shown promising results in pre-clinical models, leading to TAA-specific CTL responses and tumour protection (Bronte, Apolloni et al. 2000; Yan, Tingey et al. 2014). The use of multiple epitopes or mutated TAA DNA can further enhance anti-tumour immune responses (Guevara-Patino, Engelhorn et al. 2006). Although preclinical results have been promising, translation to the clinic has been disappointing (Guo, Manjili et al. 2013). There is room for improvement and current clinical studies are testing alternative strategies to enhance DNA tumour vaccine efficacy. A recent study suggested that intra-lymph nodal delivery could enhance TAA uptake by APCs to enhance anti-tumour T cell responses (Ribas, Weber et al. 2011).

Thus, due to the lack of TAA immunogenicity, tumour vaccines need to be combined with adjuvants or other immunostimulatory agents and immune checkpoint modulators (Tarhini, Leng et al. 2012). Viral-based tumour vaccines have the advantage that virus-specific peptide presentation could act as an immunostimulatory adjuvant during TAA presentation (Breckpot, Escors et al. 2010). Apart from encoding TAA proteins or peptides, tumour vaccine viral vectors can also incorporate immunomodulating molecules. Again, delivery of mutated TAAs can further potentiate CD8 T cell responses against multiple wild-type TAA epitopes in mouse tumour models (Liu, Peng et al. 2009). While preclinical studies showed efficacy of genetic TAA vaccines in prophylactic and therapeutic settings, success in the clinic has been poor. Although immune responses against TAAs supplied by the cancer vaccines were observed in most trials, it seldom leads to a reduction in tumour burden. This may be due to low immunogenicity of TAAs, a lack of adequate co-stimulation, and tumour-induced immune suppression. In addition, immune selective pressure induces cancer cell variants with low MHC expression and/or TAA expression, rendering TAA-targeting immune responses ineffective. Thus, effective TAA vaccines may lead to eradication of TAA-expressing cancer cells but non-immunogenic cancer cell variants are still free to expand
and thus lead to tumour progression and metastasis after a slightly extended period of remission (Bodey, Bodey et al. 2000; Guo, Manjili et al. 2013).

1.2.3. ADOPTIVE T CELL THERAPY

Rather than delivering TAAs to the patient’s immune system to initiate TAA-specific CTL responses, a different approach is to generate activated TAA-specific CTLs \textit{ex vivo}, before transfer to the patient. The objective is to generate activated CTLs with engineered receptors exhibiting a high affinity towards TAAs. Two strategies are currently applied to engineer TAA-specific CTLs \textit{ex vivo}; engineering T cells that express high-affinity T cell receptors (TCRs) or that express chimeric antigen receptors (CARs) (Fig. 1.3). Both transgene receptors are introduced into patient-specific effector T cells \textit{ex vivo} using viral vectors as gene carriers. Engineered T cells are then expanded and activated before transfer back into the patient (Coccoris, Straetemans et al. 2010; Thomas, Stauss et al. 2010). Although resting T cells are more refractory to viral vector modification than DCs, this strategy remains viable (Bobisse, Zanovello et al. 2007; Frecha, Costa et al. 2008; Perro, Tsang et al. 2010; Liechtenstein, Perez-Janices et al. 2013).

To genetically engineer T cells with high-affinity TCRs, highly reactive T cells are previously isolated from patients. These T cells can recognize and eliminate tumour cells and are used to clone their high affinity TCRs. Their TCR α and β chains are introduced into vectors for further use in T cells isolated from other patients (Johnson, Heemskerk et al. 2006). Thus, TCR transgenes are introduced into autologous T cells \textit{ex vivo}, activated, expanded, and transferred back to patient (Coccoris, Straetemans et al. 2010; Perro, Tsang et al. 2010; Thomas, Stauss et al. 2010). Genetically engineered T cells recognize TAAs in the recipient, expand upon encounter, and eliminate tumour cells. There have been promising results in pre-clinical models (Bobisse, Rondina et al. 2009) as well as success in human clinical trials (Johnson, Heemskerk et al. 2006; Morgan, Dudley et al. 2006; Parkhurst, Yang et al. 2011; Robbins, Morgan et al. 2011; Phan and Rosenberg 2013). In these trials TCRs were isolated from T cells isolated from patients undergoing remission. Thus, TCRs specific for cancer testis antigen NY-ESO and melanoma-associated antigen MELAN-A (MART-1) and gp100 (Robbins, Morgan et al.
2011; Phan and Rosenberg 2013) have been cloned. Poor persistence of adoptively transferred T cells in vivo has hampered clinical efficacy and this could be due to premature differentiation ex vivo (Perro, Tsang et al. 2010). In addition, especially in early clinical trials, on-target/off-tumour toxicity has been a problem (Lamers, Seijfer et al. 2006; Morgan, Dudley et al. 2006). Of note, there are several other problems associated with this strategy. Endogenous TAA-specific T cells frequently do not exhibit TCRs with high enough affinity. This is due to the fact that high-affinity TAA-specific T cells are eliminated by clonal deletion in the thymus or become tolerogenic upon antigen encounter in the periphery. To improve TCR affinity, further protein engineering steps are often necessary. TCRs are restricted to one HLA-type, thus only patients with the same HLA-type can be treated. Importantly, TCR-engineered T cells are still subject to immunosuppressive mechanisms in patients and as only a single TAA is targeted, non-immunogenic cancer cell variants can still be selected for tumour progression (Escors, Lopes et al. 2008; Park, Rosenberg et al. 2011; Liechtenstein, Perez-Janices et al. 2013).

To overcome some of the above-presented problems, CARs have been developed. CARs consist of intracellular T cell signalling domains (e.g. CD3 chains) fused to TAA-binding domains of antibodies. This strategy confers a higher affinity of the engineered T cells to TAAs and renders TAA recognition independent of MHC restriction (Gross, Waks et al. 1989). On the other hand, problems related to tumour immunosuppression and selection of less immunogenic cancer variants still take place. Poor persistence of transferred CAR-T cells has been observed due to immune responses against CAR-engineered T cells (Jensen, Popplewell et al. 2010; Lamers, Willemsen et al. 2011). CAR-T cell in vivo limited expansion leads to reduced efficacy in clinical trials. Interestingly, these two clinical trials had not used lentivectors for T cell gene transfer. It has been suggested that lentivector-mediated TCR transfer to T cells may be more efficient than retrovirus vector-mediated transfer. For example, this was the case for a MelanA-specific TCR clone (Bobisse, Rondina et al. 2009). In addition, there is a study suggesting that anti-transgene responses are initiated by immunogenic retroviral vector-encoded peptides (Lamers, Willemsen et al. 2011).

Nevertheless, impressive clinical results have been achieved applying CAR-T cells in human patients, especially using CD19-specific CARs in leukaemia/lymphoma (Kochenderfer, Wilson et al. 2010; Kochenderfer, Yu et al. 2010; Kalos, Levine et al.
The group of Dr. June has engineered CARs against CD19, containing the CD137 co-stimulatory domain and TCR ζ chain. T cells were engineered through lentivector transduction and their transfer led to complete remissions in a high percentage of patients but exhibiting tumour lysis syndrome and lymphopenia (Kalos, Levine et al. 2011; Porter, Levine et al. 2011). As a consequence of the success of these clinical trials there is now a good number of clinical trials using CD19-targeting CAR-T cells (https://clinicaltrials.gov). Infusion of CAR-engineered T cells is proving to be a very powerful method and care needs to be taken when selecting TAA-specificity. Infusion of CAR-T cells in a patient with metastatic colon cancer has resulted in immediate adverse symptoms and death of a patient only 5 days later. In this case, CAR-T cells were engineered to be specific for ERBB2, but showed potent off-target effects. It was suggested that CAR T cells recognized low ERBB2 expression in the lung, leading to a cytokine storm (Morgan, Yang et al. 2010).

Thus, adoptively transferring genetically engineered T cells showed clinical responses in a variety of cancer types, including melanoma, lymphoma, leukaemia, neuroblastoma, and colorectal cancer (Morgan, Dudley et al. 2006; Kalos, Levine et al. 2011) (Pule, Savoldo et al. 2008; Kochenderfer, Wilson et al. 2010; Kochenderfer, Yu et al. 2010; Park, Rosenberg et al. 2011; Parkhurst, Yang et al. 2011; Porter, Levine et al. 2011). Results such as these have revived the field of cancer immunotherapy.
**Fig. 1.3. Engineering of TAA-specific T cells for adoptive T cell therapy.** The figure depicts the introduction of TAA-specific TCRs or CARs into T cells isolated from patients. TCR α and β chains are cloned from TAA-specific T cells of patients undergoing remission. The TCR chains are amplified by PCR and inserted into viral vectors, in this case lentivectors. CARs on the other hand are generated by fusing the TAA-binding region of an antibody with TCR signalling domains. The lentiviral vectors are then used to transduce autologous T cells. \( V_H \) and \( V_L \) immunoglobulin variable regions; LTR, long terminal repeat; \( \Psi \), packaging signal; RRE, rev response element; cPPT, central polypurine flap; WPRE, woodchuck post-transcriptional element. This figure was reproduced from Liechtenstein et al., Cancers, Vol. 5(3), 815-837, 2014.
1.2.4. ADOPTIVE DC THERAPY

The recent milestone achievements of CAR technology have demonstrated that CTLs are an important target for cancer immunotherapy. But equally important targets are APCs, especially DCs, as they are crucial for the effective in vivo activation of CTLs. As DCs regulate both immunity and tolerance, they can be targeted for immunostimulatory activities but also for inducing tolerance in the case of auto-immune disorders. Upon antigen uptake and processing for presentation, DCs undergo maturation and up-regulate co-stimulatory molecules, secrete pro-inflammatory cytokines, and migrate to lymphoid organs to stimulate naïve and antigen-experienced T cells. There are several important strategies for the modification of DCs as efficient stimulators of CTL responses. The first one is to ensure TAA presentation; the second, ensuring full maturation of DCs; and the third one is to induce production of immunostimulatory molecules and cytokines by DCs (Breckpot and Escors 2009; Escors, Breckpot et al. 2012; Liechtenstein, Dufait et al. 2012; Liechtenstein, Perez-Janices et al. 2013). Successful strategies should combine all three approaches to achieve TAA presentation in the correct context.

DCs can be loaded with TAAs ex vivo by incubation with tumour cell lysates or known TAA proteins or peptides. In this way, DCs phagocytose the antigens, process them to antigen peptides, and load them onto MHC molecules for presentation on their surface (Breckpot and Escors 2009; Escors 2014) (Fig. 1.4). Alternatively, DCs can be modified to express transgenes encoding TAAs, by viral vector-mediated or other genetic modification methods. Transgenes are processed by transduced DCs and presented on MHC molecules. Of note, including the entire transgene ensures that correct peptides are loaded on MHC molecules and circumvents the need to design peptides specific for MHC genotypes. As optimal anti-tumour results are achieved through activation of both CD4 and CD8 T cells, it is beneficial that peptides are presented on MHC II and MHC I molecules (Collins and Cerundolo 2004; Figdor, de Vries et al. 2004; Liechtenstein, Perez-Janices et al. 2013; Liechtenstein, Perez-Janices et al. 2014).

To establish a potent CD8 response, DCs need to be effectively matured and activated to present antigen in the right co-stimulatory and cytokine context. Transgenic
delivery of TAAs alone is not sufficient. DCs have to mature to be fully functional, which is exhibited by high surface MHC and co-stimulatory molecule density. To ensure DC maturation intracellular DC activatory signalling pathways could be manipulated. The use of NF-κB and p38 activators, for example, can lead to DC maturation (Escors, Lopes et al. 2008; Karwacz, Mukherjee et al. 2009; Rowe, Lopes et al. 2009; Liechtenstein, Dufait et al. 2012; Liechtenstein, Perez-Janices et al. 2013). Expression of cytokines by DCs is also an effective approach, ensuring that T cells are polarized to the right phenotype during antigen presentation (Koya, Kimura et al. 2007; Huang, Ramakrishnan et al. 2012).

Immunization of mice using ex vivo transduced DCs can induce T cell responses specific for TAAs (Esslinger, Romero et al. 2002; Breckpot, Dullaers et al. 2003; Wang, He et al. 2006; Karwacz, Arce et al. 2012) and it is capable of preventing/delaying tumour growth in vivo (He, Zhang et al. 2005; Wang, He et al. 2006; Karwacz, Arce et al. 2012). Adoptive autologous transfer of modified DCs is being applied in human immunotherapy. This approach can lead to induction of tumour-specific immune responses in cancer patients that correlate with clinical response. Peripheral blood monocytes are isolated from the patient, differentiated into DCs, and genetically modified or loaded with TAA antigens ex vivo. Modified autologous DCs are then infused into the patient. In early clinical trials DC vaccines used immature rather than mature DCs, leading to immunosuppression rather than immunostimulation (Dhodapkar, Steinman et al. 2001; Dhodapkar and Steinman 2002; de Vries, Lesterhuis et al. 2003; Figdor, de Vries et al. 2004). In more recent autologous DC clinical trials, partial or complete remissions are achieved (Lesterhuis, De Vries et al. 2010; Aarnntzen, De Vries et al. 2013; Suehiro, Hasegawa et al. 2015). As this strategy is patient-specific, it is rather expensive and therefore represents a barrier for wide-spread clinical application. Alternatively, direct immunization with viral vectors (particularly lentiviral vectors) targeting DCs is an attractive alternative. Viral glycoprotein engineering to pseudotype lentivector particle can achieve selective transduction of DCs in vivo in pre-clinical models and prevent tumour growth (Yang, Yang et al. 2008). A recent clinical trial used a vaccine containing a human monoclonal antibody specific for the DC surface molecule DEC-205 fused to full-length tumour antigen NY-ESO-1. DEC-205 has a role in antigen processing and presentation, thus ensuring that NY-ESO-1 is processed and presented
on DCs *in vivo*. This strategy exhibited clinical responses in patients with advanced malignancies (Dhodapkar, Sznol et al. 2014).

Thus, autologous DC and CTL therapies have shown clinical successes but it may be advantageous to combine the two strategies as a recent clinical trial has shown. This study combined autologous tumour-lysate loaded DCs with adoptive TIL (tumour-infiltrating lymphocyte) transfer, leading to clinical responses in advanced melanoma patients (Poschke, Lövgren et al. 2014). This highlights again the efficacy of combination therapies and that will likely be employed routinely, leading to synergistic anti-tumour effects.

*Fig. 1.4. Ex vivo modification of DCs for use in immunotherapy treatments.* This figure represents the modification of ex vivo differentiated mouse and human DCs. DCs are loaded with peptides or by genetic modification. Peptide-presenting DCs are further modified to determine their activation and functional status. Activated, mature DCs (above) can be used for cancer immunotherapy and treatment of infectious diseases. While tolerogenic DCs (below) can be used to treat autoimmune disease, allergy, and in transplantation. This figure was reproduced from Escors et al., *New J Sci*, 2014.
1.2.5. IMMUNE CHECKPOINT MODULATORS

As the immune system is highly regulated to prevent auto-reactivity, there are several pathways inhibiting immune responses. These inhibitory molecule signals are generally referred to as immune checkpoints. These signals are either used by tissues to escape collateral damage from the immune system or by APCs during antigen presentation, to regulate T cell activation or restrict effector functions. Many of the immune checkpoints are mediated by ligand-receptor interactions and can therefore be blocked by antibodies (Pardoll 2012) (Fig. 1.5). Immune checkpoint modulators have become very popular recently due to clinical successes using antibodies against CTLA-4 (Cytotoxic T Lymphocyte-associated protein 4) and PD-L1/PD-1 (Programmed Death Ligand 1/Programmed Death 1).

CTLA-4 translocates to the cell membrane when T cells are activated. Expressed CTLA-4 binds molecules of the B7 family of accessory molecules on the surface of APCs. CTLA-4 binding to CD80 or CD86 diminishes T cell activation by counter-acting the positive co-stimulatory interaction delivered by CD28 binding. CTLA-4 ligation thus leads to inhibition of T cell activation and proliferation (Chambers, Kuhns et al. 2001; Peggs, Quezada et al. 2009). In addition, it also affects the Treg compartment and Treg inhibitory activities (Chambers, Kuhns et al. 2001; Henson, Macaulay et al. 2008; Wing, Onishi et al. 2008; Peggs, Quezada et al. 2009; Krummel and Allison 2011). Thus, CTLA-4 is a crucial regulatory receptor that controls immune responses and prevents autoreactivity. Ipilimumab is a monoclonal antibody against CTLA-4 approved by the FDA in 2011 for the treatment of metastatic melanoma (Mellman, Coukos et al. 2011). Systemic treatment with ipilimumab enhances anti-tumour responses in patients and exhibits prolonged survival in patients with melanoma (Hodi, Oble et al. 2008; Hodi, O'Day et al. 2010; Peggs and Quezada 2010) and non-small cell lung carcinoma (Tomasini, Khobta et al. 2012). Not surprisingly, systemic CTLA-4 blockade is accompanied with severe side effects due to induced inflammation that could be autoimmunity-related (Hodi, O'Day et al. 2010; Weber, Dummer et al. 2013).

Signalling initiated by PD-L1/PD-1 also regulates T cell activation and tolerance but T cell inhibition is regulated through a different intracellular mechanism than CTLA-4 signalling (Parry, Chemnitz et al. 2005). In addition, PD-1 is more widely expressed than...
CTLA-4, which can only be found on T cells. Thus, CTLA-4 acts mainly on T cell regulation in lymphoid organs (Nishimura and Honjo 2001; Keir, Butte et al. 2008; Francisco, Salinas et al. 2009; Francisco, Sage et al. 2010; Liechtenstein, Dufait et al. 2012). Importantly, a wide range of human cancer cells up-regulate PD-L1 expression in patients, in this way inhibiting anti-cancer immune responses. PD-L1 is therefore an important target for cancer immunotherapy (Blank, Gajewski et al. 2005; Zhang, Gajewski et al. 2009; Samimi, Benoit et al. 2010; Zhou, Munger et al. 2010; Andorsky, Yamada et al. 2011). Recent clinical trials showed clinical efficacy administering systemic anti PD-L1 or PD-1 antibodies. Clinical responses were observed in various advanced cancer types, including melanoma, breast, lung, pancreatic, colon, renal, and gastric cancer (Brahmer, Drake et al. 2010; Brahmer, Tykodi et al. 2012; Topalian, Hodi et al. 2012).

CTLA-4 and PD-1 regulate distinct inhibitory pathways. It has therefore been suggested that combination therapies blocking both molecules may exhibit enhanced efficacy. Recent clinical trials administering ipilimumab (anti-CTLA-4) in combination with nivolumab (anti-PD-1) were conducted in patients with advanced melanoma (Wolchok, Kluger et al. 2013; Postow, Chesney et al. 2015). Co-administration of both antibodies led to greater objective response rate (61% versus 11%) and progression-free survival as compared to treatment with ipilimumab alone. In addition, 22% of patients treated with nivolumab and ipilimumab were reported to have complete responses while none of ipilimumab-treated patients exhibited complete responses (Postow, Chesney et al. 2015). Further combining CTLA-4 and PD-L1/PD-1 targeting strategies with tumour vaccines may lead to enhanced efficacy, a strategy that has already yielded promising pre-clinical results (Curran, Montalvo et al. 2010; Duraiswamy, Kaluza et al. 2013). However, systemic inhibition of CTLA-4 and PD-1 signalling may enhance side effects due to induction of auto-immunity. A safer strategy would be to target immune checkpoints locally rather than systemically, as will be discussed in later chapters.
Fig. 1.5. Immune checkpoints mediated by ligand-receptor interactions between T cells and APCs during antigen presentation. Scheme depicts antigen presentation by APCs (left) to T cells (right). Signal 1 of T cell activation is mediated by the binding of the peptide-MHC complex to the TCR. The depicted co-stimulatory and co-inhibitory ligands on APCs bind to their receptors on T cells, determining the activation status of the T cell in response to the antigen. These interactions represent possible targets for cancer immunotherapy and several strategies for their inhibition are being developed. Figure adapted and modified from Pardoll, Nat Rev Cancer, Vol. 12(4), 252-64, 2012.
1.2.6. DEPLETION OF IMMUNOSUPPRESSIVE LEUKOCYTES

DCs and T cells are the main targets when the desired outcome is to activate the immune system in cancer immunotherapy. But many cancer immunotherapy treatments are ineffective as TAA-specific T cells are frequently inactivated in cancer patients. Immunosuppressive cell types therefore need to be considered important targets and extensive research is dedicated to finding strategies to selectively deplete them. Particularly successful could be the combination of T and DC activating strategies with suppressive cell-depleting regimens. Two important immunosuppressive cell types have been the target of such depletion strategies; Tregs and MDSCs. Thus, depletion of Tregs and MDSCs has been shown to enhance cancer immunotherapy in mouse models and patients. Sunitinib (multitargeted tyrosine kinase inhibitor), for example, has been shown to reduce Treg and MDSC numbers in treated human patients, thus enhancing its anti-tumour effects (Xin, Zhang et al. 2009). It is becoming increasingly clear that certain chemotherapeutic treatments, including gemcitabine, 5-fluorouracil, docetaxel, and doxorubicin, work well in clinical practice because they inhibit cancer cell growth and decrease MDSC numbers (Suzuki, Kapoor et al. 2005; Kodumudi, Woan et al. 2010; Vincent, Mignot et al. 2010; Alizadeh and Larmonier 2014; Alizadeh, Trad et al. 2014). Therefore, there is a high interest in developing strategies to specifically target these immunosuppressive cell types.

Treg depletion has been approached through three main strategies; Treg cell death induction through chemotherapeutic agents, Treg depletion by antibodies targeting receptors (CD25, CTLA-4, PD-1, GITR), or targeting Treg trafficking (CCR4 antagonist) (Wainwright, Dey et al. 2013). In mice, depletion of Tregs with CD25-specific antibodies leads to tumour regression, but only during the early phase of tumour growth (Onizuka, Tawara et al. 1999). Similarly, combining anti-CTLA-4 antibody with CD25-depleting antibody leads to tumour rejection in a B16 mouse melanoma model (Sutmuller, van Duivenvoorde et al. 2001). CTLA-4 and PD-1 blocking antibodies can be combined, leading to inhibition of Treg function and tumour rejection. This effect could be augmented when combined with a tumour vaccine, demonstrating again the advantages of combination therapies (Duraiswamy, Kaluza et al. 2013). In the clinical practice successes with anti-CTLA-4 and anti-PD-1 antibodies have been shown, as
discussed before (Brahmer, Drake et al. 2010; Hodi, O'Day et al. 2010; Peggs and Quezada 2010; Brahmer, Tykodi et al. 2012; Tomasini, Khobta et al. 2012; Topalian, Hodi et al. 2012). Targeting GITR (glucocorticoid-induced TNFR-related protein) and CD25 may be more problematic in human patients due to the existence of additional targets (such as activated T and B cells) and their high toxicity (Wainwright, Dey et al. 2013).

Treg cells are particularly important during early phases of tumour establishment. This is supported by the observation that Treg depletion is only effective in animals during early phases of tumour progression or before tumour establishment (Onizuka, Tawara et al. 1999; Elpek, Lacelle et al. 2007). As MDSCs can hamper T cell activities at all levels using antigen-specific and unspecific immunosuppressive mechanisms, they also represent an attractive target to enhance anti-tumour immune responses. Several studies in mouse models have shown the efficacy of MDSC-depleting strategies. Murine MDSCs have been depleted in vivo by targeting GR-1 with monoclonal antibodies. This strategy restored T cell-mediated anti-tumour immune responses and prevented tumour recurrence. Of note, anti-GR-1 antibodies also deplete neutrophils, rendering the subject more susceptible to opportunistic infections (Terabe, Matsui et al. 2003; Mundy-Bosse, Lesinski et al. 2011). Further pre-clinical studies have shown that targeting SCF (stem cell factor) (Pan, Wang et al. 2008), IL4-Rα (Roth, De La Fuente et al. 2012), S100 proteins (Qin, Lerman et al. 2014), and treatment with zoledronic acid (Porembka, Mitchem et al. 2012) decreases tumour-infiltrating MDSC numbers. This restores tumour-infiltrating T cell proliferation, decreases Treg numbers, and inhibits angiogenesis. In this way, these treatments limit tumour progression. MDSC suppressive activities can be inhibited by administration of phosphodiesterase inhibitors. This reduced the immunosuppressive mechanisms of MDSCs in tumour mouse models by reducing arginase-1 and iNOS activity (Serafini, Meckel et al. 2006).

All-trans-retinoic acid (ATRA) has been successfully used to deplete MDSCs in tumour mouse models and patients. ATRA reduces MDSC numbers in tumour-bearing mice and thus enhanced and prolonged the efficacy of tumour vaccines (Kusmartsev, Cheng et al. 2003; Weiss, Vitacolonna et al. 2009; Lee, Seo et al. 2012). ATRA treatment of renal cell carcinoma patients decreased MDSC numbers in peripheral blood and improved the ratio of myeloid to lymphoid DCs, DC function, and antigen-specific T cell responses (Mirza, Fishman et al. 2006). Co-treatment of ATRA with a cancer vaccine (DCs
transduced with p53) significantly improved immune response to vaccination (Iclozan, Antonia et al. 2013). Mechanistically it was shown that ATRA treatment of CD33+ MDSCs from patients with renal cell carcinoma induced their differentiation into APC precursors. Abrogation of MDSC immunosuppressive activity thus leads to improved T cell function in vitro (Kusmartsev, Su et al. 2008). Treatment with ATRA caused apoptosis of G-MDSCs and differentiation of M-MDSCs into APCs (Kusmartsev, Cheng et al. 2003; Kusmartsev, Su et al. 2008).

Sunitinib, on the other hand, does not induce MDSC maturation to APCs but reduces MDSC suppressive function and viability in vitro. In addition, sunitinib reduces MDSC and Treg numbers in peripheral blood of renal cell carcinoma patients (Ko, Zea et al. 2009). Depletion of Tregs and MDSCs would be favourable rather than depleting only a single immunosuppressive cell type. Thus, one study combined anti-CD25 treatment with ATRA to control Treg and MDSC expansion. Further combination with an IL-1α vaccine increased survival of tumour-bearing mice (Weiss, Vitacolonna et al. 2009).

As the immune system can recognize, control, fight, and eliminate cancer cells (DuPage, Mazumdar et al. 2012; Tran, Burt et al. 2013), it is important to overcome immunosuppressive mechanisms and cell types so that TAA-specific CTLs effectively recognize and eliminate cancer cells. Thus, pre-clinical research should assess whether cancer immunotherapy treatments can overcome immunosuppressive cells.
1.3. LENTIVIRAL VECTORS

As there is a high demand for more targeted and effective anti-cancer therapies, gene therapy approaches have become a focus for cancer immunotherapy research. Gene therapy is based on the transfer of genetic material to target cells and usually aims at inserting therapeutic genes or silencing disease-causing genes. Several techniques to deliver therapeutic genes have been developed over the past decades, enhancing the field of gene therapy and paving the way to its use in the clinical practice (Escors, Breckpot et al. 2012). The aim of cancer immunotherapy is not to correct a disease-causing gene but rather to boost or induce potent and long-lasting CTL responses to TAAs. Hence, through the use of gene carrier vehicles, TAAs can be expressed or specifically targeted. Additionally, intracellular signalling pathways of interest can be altered in various immune cell types. Many pre-clinical and clinical studies use genetically modified DCs and T cells for cancer immunotherapy. For this purpose, viral vectors are most effective, leading to specific, efficient, stable, and long-term transgene expression.

A milestone in gene therapy was the clinical trial to correct X-linked severe combined immunodeficiency (X-SCID) in children using a mouse leukemia virus (MLV) vector (Cavazzana-Calvo, Hacein-Bey et al. 2000; Hacein-Bey Abina, Le Deist et al. 2002; Thrasher, Gaspar et al. 2006). Unfortunately several of the children developed leukaemia due to insertional mutagenesis. Therapeutic efficacy of cancer gene therapy targeting the immune system was first demonstrated in a clinical trial for melanoma. In this study a retrovirus encoding a MART-1 (Melanoma-associated antigen recognized by T cells)-specific TCR was engineered for autologous T cell therapy. Objective melanoma regression and durable persistence of engineered cells in patients demonstrated the therapeutic potential of genetically engineered cells for cancer immunotherapy (Morgan, Dudley et al. 2006). Nowadays, the most popular viral vectors for DC and T cell transduction are γ-retroviral vectors, adenoviral vectors, and lentiviral vectors (Yang, Yang et al. 2008).

As lentiviral vectors (lentivectors) were used in this thesis, they will be discussed in detail here. Most lentivectors are based on the HIV-1 (Human Immunodeficiency Virus type 1) virus, and thus HIV structure and life cycle will also be described. In addition, the
advantages of using lentivectors will be discussed, justifying the choice of using these vectors as gene transfer vehicles in this thesis.

1.3.1. HIV STRUCTURE AND LIFE CYCLE

RNA retroviruses, such as lentiviruses, integrate a cDNA copy of their RNA genome into host cell chromosomes and use the host cell’s machinery to replicate themselves. This is achieved through reverse transcription and integration by enzymes supplied by the lentivirus itself. Lentiviruses are part of the *retroviridae* family. The *retroviridae* are diploid ssRNA (single stranded RNA) viruses, and their genome is organized from the 5’ to the 3’ end (positive sense) as follows; Genes gag, pol, and env are encoded in the genomic RNA of all members of the *retroviridae* family (Fig. 1.6.A). Gag encodes the structural proteins of the virus, including matrix, capsid, and nucleocapsid proteins (Fig. 1.6.B). The ssRNA is complexed to nucleocapsid proteins in the viral particle. Pol encodes the enzymes reverse transcriptase, integrase, and protease. These enzymes are used for reverse transcription, integration, maturation, and replication. The envelope glycoprotein is encoded by env and it is inserted in the viral membrane that surrounds the particle. In addition, the envelope glycoprotein confers cell and tissue tropism to the viral particle. The *retrovirida* can be divided into simple and complex retroviruses. Simple retroviruses are onco-retroviruses, such as mouse leukemia virus (MLV). Complex retroviruses, such as HIV, also contain accessory genes in their RNA genome (vif, vpr, vpu, nef, rev, tat) that are linked to pathogenesis, virulence, and regulation of gene expression (Fig. 1.6.A). In addition, they contain other cis-acting RNA elements to regulate gene expression, reverse transcription, genome packaging, and particle assembly (Escors and Breckpot 2010; Liechtenstein, Perez-Janices et al. 2013).

The retrovirus life cycle begins when the viral envelope glycoproteins bind to their receptor on the host cell (Fig. 1.6.C). This induces a conformational change in the virus envelope glycoprotein, leading to the exposure of a fusion peptide, which induces the fusion of the virus and cell membranes (Wild, Greenwell et al. 1993). Depending on the type of virus, the particle can enter the cell directly or through endocytosis. The two ssRNA copies are reverse transcribed into one dsDNA in the viral core before the virus
enters the cellular nucleus. Simple retroviruses can only enter the nucleus during mitosis while it is disassembling. The core of complex retroviruses on the other hand can be actively transported into the nucleus and thus enter at any time of the cell cycle (Bukrinsky, Haggerty et al. 1993; Lewis and Emerman 1994). Once in the nucleus, the virus dsDNA is integrated into the host cell chromosomes as a provirus. The U3 virus promoter then directs gene transcription of the provirus. The virus mRNA encoding the structural and enzymatic proteins is translated within the cytoplasm of the cell. Upon recognition of the packaging signal (Ψ) by the nucleocapsid domain of gag, the untranslated full-length viral genomic RNA transcript is packaged and assembled into viral particles. The genomic RNA complexed to gag then assembles at the plasma membrane by interaction with the C-terminus of env. As the viral particles bud off the host cell, they further mature by the activation of the HIV protease within the core which processes gag into each separate structural protein. This leads to the release of fully infectious HIV, restarting their viral life cycle, progressively producing more of its own progeny (Palu, Parolin et al. 2000; Escors and Breckpot 2010; Liechtenstein, Perez-Janices et al. 2013).
**Fig. 1.6. HIV-1 virion structure, genome, and life cycle.** (A) HIV-1 virion is represented here. The lipid envelope contains the envelope transmembrane glycoproteins (ENV). The matrix proteins (MA) form a shell enclosing the nucleocapsid (NC) proteins. The nucleocapsid is made of the RNA genome complexed to the nucleocapsid proteins. The conical HIV-1 core consists of the nucleocapsid, matrix, HIV-1 enzymatic priteins, and cellular proteins. (B) The organization of the HIV-1 genome is schematically represented here as the 5’ to 3’ end organization of the integrated provirus. The 5’ LTR (long-terminal repeat) contains three functional regions; the U3 (HIV promoter), the R and U5 regions, which are involved in RNA replication and transcription. The packaging signal (Ψ) directs the packaging of the HIV genome into lentivirus particles. Following the packaging signal are the genes Gag-Pro-Pol and Env genes. The HIV-1 regulatory/virulence accessory genes vif, vpr, vpu, nef, rev, and tat are distributed along the genome. RRE represents the rev response element. (C) The scheme depicts the retroviral life cycle. R indicates retrovirus engagement with cellular receptor on target cell membrane. This leads to fusion and retrovirus entry, either by direct fusion or endocytosis. After reverse transcription, a cDNA copy is transported to the nucleus and integrated into host cell DNA as a provirus. The host cell’s machinery is used for provirus transcription and translation within the cytoplasm. New virus particles are assembled and released from host cell. Figures adapted or reproduced from Liechtenstein et al., Cancers, Vol. 5(3), 815-837, 2013.
1.3.2. LENTIVECTOR DESIGN AND BIOSAFETY

The capacity of viruses to efficiently deliver and transcribe their genes in host cells has been taken advantage of for gene therapy techniques. Using recombinant DNA cloning techniques pathogenic and virulent genes are removed and replaced with therapeutic genes. For this purpose a wide range of virus species have been used, including adenoviruses, herpes viruses, retroviruses, and lentiviruses. Nevertheless, vectors based on the *retroviridae* family are most widely used nowadays, as they lead to stable and long-term transgene expression in target cells, making them ideal gene carriers. In the 1980s retroviruses were among the first virus vectors to be developed for gene therapy (Mann, Mulligan et al. 1983), leading to their successful application for human gene therapy in the 2000s. The first successful (from a therapeutic point of view) clinical trials using viral vectors were conducted with γ-retroviral vectors. Due to insertional mutagenesis severe genotoxicity developed in several patients indicating that the biosafety of viral vectors had to be further improved for use in human therapy (Cavazzana-Calvo, Hacein-Bey et al. 2000).

Lentivectors were first developed and applied in mouse models in the 1990s (Naldini, Blomer et al. 1996; Naldini, Blomer et al. 1996) and their biosafety has steadily increased since then. In the beginning retroviral and lentiviral vectors were cloned as full-length infectious cDNA clones, in which only the env gene was deleted and provided in trans by a second plasmid during co-transfection (Mann, Mulligan et al. 1983). Biosafety was increasingly improved by providing Gag-Pol genes in a third expression plasmid and eliminating non-essential virulence genes (Fig. 1.7). Thus, lentivectors are usually generated by co-transfection of three plasmids, leading to the release of virus-like particles carrying the defective non-replicating genome. These three plasmids are the transfer vector itself, the packaging plasmid, and the envelope plasmid (Fig. 1.7.A). The transfer vector plasmid encodes the defective virus genome and the transgene of interest, while the packaging plasmid encodes Gag-Pol genes. Finally, the envelope plasmid encodes the envelope glycoprotein. A widely used heterologous (non-HIV env) envelope glycoprotein is VSV-G (vesicular stomatitis virus G protein), which confers a wide species and cell tropism to the lentivector particles (Yee, Friedmann et al. 1994; Akkina, Walton et al. 1996; Naldini, Blomer et al. 1996; Naldini, Blomer et al. 1996). The
2nd generation lentivectors still contain the gag-pol, rev, and tat genes within the packaging plasmid but the viral accessory genes (vif, vpr, vpu, nef) have been removed (Zufferey, Nagy et al. 1997) (Fig. 1.7.A). The 3rd generation lentivectors are tat-independent as the U3 HIV promoter of the transfer vector is replaced with a strong constitutive promoter, such as the CMV (early-intermediate cytomegalovirus) promoter. 3rd generation lentivectors are still rev-dependent but rev is provided \textit{in trans} in a fourth plasmid. Rev interacts with the rev response element (RRE) of the viral/transfer vector transcript and thereby induces nuclear export of this mRNA (Dull, Zufferey et al. 1998; Breckpot, Aerts et al. 2007; He, Munn et al. 2007; Escors and Breckpot 2010; Liechtenstein, Perez-Janices et al. 2013)(Fig. 1.7.B). 3rd generation lentivectors have been further modified to increase their biosafety. By removing large parts of the U3 region within the 3’ LTR (Long Terminal Repeat) containing most of the transcriptional enhancers, it was assured that they are replication defective. After integration into the host chromosomes, the defective 3’LTR is copied to the 5´region of the provirus. Thus, as the defective LTR in the provirus does not act as a promoter, its activities do not interfere with the internal expression cassette, which will be fully active. These vectors are thus termed self-inactivating lentivectors (Zufferey, Dull et al. 1998). Elimination of the above-listed virus elements reduces the likelihood of lentivector recombination with other retroviruses or retrovirus-like elements that could reconstitute an infectious HIV-like retrovirus (production of replication-competent virus). Further work has generated integration-deficient lentivectors that contain mutations in the integrase gene, thus transgenes remain in the nucleus as episomes, circumventing the possibility of insertional mutagenesis (Yanez-Munoz, Balagga et al. 2006; Karwacz, Mukherjee et al. 2009; Hu, Dai et al. 2010).

Lentivectors display several advantages over other viral vectors. As lentiviruses are derived from complex retroviruses they can transduce cells irrespective of their division status (Bukrinsky, Haggerty et al. 1993; Naldini, Blomer et al. 1996; Naldini, Blomer et al. 1996). Thus, these vectors can efficiently transduce a wide range of cell types. They are frequently pseudotyped with VSV-G, which renders them structurally stable and leads to generation of relatively high titers. Due to stable genome integration within transcriptionally active chromatin sites, lentivector transduction leads to prolonged and high transgene expression (Mitchell, Beitzel et al. 2004). Additionally,
lentivectors can accommodate relatively large gene inserts and transduce human and mouse DCs with a superior efficiency than adenoviral vectors (Esslinger, Romero et al. 2002). Furthermore, lentivectors induce less anti-vector immunity than other viral vectors, such as adenoviral vectors, a crucial asset for gene therapy (Barouch and Nabel 2005) (Esslinger, Romero et al. 2002; He and Fallo 2006; He, Zhang et al. 2006). Importantly, they still display significant T cell adjuvant activities, which could be due to the provision of TLR (Toll-like receptor) ligands to APCs (Esslinger, Chapatte et al. 2003; Breckpot, Emeagi et al. 2007; Breckpot, Escors et al. 2010; Rossetti, Gregori et al. 2011). Nevertheless, it has also been argued that adjuvant capacities may in some cases stem from contaminants of vector preparations (Pichlmair, Diebold et al. 2007).

Depending on the use of lentivectors, target cell types vary and hence the ability to target them specifically is important (Dufait, Liechtenstein et al. 2012; Dufait, Liechtenstein et al. 2013). Targeting can be achieved by several methods, including the incorporation of different promoters in the transfer plasmid. In this way it is assured that the transgene is expressed in a cell-specific way (Escors and Breckpot 2010). The use of various envelope glycoproteins from different viruses with various tropisms (pseudotyping) ensures that only some specific cell types are transduced (Morizono, Xie et al. 2005; Yang, Yang et al. 2008; Frecha, Levy et al. 2010). Further, antibodies can be incorporated that recognize specific surface receptors into the lentivector envelope membrane, thus leading to preferential transduction of specific cell types (Yang, Bailey et al. 2006; Gennari, Lopes et al. 2009; Morizono, Pariente et al. 2009). These strategies can also be combined to increase specificity (Pariente, Morizono et al. 2007; Pariente, Mao et al. 2008; Yang, Yang et al. 2008).

As there is increasing evidence suggesting that lentivectors are less genotoxic, they have started replacing γ-retroviral vectors in human therapy in recent years (Hematti, Hong et al. 2004; Modlich, Bohne et al. 2006; Bokhoven, Stephen et al. 2009; Modlich, Navarro et al. 2009) (Montini, Cesana et al. 2006; Howe, Mansour et al. 2008; Arumugam, Higashimoto et al. 2009; Kustikova, Schiedlmeier et al. 2009; Montini, Cesana et al. 2009; Biffi, Bartolomae et al. 2011; Cesana, Sgualdino et al. 2012; Liechtenstein, Perez-Janices et al. 2013). Nevertheless, there are still more clinical trials using retroviral or adenoviral vectors than lentivectors (https://clinicaltrials.gov). The genotoxic events in early human therapy trials using MLV-based γ-retroviral vectors was
shown to be due to integration of retroviral vectors close to the proto-oncogene LMO2 (LIM domain only 2) (Hacein-Bey-Abina, Von Kalle et al. 2003). MLV preferentially integrate into regulatory sequences such as transcription start sites. Lentivectors on the other hand tend to integrate into transcription units and do not integrate upstream of the transcriptional start (Wu, Li et al. 2003; Hematti, Hong et al. 2004). In contrast to γ-retroviral vectors, lentivectors do not preferentially insert close to proto-oncogenes or cell cycle genes and do not accelerate tumour growth in tumour-prone mice (Montini, Cesana et al. 2006; Kustikova, Schiedlmeier et al. 2009; Montini, Cesana et al. 2009; Biffi, Bartolomae et al. 2011). Apart from one exception, there has so far not been observed any serious genotoxicity in clinical trials using lentiviral vectors (Levine, Humeau et al. 2006; Cartier, Hacein-Bey-Abina et al. 2009; Cavazzana-Calvo, Payen et al. 2010; DiGiusto, Krishnan et al. 2010; Biffi, Bartolomae et al. 2011; Kalos, Levine et al. 2011; Porter, Levine et al. 2011; McGarrity, Hoyah et al. 2013; Tebas, Stein et al. 2013; Maude, Frey et al. 2014). In a clinical trial for β-thalassaemia, lentivector integration led to the transcriptional activation of HMGA2 in erythroid cells with further increased expression of a truncated HMGA2 mRNA insensitive to degradation by let-7 microRNAs. This aberrant splicing event appeared to cause clonal dominance (Cavazzana-Calvo, Payen et al. 2010; Cesana, Sguadino et al. 2012). Lentivectors have even been used in patients with HIV and no insertional mutagenesis or lentivector-induced tumours were observed in these patients even in the presence of wild-type HIV (Levine, Humeau et al. 2006). Of note, genotoxicity may in some cases result from the choice of vector transcriptional regulatory elements rather than the vector itself (Maruggi, Porcellini et al. 2009; Ginn, Liao et al. 2010; Knight, Bokhoven et al. 2010). There are two major fears concerning viral vector use in patients, the first is oncogenic potential through insertional mutagenesis and genetic instability while the second is production of replication-competent virus. Even though lentivectors appear to induce less insertional mutagenesis, the use of non-integrating lentivectors can further reduce genotoxicity potential in human therapy (Yanez-Munoz, Balaggan et al. 2006; Karwacz, Mukherjee et al. 2009; Hu, Dai et al. 2010). Generation of replication-competent virus could occur through homologous recombination. So far, no replication-competent lentiviruses have been found in human clinical trials with lentivectors. Importantly, lentivectors have also
been used in HIV patients where the risk of recombination with wild-type HIV virus should be much higher (Levine, Humeau et al. 2006).

**Fig. 1.7. Increased biosafety through advanced generations of lentivector systems.** (A) The figure depicts the second generation lentivector system. Packaging and envelope plasmids contain a strong constitutive promoter, such as CMV (Cytomegalovirus) promoter. The transfer vector encodes a defective HIV-1 genome as lacking genes are provided by the packaging and envelope plasmids. Depicted here is a transfer vector with two LTRs (Long Terminal Repeats, U3-R-U5) and the expression cassette containing an internal promoter and the desired transgene (gene). \(\Psi\), packaging signal; RRE, rev response element. (B) This scheme depicts the third generation lentivector system. In this generation, rev and tat genes are removed from the packaging plasmid. The 5’ HIV U3 region has been replaced by a strong constitutive promoter, such as CMV promoter, which is tat-independent. (C) The third generation of lentivectors can be further modified to increase biosafety and efficacy. Further improvements are indicated as stars. These include the addition of the cPPT (central polypurine flap) and the WPRE (woodchuck post-transcriptional regulatory element). The removal of enhancers from the 3’ HIV U3
region generates self-inactivating lentivectors (ΔEnh). Introducing point mutations or deletions within the integrase attachment sites in the 5’ U3 region of the transfer vector plasmid, or in the integrase gene itself, non-integrating lentivectors are engineered. Figure reproduced from Liechtenstein et al., Cancers, Vol. 5(3), 815-837, 2013.

1.3.3. LENTIVECTOR USE IN CANCER IMMUNOTHERAPY

The clinical efficacy of lentivectors in human gene therapy has been established in treatment of HIV, X-linked adrenoleukodystrophy, β-thalassaemia, and advanced leukaemia in the past years (Levine, Humeau et al. 2006; Cartier, Hacein-Bey-Abina et al. 2009; Cavazzana-Calvo, Payen et al. 2010; Kalos, Levine et al. 2011; Porter, Levine et al. 2011; Tebas, Stein et al. 2013). We have discussed in previous chapters that it is crucial to initiate potent and long-lasting CD8 T cell mediated immunity for cancer immunotherapy. Thus, viral vectors are effective gene delivery vehicles inducing potent T cell responses (Barouch and Nabel 2005; Rowe, Lopes et al. 2006; He and Falo 2007; He, Munn et al. 2007), possibly due to their adjuvant activities (Pichlmair, Diebold et al. 2007; Breckpot, Escors et al. 2010; Liechtenstein, Perez-Janices et al. 2013). Recently, the first clinical trials using lentivector-mediated cancer immunotherapy treatments showed clinical efficacy (Kalos, Levine et al. 2011; Porter, Levine et al. 2011). In these clinical trials T cells were transduced with lentivectors ex vivo to express CARs against CD19 and were thus applied in leukemia patients. Due to the highly promising results, this same strategy is now being employed in a variety of different leukemias. In one of the most recent clinical trials complete remissions in 90% of relapsed and refractory acute lymphoblastic leukemia (ALL) patients was achieved. All patients acquired cytokine-release syndrome but this could be treated effectively with anti-IL6 antibodies (Maude, Frey et al. 2014).

In these recent anti-cancer clinical successes, T cells were the target of lentivector transduction, but significant research is also employed in targeting DCs for cancer immunotherapy. Lentivectors, in contrast to retroviral vectors, can transduce non-dividing cells such as DCs (Bukrinsky, Haggerty et al. 1993; Naldini, Blomer et al. 1996). Importantly for cancer immunotherapy, transduction of DCs with lentivectors does not impair their capacity to stimulate T cell responses. Lentivector-transduced DCs
can thus effectively stimulate antigen-specific CTL responses (Gruber, Kan-Mitchell et al. 2000; Dyall, Latouche et al. 2001; Zarei, Leuba et al. 2002; Breckpot, Dullaers et al. 2003; He, Zhang et al. 2005; He and Falo 2006; Karwacz, Arce et al. 2012). Other viral vectors are not as effective at transducing DCs without affecting their viability or antigen presenting capabilities (He and Falo 2006; He and Falo 2007; He, Munn et al. 2007). Lentivector transduction was shown to result in prolonged in vivo antigen presentation in mouse models, thus increasing the potency and duration of CTL responses (He and Falo 2006). Thus, pre-clinical studies using ex vivo manipulated DCs for cancer immunotherapy have yielded promising results. However, translation into clinical practice has yielded rather disappointing results so far (Escors 2014). This may partly be due to the fact that transferred DCs are subjected to the immunosuppressive tumour environment, once transferred to the patient. Nevertheless, DCs, as the main regulators of adaptive immunity, represent a major gene therapy target for cancer immunotherapy and lentivectors are an efficient tool for their manipulation. In addition, the increasing knowledge of immunosuppressive cell types that hamper current immunotherapeutic strategies may indicate these cells as novel targets for genetic modification.
1.4. PhD AIMS

The initial objective of this thesis was the development and assessment of lentivector vaccines delivering TAAs while modulating cytokine secretion and co-stimulation during antigen presentation. It was hypothesized that presentation of TAAs in a positive co-stimulatory context could potentiate in vivo anti-TAA immune responses. After engineering of the lentivector constructs the first step was to assess the ability of the lentivector vaccines to stimulate TAA-specific T cell responses in vitro and in vivo. Furthermore, lentivector vaccines were evaluated in murine tumour models, in prophylactic as well as therapeutic vaccination schemes.

Initially I thought of DCs as the main target for lentivector vaccination. As DCs are defective in established tumours, I further wanted to test the lentivector vaccines in an immunosuppressive myeloid cell type. Thus, the second part of this thesis deals with the development of an ex vivo MDSC differentiation system. Ex vivo differentiated MDSCs were extensively characterized and their T cell stimulatory activities tested upon lentivector transduction.

The three main objectives of this PhD thesis can be summarized as the following:

- Engineering and testing of lentivector vaccines in the context of vaccination and cancer therapy.
- Development and evaluation of an ex vivo MDSC differentiation system.
- Use of ex vivo differentiated MDSCs in in vitro assays and high throughput studies to identify new molecular targets for future therapeutic intervention.
CHAPTER 2: MATERIALS AND METHODS

2.1. MOLECULAR BIOLOGY

2.1.1. SOLUTIONS AND BUFFERS USED FOR CLONING

<table>
<thead>
<tr>
<th>SOLUTION/BUFFER</th>
<th>COMPOSITION</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x TAE buffer</td>
<td>40mM Tris (pH 7.8), 20mM sodium acetate, 1mM EDTA</td>
</tr>
<tr>
<td>6x gel loading buffer</td>
<td>0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in H₂O</td>
</tr>
<tr>
<td>Transformation buffer</td>
<td>TFB-I</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Transformation buffer</td>
<td>TFB-II</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1. Solutions and buffers used for molecular biology

2.1.2. PCR REACTION

PCR reactions were performed using GoTaqFlexi DNA Polymerase (non-proof-reading, Promega) or Phusion Hot Start II High-Fidelity DNA Polymerase (proof-reading, 2U/μl, Thermo Scientific) in a C1000 thermal cycler (Bio Rad). PCR reaction reagents and cycling conditions are listed below (Tables 2.2 and 2.3). Forward and reverse primers were ordered from Sigma. PCR products were analysed by gel electrophoresis and sequencing.
<table>
<thead>
<tr>
<th>REAGENT</th>
<th>VOLUME (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x Green Go Taq Flexi Buffer (Promega)/</td>
<td>10μl</td>
</tr>
<tr>
<td>5x Phusion HF Buffer (Thermo Scientific)</td>
<td></td>
</tr>
<tr>
<td>MgCl₂ (25mM, Promega)</td>
<td>4μl</td>
</tr>
<tr>
<td>DTT (100mM)</td>
<td>0.5μl</td>
</tr>
<tr>
<td>dNTP Mixture (2.5mM, TakaRa)</td>
<td>1.5μl</td>
</tr>
<tr>
<td>Forward primer (50ng/μl)</td>
<td>1μl</td>
</tr>
<tr>
<td>Reverse primer (50ng/μl)</td>
<td>1μl</td>
</tr>
<tr>
<td>H₂O</td>
<td>Up to 50μl</td>
</tr>
<tr>
<td>Taq polymerase / Phusion polymerase</td>
<td>0.5μl</td>
</tr>
<tr>
<td>DNA template (50ng-1μg)</td>
<td>1μl</td>
</tr>
</tbody>
</table>

*Table 2.2. PCR reaction reagents*

<table>
<thead>
<tr>
<th>STEP</th>
<th>TIME Taq</th>
<th>TIME Phusion</th>
<th>TEMPERATURE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymerase activation</td>
<td>10min</td>
<td>2min</td>
<td>95°C</td>
</tr>
<tr>
<td>1. Denaturing</td>
<td>30sec</td>
<td>30sec</td>
<td>94°C</td>
</tr>
<tr>
<td>2. Annealing</td>
<td>30sec</td>
<td>30sec</td>
<td>Usually 55°C (but depends on template size and GC content)</td>
</tr>
<tr>
<td>3. Elongation</td>
<td>40sec/kb of product</td>
<td>1.5min/kb of product</td>
<td>72°C</td>
</tr>
<tr>
<td>Repeat 1.-3.</td>
<td>25 cycles</td>
<td>35 cycles</td>
<td></td>
</tr>
<tr>
<td>Final elongation</td>
<td>10 minutes</td>
<td>10 minutes</td>
<td>72°C</td>
</tr>
</tbody>
</table>

*Table 2.3. Polymerase reaction cycling conditions*
2.1.3. RESTRICTION ENZYME DIGESTION

Restriction enzyme digestion for cloning was usually performed with 10μg of DNA in a total volume of 30μl. 3μl of 10x digestion buffer, 1μl of each restriction enzyme, DNA, and distilled, autoclaved H₂O were incubated at 37°C for 2 hours. Agarose gel electrophoresis was then used to analyse and purify digested DNA fragments. Restriction enzymes and digestion buffers were purchased from Promega, New England Biolabs, and Thermo Scientific.

2.1.4. AGAROSE GEL ELECTROPHORESIS FOR ANALYSIS AND PURIFICATION

1% agarose gels were prepared by dissolving agarose (D1 agarose low EEO, Conda) in 1x TAE buffer (Table 2.1). After cooling, 0.5μg/ml of ethidium bromide (Invitrogen) or RedSafe (20’000x, iNtRON) was added. 6x loading buffer was added to DNA samples. After agarose gel solidification DNA samples were loaded. A 1kb Plus DNA ladder (Invitrogen) was used as size standards. Gel visualization was performed in a Gel Doc™ 1000 Single wavelength mini-transilluminator (Bio-Rad) or ChemiDoc XRS+ system (Bio-Rad). DNA fragments were cut from gels using a scalpel. DNA fragments were purified using the Gel Extraction kit (Qiagen) following the manufacturers’ instructions.

2.1.5. LIGATION REACTION

Ligation reactions of DNA fragments was performed using T4 DNA ligase (1μl, Promega), 10x ligation buffer (1μl), DNA vector (4μl), and DNA insert (4μl), in a final volume of 10μl. Reactions were incubated either for one hour at RT (Room Temperature) or overnight at 4°C.
2.1.6. pGEM-T CLONING AND SEQUENCING

For sequencing of PCR products amplified with Taq polymerase, PCR products were ligated to the pGEM-T vector using the PCR cloning kit pGEM-T (Promega). Ligations were performed in 10μl of final volume, containing 1μl of T4 DNA ligase (3U/μl, Promega), 5μl of 2x ligation buffer (Promega), 1μl pGEM-T plasmid vector, and 2μl DNA insert to be sequenced. Sequencing was performed by UCL (University College London) or CIMA (Centro de Investigación Médica Aplicada, Pamplona) DNA sequencing services.

2.1.7. COMPETENT BACTERIA PREPARATION

LB-Agar plates were prepared by dissolving LB-Agar (Lennox L agar, Invitrogen) in distilled water according to manufacturer’s instructions. Using a LB-Agar plate containing tetracycline (10μg/ml), XL1 blue E. coli were grown overnight at 37°C. The following day a single colony was picked and grown overnight in a shaker at 37°C in 10ml of LB broth (Lennox Broth base, Invitrogen) containing tetracycline (10μg/ml). 1ml of overnight culture (starter culture) was then grown in 100ml LB (without tetracycline) for 2 hours in a shaker at 37°C without tetracycline. The bacterial culture was then cooled for 5 minutes on ice and centrifuged for 10 minutes at 4°C and 3000rpm. Gentle resuspension of cell pellet in 50ml ice-cold TFB-I buffer (Table 2.1) was followed by a 5-minute incubation on ice. The centrifugation step was repeated, followed by gentle resuspension performed with 4ml of ice-cold TFB-II buffer, and left on ice for 15 minutes. The bacterial suspension in TFB-II buffer were aliquoted and stored at -80°C until use.

2.1.8. TRANSFORMATION OF COMPETENT BACTERIA

Competent bacteria were thawed on ice for 20 minutes. Purified plasmid DNA (1μl) or ligation reaction (3μl) was added to bacteria and incubated on ice for 20 minutes. Heat shock transformation was performed by incubating bacteria for 2 minutes at 37°C and immediately transferred to ice for 2 additional minutes. Bacteria were then
plated on LB-Agar (Invitrogen) plates containing ampicillin (100μg/ml) and incubated overnight at 37°C.

2.1.9. PLASMID PREPARATION

Single colonies from transformations were grown overnight in LB medium containing ampicillin (100μg/ml). LB volumes depended on quantity of DNA required, 4ml were used for miniprep and 100ml for midiprep preparation. Plasmid Mini kit (Qiagen) was used for miniprep plasmid preparations, which could be used for further cloning. Plasmid Midi Kit (Qiagen) was used for midiprep preparations, which could be used for further application in transfection. DNA was quantified by spectrophotometry using NanoDrop (ND-1000; NanoDrop Technologies). As a quality control for each plasmid preparation, a restriction analysis was performed usually with HindIII. When required, plasmids were cut with appropriate restriction enzymes and DNA fragments separated by agarose gel electrophoresis. ApE programme (an Open Access plasmid Editor, Utah University; http://biologylabs.utah.edu/jorgensen/wayned/ape/) was used for restriction enzyme selection and analysis.
2.2. LENTIVIRAL VECTORS

2.2.1. LENTIVECTOR CONSTRUCT PREPARATION

Figure 2.1. shows schematic representations of the lentivector, packaging, and envelope plasmid constructs used in this work. Lentivector expression constructs used were self-inactivating HIV-derived vectors. Dual lentivector plasmids (pDUAL, Fig.2.1.A) were constructed, co-expressing two genes, as previously described (Arce, Breckpot et al. 2011; Karwacz, Bricogne et al. 2011). Mouse cytokine genes were expressed under the transcriptional control of the spleen focus-forming virus promoter (SFFV p). The two chains of the IL12 and IL23 cytokine genes were fused by a linker sequence (GGCAGTACTTCGGGCAGTGGTAAGCCTGGTAGTGGTGAGGGTAGTACCTCAGGGGT). The second transgene was inserted under the transcriptional control of the human ubiquitin promoter (UBIQ p). Second transgenes used in this study were the reporter gene GFP, xeno-antigen HA-tagged IiOVA, or tumour-antigen HA-tagged IiTRP1. While HA-tagged IiOVA had been previously constructed and used in our laboratory (Escors, Lopes et al. 2008; Arce, Breckpot et al. 2011), HA-tagged IiTRP1 was engineered for this study. HA-tagged IiTRP1 gene was engineered as a fusion between the coding regions of the N-terminus of the MHC II invariant chain (ii) with the full-length HA-tagged mouse tyrosinase related protein (Trp1) gene, a known melanoma-associated tumour antigen.

For the generation of stable cell lines, the second transgene encoded an antibiotic resistance gene such as puromycin resistance (puroR). When indicated, dual lentivector plasmids contained the PD-L1-specific short-hairpin RNA (shRNA) designated p1, flanked by NotI restriction enzyme sites (Karwacz, Bricogne et al. 2011). The sequence for shRNA p1 was 5’-AAGGTTATATTGCTGTTGACAGTGAGCGCAACCGAAATGATA CACAATTCTAGTGAAGGCCACAGAGTAGAATTGTGTATCTATTTCTGGTCTACTGCCTCG-3’. When indicated, the target sequence for haematopoietic endogenous microRNA 142 3p (Brown, Cantore et al. 2007) was inserted in dual lentivectors, in the XhoI restriction site. The microRNA 142 3p target sequence was 5’-CGGCCGCACTCTAGAGTCGACTCCATAAA GTAGGAAACACTACACTCAGTTCCATAAAGTAGGAAACACTACAACCCGGTTCATAAAGTAGGAAA CACTACATCCTCCATAAAGTAGGAAACACTACACTAGAGTCGACCTGCAGGCGGCAGGATAT CACTAGTGATTTGGCGCGAGTCGACCTGCAGGGCATGCAAGCTTGGATATC-3’.

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For the cloning of P450R-specific shRNA, pHIV-SIREN was used as a backbone (Fig. 2.1.B). The pHIV-SIREN system is not commercial, its construction has been described in (Lanna, Henson et al. 2014). Three shRNA target sequences were used as follows; target 1 (CGGAGGCACATCCTAGCCATT), target 2 (GCATCTAATGCACCTGGAATT), and target 3 (CCTGACCTACTGGTCATCTT).
Fig. 2.1. Schematic representation of plasmid constructs used in this PhD thesis. (A-B) Transfer lentivector plasmids. (A) pDUAL lentivector plasmids. (B) pHIV-SIREN lentivector plasmid. (C) Packaging (p8.91) and envelope (pMD-G) plasmids, co-transfected together with lentivector expression plasmids. LTR, Long terminal repeat; RRE, rev response element; cPPT, central polypurine tract; SFFV p, spleen focus-forming virus promoter; CYTOK, cytokine gene; WPRE, Woodchuck hepatitis virus post-transcriptional regulatory element; UBI p, ubiquitin promoter; puroR, puromycin resistance gene; SIN, self-
inactivating LTR; CMV, cytomegalovirus promoter; Gag, Pro-Pol, rev, tat, structural and non-structural HIV-1 genes required for lentivector production and packaging.

2.2.2. LENTIVector PREPARATION

Lentivectors were prepared by transient transfection of 293T cells using the Fugene-6 transfection reagent (Promega). The expression plasmid (containing desired transgenes, Fig. 2.1.A,B) was mixed and co-transfected with the packaging plasmid (p8.91, containing HIV-1 structural genes) and VSV-G envelope plasmid (pMD-G)(Fig.2.1.C). 1.5μg of each p8.91 (Plasmid Factory) and pMD-G (Plasmid Factory) were mixed with 3μg of expression vector plasmid in a final volume of 50μl ultrapure water. 25μl of Fugene-6 in 100μl Dulbecco’s Modified Eagle Medium (DMEM, Gibco) were added to the plasmid mixture, followed by a 15 minute incubation time at RT. The transfection mixture was then added to 80% confluent 293T cells and supernatant containing lentivector particles was collected every 24 hours for 72 hours. Supernatants were filtered (45μm filters) and concentrated 100-fold by ultracentrifugation (1.5-2 hours at 22000rpm) through a 20% sucrose cushion in phosphate-buffered saline (PBS 1x, Lonza). Lentivectors were resuspended in PBS containing 10% glycerol, aliquoted, and stored at -80°C.

2.2.3. LENTIVector TITRATION

3*10^5 293T cells were transduced with 1μl of concentrated lentivectors and harvested 72 hours later. Lentivectors containing the reporter gene GFP were titrated by flow cytometry. The percentage of GFP^+ cells represented the percentage of transduced cells. In this way, the lentivector titer (infectious units/ml) could be estimated by the following equation:

\[
\text{Titer (infectious units/ml)} = \text{Number of cells} \times (\%\text{GFP}^+\text{cells}/100) \times (1000/\mu l \text{ used})
\]

Lentivectors containing no fluorescent reporter gene were titrated by quantitative PCR using a TaqMan^® probe (fluorescent reporter molecule, Applied Biosystems), as previously described (Towers, Stockholm et al. 1999; Rowe, Lopes et al.}
2006). For this purpose, transduced 293T cells were harvested and genomic DNA was purified using the DNeasy Blood and Tissue Kit (Qiagen). The TaqMan® gene expression assay (Applied Biosystems) was used to estimate the number of integrated HIV-1-derived vectors in transduced cells. Primer/probe sequences used were Primer 1 (GAGTCCTGCGTCGAGAGC), primer 2 (TGTGTGCCGTCTGTTGTGT), and probe (CAGTGGCGCCGAACAGGGA) complexed to FAM-TAMRA, were used. Dilutions of salmon testes DNA (Invitrogen) were added to standardise total DNA concentration in each sample if required. Reactions were prepared to a final volume of 25μl, containing 1.5μl of 200 μM primer 1, 1.5μl of 200 μM primer 2, 0.75μl of 200mM probe, 12.5μl of TaqMan® master mix (Applied Biosystems), and ultrapure water up to the final volume. Cycling conditions were applied according to manufacturer’s instructions and quantitative PCR was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems).
2.3. **IN VITRO CELL CULTURE**

2.3.1. **CELL LINE MAINTENANCE AND TRANSDUCTION**

293T and B16 cell lines were grown in Dulbecco’s Modified Eagle Medium (DMEM, Gibco), supplemented with 10% FBS (Foetal bovine serum, Gibco), L-glutamine (2mM, Gibco), penicillin (100U/ml, Gibco), and streptomycin (100μg/ml, Gibco). Cells were grown and maintained at 37°C in a humified 5% CO₂ incubator and split every 2-3 days using trypsin EDTA (Gibco). The H1299 human non-small cell lung carcinoma cell line was grown in the same conditions but using the RPMI 1640 medium (Gibco). The 293T cell line is derived from human embryonic kidney cells and contains the large T antigen from simian virus 40. 293T cells were primarily used for lentiviral preparation and titration. B16 cells are mouse melanoma cells and were primarily used for *in vivo* tumour experiments. 293T cells were purchased from the American Type Cell Culture Collection (ATCC) and generously donated by Prof. Mary Collins (UCL). B16 cells were obtained from UCL’s cancer cell repository and H1299 cells were kindly donated by Dr. Lorena Maestre (CNIO, Madrid, Spain).

293T-GMCSF and B16-GMCSF cell lines were generated by transducing 293T and B16 cells with lentivectors (multiplicity of transduction of 50) co-expressing genes encoding murine GM-CSF and puromycin resistance. Transduced cells were then selected by addition of 3μg/ml puromycin (CAYLA-InvivoGen). H1299-huIL4-huGMCSF cells were generated by transducing H1299 cells with two lentivectors. The first lentivector co-expressed human GMCSF and puromycin resistance while the second lentivector co-expressed human IL4 and blasticidin resistance. Transduced cells were selected with 3μg/ml puromycin (CAYLA-InvivoGen) and 5μg/ml blasticidin (Invitrogen).

2.3.2. **PREPARATION OF BONE-MARROW-DERIVED DENDRITIC CELLS AND MYELOID-DERIVED SUPPRESSOR CELLS**

Tibia and femur of C57BL/6 mice were used for collection of bone marrow (BM). BM cells were collected by flushing tibia and femur with Hank’s balanced salt solution (HBSS, Gibco), supplemented with 2% FCS, using 25G needles (BD Microlance) attached to syringes. Red blood cells were lysed using the Red Blood Cell Lysis Buffer (Sigma-
Aldrich). For preparation of BM-DCs (Bone marrow-derived DCs), BM cells were resuspended in Iscove’s Modified Dulbecco’s Medium (IMDM, Gibco) and supplemented with 10% FBS (Gibco), L-glutamine (2mM, Gibco), penicillin (100U/ml, Gibco), streptomycin (100μg/ml, Gibco), recombinant GM-CSF (50ng/ml, MiltenyiBiotec), and 2-Mercaptoethanol (50μM, Sigma-Aldrich). BM-DCs were left in culture for a minimum of 4 days before transduction or use in in vitro assays. To induce maturation of immature BM-DCs, cells were treated with 100ng/ml LPS (Sigma-Aldrich) at least 4 hours before use in in vitro assays. For preparation of myeloid-derived suppressor cells (MDSCs) a similar protocol was followed. Purified BM cells were cultured for 4 days in 75% conditioning medium (CM<sup>293T</sup> and CM<sup>B16</sup>) before transduction or use in in vitro assays. CM<sup>293T</sup> and CM<sup>B16</sup> were generated by collecting supernatants from confluent 293T-GMCSF and B16-GMCSF cells and removing cell debris by centrifugation. If required, monocytic (M)-MDSC and granulocytic (G)-MDSC populations were isolated from MDSC cultures using the mouse Myeloid Derived Suppressor Cell Isolation Kit (MiltenyiBiotec), according to manufacturer’s conditions.

### 2.3.3. PREPARATION OF HUMAN MDSCs FROM WHOLE BLOOD

Whole blood was centrifuged for 15 minutes at room temperature at 3000rpm. The white layer containing the white blood cells was retrieved and diluted with RPMI medium. Ficoll-Paque™ PLUS (GE Healthcare) was added to Falcon tubes and the diluted blood cells were added gently on top (without disturbing Ficoll surface). Tubes were centrifuged for 45 minutes at room temperature at 1500rpm, without a brake. The Peripheral Blood Mononuclear Cell (PBMC) layer was collected (underneath plasma and above Ficoll and red blood cell layers) and washed with RPMI. Ten million PBMCs per well were then plated in M6 well plates and left at 37°C for 4 hours. Floating cells (lymphocyte fraction) and attached cells (monocytes) were aliquoted and frozen separately at -80°C. Alternatively, the monocytic fraction was washed with PBS (room temperature) and cultured with CM<sup>H1299</sup> for three to five days. CM<sup>H1299</sup> was generated by collecting supernatants from H1299-huGMCSF-huIL4 cells and removing cell debris by centrifugation.
2.3.4. SURFACE AND INTRACELLULAR STAINING FOR FLOW CYTOMETRY

Stainings were performed on ice in the dark. BM-DCs and MDSCs were blocked for 20 minutes with mouse Fc Block™ (anti-CD16/CD32, BD Biosciences) in PBS supplemented with 20% FBS. Antibodies directed against surface markers (Table 2.3) were diluted to the required concentration in PBS supplemented with 20% FBS and mouse Fc Block™. Cells were incubated with antibody for 10 minutes and washed. Cells could then be used to perform fluorescent activated flow cytometry (FACS). To perform intracellular stainings, cells were fixed for 20 minutes (Cytoperm/Cytofix, BD Biosciences). For staining of transcription factors or nuclear proteins the FoxP3/Transcription Factor Staining Buffer Set (eBioscience) was used. Cells were washed and incubated with buffer from appropriate kit containing desired antibody concentrations (Table 2.4). Cells were washed and analysed by flow cytometry using FACS Calibur, Canto, or LSRFortessa (BD Biosciences). Results were analysed using FlowJo 7.6 software.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Alternate Name</th>
<th>Fluorochrome</th>
<th>Clone</th>
<th>Company</th>
<th>Dilution</th>
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## Table 2.4. Antibodies used for FACS staining of mouse cells

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*Table 2.5. Antibodies used for FACS staining of human cells*

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</table>

*Table 2.6. Other antibodies or dyes used for FACS staining*
2.3.5. SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) AND WESTERN BLOT ANALYSIS

Protein extraction was performed on ice and all reagents are shown in Table 2.7. Cells were washed with PBS, lysed (1% Igepal in PBS, Sigma-Aldrich,) containing protease inhibitors as recommended by the manufacturer (Protease Inhibitor Cocktail tablets, Roche Life Science) and incubated on ice for 15 minutes. Nuclei were removed by centrifugation in a microfuge at maximum speed for 1 min, and reducing protein buffer (Laemmli Buffer) was added to supernatant. Samples were heated at 90°C for 5 minutes before freezing (-80°C) or loaded in 10% polyacrylamide gels. Total protein was then separated using a 10% denaturing SDS-polyacrylamide gel in SDS running buffer. Separated proteins were then transferred to a Hybond ECL nitrocellulose membrane (Amersham) using transfer buffer following standard conditions. Membranes were blocked for 1 hour at room temperature with blocking buffer and incubated overnight with primary antibody (4°C). Membranes were washed with washing buffer and incubated with HRP-conjugated secondary antibodies. Membranes were washed and developed using ECL (Enhanced chemiluminescence) substrate solution containing 1μl hydrogen peroxide. Western Blots were developed either on radiographic film or in a ChemiDoc XRS+ System (Bio-Rad).

Transgene expression in transduced cells was detected using HA tag-specific rabbit antibody (Sigma). For detection of tumour-associated antigens in mouse tumour tissue, mouse anti-tyrosinase, TRP1, TRP2, and Gp100-specific antibodies were used (Abcam). In addition, we used mouse anti-iNOS antibody (Cell Signaling), polyclonal anti-P450R antibody (Abcam), and anti-GADPH (Calbiochem) for analysis of myeloid cells and loading control. HRP-conjugated anti-rabbit and anti-mouse antibodies (DAKO) were used as secondary antibodies.
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<th>BUFFER/GEL</th>
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</tr>
<tr>
<td>Running Buffer (1x)</td>
<td>25mM Tris (pH8.5), 200mM glycine, 0.1% SDS</td>
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<tr>
<td>Transfer Buffer (1x)</td>
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<tr>
<td>Blocking Buffer (1x)</td>
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<td>Washing Buffer (1x)</td>
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<tr>
<td>10% separating polyacrylamide gel</td>
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<td>7% concentrating gel</td>
<td>7% acrylamide, 125mM Tris-HCl (pH6.8), 10% SDS, 0.1% TEMED, 1% APS</td>
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*Table 2.7. Composition of buffers, solutions, and gels used for SDS-PAGE.*
2.3.6. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

BD OptEIA™ Set Mouse GM-CSF and BD OptEIA™ Set Mouse IL-10 were purchased from BD Biosciences and mouse IL12 and IFNγ ELISA kits were purchased from eBioscience. ELISAs were performed according to manufacturer’s instructions. Briefly, 96-well Nunc-Immuno polystyrene Maxisorp ELISA flat bottom plates (Thermo Scientific) were coated overnight with capture antibody in PBS. The next day the plate was washed (Wash Buffer, 0.05% Tween-10 in PBS) and blocked with assay diluent (10% FBS in PBS) at room temperature for one hour. After washing plate was incubated with standards and samples in the corresponding wells (diluted in assay diluent) for two hours. The plate was washed and working detector (detection antibody and SAv-HRP reagent) was added. After one hour of incubation, plate was washed and substrate solution (TMB Substrate Reagent, BD Pharmingen) was added. Incubation time varied depending on antibody used. To finish the enzymatic colourimetric reaction, Stop Solution (Sigma) was added and plate read at 450nm absorbance in a microplate reader (Thermo Scientific).

2.3.7. ELISpot ASSAY

The ELISpot assay was carried out as described (Escors, Lopes et al. 2008). Briefly, ELISpot 96 well plates coated with nitrocellulose membranes were coated with a solution of IFNγ capture antibody (1 μg/ml concentration) overnight at 4°C. Then, the wells were blocked with 20% FCS in PBS and half a million splenocytes from the appropriate treated mice were incubated overnight in complete IMDM medium containing 100 ng class I or class II OVA peptides per ml in the corresponding wells. Then, wells were washed twice with PBS and incubated with alkaline phosphatase-conjugated anti-IFNγ antibody for one minute at RT. AP developing solution was added and after the appearance of spots, the reaction was stopped with 0.1% sulphuric acid. Spots were counted in an ELISpot reader.
2.3.8. BIOACTIVE TGFβ BIO-ASSAY

Bioactive TGFβ within supernatants could be measured by using a TGFβ reporter cell line (SMAD-GFP), previously developed by our laboratory (Arce, Breckpot et al. 2011). Supernatants were cultured with SMAD-GFP cells and concentration standards were prepared using recombinant human TGFβ (Abcam).
2.4. **EX VIVO ASSAYS AND IN VIVO EXPERIMENTS**

2.4.1. **MOUSE STRAINS**

C57BL/6 and BALB/c mice were used for extraction of bone marrow and splenocytes. C57BL/6 mice were used for vaccination and tumour experiments. OT-I and OT-II mice were used for ex vivo antigen-specific assays as these mice contain OVA-specific CD8 and CD4 T cells, respectively (Hogquist, Jameson et al. 1994; Barnden, Allison et al. 1998). Approval for the animal studies was obtained from the University College London Animal Ethics Committee, the Animal Ethics Committee of the University of Navarra, and from the Government of Navarra.

2.4.2. **HARVESTING OF SPLEENS AND PURIFICATION OF CD4 AND CD8 T CELLS**

Spleens were collected from C57BL/6 and mashed through a nylon mesh (70μm, BD Falcon) and resuspended and washed in HBSS. Red blood cells were lysed with Red Blood Cell Lysis Buffer (Sigma-Aldrich), washed, and counted. CD4 and CD8 T cells could then be purified or whole splenocyte populations cultured in IMDM, supplemented with 10% FBS (Gibco), L-glutamine (2mM, Gibco), penicillin (100U/ml, Gibco), streptomycin (100μg/ml, Gibco), recombinant IL-2 (10U/ml, Peprotech), and 2-Mercaptoethanol (50μM, Sigma-Aldrich). CD4 and CD8 T cells were purified using the CD4+ T cell isolation kit, mouse and the CD8a+ T cell isolation kit, mouse (MiltenyiBiotec), according to manufacturer instructions. Purified CD4 and CD8 T cells could then be analysed by FACS for purity and cultured in above medium.

2.4.3. **T CELL SUPPRESSION ASSAY**

Purified CD8 T cells were plated at $10^5$ cells/well in 96-well plates and were stimulated with anti-CD3/anti-CD28 coated beads (Dynabeads® Mouse T-activator CD3/CD28, Gibco) following the manufacturer’s recommendations. When indicated, CD8 T cells were previously stained with 0.5μM CFSE (Carboxyfluoresceinsuccinimidyl ester, Invitrogen), according to manufacturers instructions. M-MDSC, G-MDSC, or bulk MDSC were added to activated T cells at various ratios. When indicated, supernatants containing IL12 were added at 250pg/well. Monoclonal antibodies against CTLA-4, PD-1,
and control hamster IgG (BioXCell, UC10-4F10-11, BE0033-2, BE0091) were added at 10μg/ml per well. To evaluate T cell proliferation, cells were stained with anti-CD3 antibody and CFSE dilution in CD3+ cells was assessed 72 hours later by flow cytometry. Alternatively, cell cultures were stained with CD11b, CD8, and Ki67 FACS antibodies. Supernatants were collected and IFNγ concentrations were analysed using ELISA (eBioscience).

Suppression assays were performed in a similar way using human H1299-MDSCs. For this purpose, Dynabeads® Human T-activator CD3/CD28 (Gibco) were used to activate thawed human lymphocytes, previously purified from whole blood and kept frozen at ~80 degrees until use. After 4 hours of incubation with activation beads, H1299-MDSCs were added at various ratios. 72 hours later cell cultures were stained with CD4, CD8, and Ki67 FACS antibodies and analysed by flow cytometry.

2.4.4. MIXED LYMPHOCYTE REACTION

Spleens were collected from BALB/c mice and CD8 and CD4 T cells purified, as described previously. T cells were co-cultured with MDSCs differentiated from BM of C57/BL6 mice at a ratio of 10:1. 72 hours later, cells were stained with CD4, CD8, Ki67, and IFNγ antibodies and analysed by flow cytometry.

2.4.5. ANTIGEN SPECIFIC CO-CULTURE ASSAY

BM-DCs and MDSCs were transduced with IiOVA-containing lentivectors for 3 days before co-culture with OVA-specific purified CD4 and CD8 T cells (from OT-II and OT-I mice). 3 days later cells were stained with anti-CD4, CD8, and IF-γ antibodies and analysed by flow cytometry.

2.4.6. TREATMENT WITH CHEMOTHERAPEUTIC AGENTS

BM-DCs and MDSCs were incubated with Paclitaxel (200nM), Docetaxel (50nM), and Irinotecan (32μM) at day 5 of culture for 72 hours. Cell viability was assessed via trypan blue staining (Sigma-Aldrich). Chemotherapeutic agents were obtained from the Pharmacy department of the Hospital de Navarra, Pamplona, Spain.
2.4.7. DIRECT LENTIVECTOR VACCINATION AND ANALYSIS OF IN VIVO T CELL RESPONSES

C57BL/6 mice were subcutaneously (s.c.) injected at the base of the tail with concentrated lentivectors (1*10^7 infectious units/mouse). 5 naive mice were used per group. Two weeks later spleens could be harvested and isolated for analysis of T cell responses. Splenocytes of mice vaccinated with IiOVA-containing lentivectors were isolated and cultured overnight in the presence of class I and II OVA peptides, as previously described (Arce, Breckpot et al. 2011). Class I OVA peptide (SIINFEKL) and class II OVA_{323-339} peptide were generously donated by Prof. Mary Collins. 0.1ng/μl class I OVA peptide and 2μg/ml class II OVA_{323-339} peptide were used. Th1/2/17, Treg, and CTL responses were assessed by flow cytometry using the appropriate antibody combinations.

Splenocytes from mice vaccinated with IiTRP1-containing lentivectors were isolated and cultured overnight with BM-DCs that were previously transduced with IiTRP1-containing lentivectors and activated with LPS (100ng/ml). Th1 and CTL responses were assessed by flow cytometry using the appropriate antibody combinations. All vaccination experiments were repeated independently at least twice, unless stated otherwise.

2.4.8. TUMOUR EXPERIMENTS ASSESSING LENTIVECTOR VACCINES

Prophylactic experiments were performed by vaccination of healthy mice with lentivectors (1*10^7 infectious units/mouse). Two weeks later B16 cells were subcutaneously transferred at the base of the tail. Tumour growth and survival was monitored and mice sacrificed when tumour sizes grew above 150mm^2. Long-term survivors were re-challenged with tumour cells.

Therapeutic experiments were performed by transferring 3*10^5 B16 cells/mouse and tumours were allowed to grow until they reached sizes between 50 and 200mm^3. Lentivectors (1*10^7 infectious units/mouse) were intra-tumourally injected and tumour growth and survival was monitored. Mice were sacrificed when tumour volumes reached levels above 2500mm^3. 5 mice per group were used and experiments repeated independently at least twice.
2.4.9. PROTEIN EXTRACTION AND SINGLE CELL PURIFICATION FROM TUMOUR SAMPLES

Tumours were resected, cut into pieces, and dissociated with the GentleMACS dissociator (MiltenyiBiotec) in 5ml PBS. Dissociation program m_impTumor_02 was run for 37 seconds. Collagenase I (150U, Sigma-Aldrich) and Dispase II (4.8mg, Sigma-Aldrich) were added and incubated at 37°C for 40 minutes, using the MACSmix tube rotator (MiltenyiBiotec). DNase I was added (10U, Sigma-Aldrich) and dissociated with GentleMACS dissociator using the same program. Cell suspension was transferred to a 70μm nylon mesh and washed with PBS. Cell pellet was subjected to Red Blood Cell Lysis Buffer, incubated, and washed. Cells could then be resuspended in medium, counted, and stained with flow cytometry antibodies. For immunoblot analyses of tumour samples, tumours were cut into small pieces and placed in an eppendorf tube. Then, 200 μl of laemmli buffer was added and incubated at 90 degrees for 10 min. Then, proteins were resolved by SDS-PAGE and analysed by immunoblotting with the indicated antibodies.

2.4.10. IN VIVO MDSC SUPPRESSIVE ACTIVITY AND BIOLUMINESCENCE IMAGING

B16-MDSCs were differentiated ex vivo and used for s.c. injection on day 5/6 of culture. Naïve C57/BL6 mice were s.c. challenged with either 1*10⁶ B16 cells alone, or a mixture of 1*10⁶ B16 cells (control mice) and 2*10⁶ B16-MDSC cells (MDSC mice). Every week (during the following 2 weeks) MDSC mice were s.c. or intra-tumourally injected with 2*10⁶ B16-MDSC cells. Tumour growth and survival was monitored and mice sacrificed when tumour sizes grew above 150mm². 5 mice per group were used and experiments repeated independently at least twice. When indicated, B16-Fluc cells (expressing the firefly luciferase gene, kindly donated by Dr. Frederick Arce, UCL) were used. In this way, we assessed tumour size also via bioluminescence imaging. Upon intraperitoneal injection of luciferin (3mg/mouse, VivoGlo™ Luciferin, Promega) bioluminescence was recorded with a Photon Imager Optima bioluminescence device (Biospace Lab). Images were processed and bioluminescence signal quantified using the M3 vision software (Biospace Lab). Regions of interest (ROIs) were defined as regions
above threshold. Photon emission intensity (photons/s/cm²/sr) was calculated from
data of emitted photons from the respective ROIs by using M3 vision software.
2.5. MASS SPECTROMETRY-BASED QUANTITATIVE PROTEOMICS

Mass spectrometry and sample preparation were carried out by Navarrabiomed-FMS proteomics platform.

2.5.1. SAMPLE PREPARATION FOR PROTEOMIC ANALYSIS

Three biological replicates were prepared for each experimental condition. B16-MDSCs, 293T-MDSCs, and conventional immature BM-DCs were collected on day 8 of culture and pelleted by centrifugation. Cell pellets were lysed using a buffer containing 7 M urea, 2 M thiourea, 4% (v/v) CHAPS, and 50 mM DTT. Homogenates were centrifuged for 1 hour at 14000rpm at 15°C. Protein concentration was then measured using the Bradford assay kit (Bio-Rad).

2.5.2. PEPTIDE LABELLING FOR iTRAQ (ISOBARIC TAGS FOR RELATIVE AND ABSOLUTE QUANTITATION) PROTEOMIC ANALYSIS

The iTRAQ system was used to perform a shotgun comparative proteomic analysis of total cell extracts (Unwin, Griffiths et al. 2010). 160µg of protein extracts were precipitated with methanol/chloroform and pellets dissolved in 7 M urea, 2 M thiourea, and 4% (v/v) CHAPS. iTRAQ labeling was performed according to the manufacturer’s instructions (ABSciex). Briefly, 80µg of protein from each sample was reduced with 50mM tris (2-carboxyethyl)phosphine (TCEP) at 60°C for 1 hour. Cysteine residues were then alkylated with 200mM methylmethanethiosulfonate (MMTS) at room temperature for 15 minutes and proteins digested using trypsin (Promega; 1:20, w/w) at 37°C for 16 hours. Each sample was then labelled with one isobaric amine-reactive tag (Table 2.8) and incubated for 1 hour. Samples were then mixed and evaporated in a vacuum centrifuge until <40µl.
<table>
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<tr>
<th>Experiment</th>
<th>Sample</th>
<th>Isobaric Tag</th>
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<td>DC-1</td>
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<td>DC-2</td>
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<td></td>
<td>DC-3</td>
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<td></td>
<td>B16-1</td>
<td>Tag\textsubscript{116}</td>
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<td>B16-3</td>
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<tr>
<td>293T-MDSCs vs B16-MDSCs</td>
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<td></td>
<td>B16-3</td>
<td>Tag\textsubscript{115}</td>
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Table 2.8. iTRAQ isobaric labeling of peptide samples

2.5.3. PEPTIDE FRACTIONATION

Peptides were then fractionated to increase proteome coverage. Peptide pool was injected into an Ettan LC system with an X-Terra RP18 pre-column (2.1mm x 20mm) and a high pH stable X-Terra RP18 column (C18; 21mm x 150mm; 3.5μm) (Waters) at a flow rate of 40 μl/min. Peptides were eluted with a mobile phase B of 5-65% linear gradient over 35 minutes (A, 5mM ammonium bicarbonate in water, pH 9.8; B, 5mM ammonium bicarbonate in acetonitrile, pH 9.8). 8 fractions were collected and evaporated under vacuum. Samples were reconstituted in 20μl of 2% acetonitrile, 0.1% formic acid, 98% MilliQ-H\textsubscript{2}O and were now ready for mass spectrometric analysis.
2.5.4. TRIPLE-TOF 5600 MASS SPECTROMETRY

Peptide mixtures were separated by reverse phase chromatography using an Eksigent nanoLC ultra 2D pump, fitted with a 75µm ID column (Eksigent 0.075 x 150). Samples were desalted and concentrated by loading in a 0.5cm length 300µm ID pre-column (packed as separating column). Mobile phases were 100% water 0.1% formic acid 0.1% formic acid (FA) (buffer A) and 100% Acetonitrile 0.1% FA (buffer B). Development of column gradient was performed in a 70 minute two step gradient. Gradient was developed from 2% B to 30% B in 60 minutes and 30% B to 40% B in 10 minutes. Column was then equilibrated using 95% B for 5 minutes and 2% B for 15 minutes. Importantly, pre-column was always in line with column and flow was maintained all along the gradient at 300 nl/min. Eluting peptides were analysed using the AB Sciex 5600 TripleTOF™ system. Information data was acquired upon a survey scan performed in a mass range from 350 m/z up to 1250 m/z in a scan time of 250 ms. Top 25 peaks were selected for fragmentation and a minimum accumulation time for MS/MS was set to 75 ms giving a total cycle time of 2.1 seconds. Product ions were scanned in a mass range from 100 m/z up to 1700 m/z and excluded for further fragmentation during 15 seconds.

2.5.5. DATA ANALYSIS USING ProteinPilot™ 4.5

Data files were then processed using ProteinPilot™ 4.5 software from AB Sciex. ProteinPilot™ 4.5 uses the algorithm Paragon™ (v.4.0.0.0) (Shilov, Seymour et al. 2007) for database search and Progroup™ for data grouping. Each MS/MS spectrum was searched against a database of murine protein sequences (Uniprot complete mouse proteome), as described previously (Liechtenstein, Perez-Janices et al. 2014). The search parameters allowed for cysteine modification by MMTS and biological modifications programmed in the algorithm (i.e. phosphorylations, amidations, semitryptic fragments, etc.). Reporter ion intensities were bias corrected for the overlapping isotope contributions from the iTRAQ tags according to the certificate of analysis provided by the reagent manufacturer (ABsciex). The peptide and protein selection criteria for relative quantitation were performed as follows. Only peptides unique for a given
protein were considered for relative quantitation, excluding those common to other isoforms or proteins of the same family. Proteins were identified on the basis of having at least one peptide with an ion score above 99% confidence. Among the identified peptides, some of them were excluded from the quantitative analysis for one of the following reasons: (i) The peaks corresponding to the iTRAQ labels were not detected; (ii) the peptides were identified with low identification confidence (<1.0%); (iii) the sum of the signal-to-noise ratio for all of the peak pairs was <6 for the peptide ratios. The protein sequence coverage (95% conf.) was estimated for specific proteins by the percentage of matching amino acids from the identified peptides having confidence greater than or equal to 95% divided by the total number of amino acids in the sequence. Several quantitative estimates provided for each protein by ProteinPilot were utilized: the fold change ratios of differential expression between labelled protein extracts; the p-value, representing the probability that the observed ratio is different than 1 by chance. A decoy database search strategy was also used to estimate the false discovery rate (FDR), defined as the percentage of decoy proteins identified against the total protein identification. The FDR was calculated by searching the spectra against the decoy database generated from the target database. The results were then exported into Excel for manual data interpretation. Although relative quantification and statistical analysis were provided by the ProteinPilot software, an additional 1.3-fold change cutoff for all iTRAQ ratios (ratio <0.77 or >1.3) was selected to classify proteins as up- or down-regulated. Proteins with iTRAQ ratios below the low range (0.77) were considered to be under-expressed, whereas those above the high range (1.3) were considered to be over-expressed.

False discovery rate was performed using a non-lineal fitting method and results displayed corresponded to those reporting a 1% Global False Discovery Rate (FDR) or better. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) (Vizcaíno, Deutsch et al. 2014) via the PRIDE partner repository with the data set identifiers PXD001103 and PXD001106.
2.5.6. BIOINFORMATIC ANALYSIS

The proteomic information could then be analysed using various bioinformatic tools, including DAVID (Database for Annotation, Visualization and Integrated Discovery) Bioinformatics Resources (v6.7) and PANTHER (Protein annotation through evolutionary relationship) (http://www.pantherdb.org/) software tools (Huang da, Sherman et al. 2009; Mi, Muruganujan et al. 2013). These programs can detect and infer differentially activated/deactivated pathways as a result of cell type differences. UniProtKnowledge base (Universal Protein Resource, http://www.uniprot.org/) was used to obtain protein functional information. In addition, STRING (Search Tool for the Retrieval of Interacting Genes) software (v.9.1) (http://stringdb.org/)(Franceschini, Szklarczyk et al. 2013) was used to identify specifically up- or dysregulated regulatory/metabolic networks in MDSCs.
2.6. STATISTICAL ANALYSIS

GraphPad Prism, Salstat and SPSS software packages were used for plotting data and statistical analyses. No data was considered an outlier. All experiments were repeated independently at least twice, unless stated otherwise. Means were compared using an unpaired T-student test or one-way ANOVA. Flow cytometric data from surface or intracellular staining from multiple groups were analysed by one-way ANOVA followed by a Tukey’s \textit{a posteriori} test. Percentages of T cells in \textit{in vitro} assays were normally distributed and analysed with one-way ANOVA followed by a Tukey’s \textit{a posteriori} test. Three independent reactions per group were used for most \textit{in vitro} assays. Data from independent experiments was pooled to increase statistical sensitivity, unless stated otherwise.

For \textit{in vivo} experiments, 5 mice per group were used following the results of Mead’s resource equation for power analysis. Survival data from tumour experiments was compared using the Log-Rank test. Tumour sizes and lifespan were compared with the non-parametric tests U of Mann-Whitney or Kruskall Wallis.

Significant, very significant and highly significant differences were indicated in the figures with *, ** or *** when the associated probability was P<0.05 (two-tailed, alpha=0.05, n≥5), P<0.01 (two-tailed alpha=0.01, n≥5) and P<0.001 (two-tailed alpha=0.001, n≥5), respectively.
CHAPTER 3: IL12 EXPRESSION AND PD-L1 SILENCING INDUCES TRP1-SPECIFIC T CELL RESPONSES AND DELAYS MELANOMA GROWTH IN VIVO IN MICE

3.1. INTRODUCTION

Cancer immunotherapy treatments aim at eliminating cancer cells by stimulating the immune system. Especially important in this regard is the activation and expansion of cytotoxic CD8^+ T lymphocytes (CTLs). T cells specific for tumour-associated antigens (TAAs) already circulate systemically in naïve and tumour-bearing individuals but are mainly unresponsive (Novellino, Castelli et al. 2004; Boon, Coulié et al. 2006). Importantly, unresponsiveness of anti-tumour T cell responses can be reversed by vaccination and thus initiate tumour regression (Germeau, Ma et al. 2005; Boon, Coulié et al. 2006; Morgan, Dudley et al. 2006; Cipponi, Wieers et al. 2011). Various TAAs have been identified for many tumour types, including melanoma, thus providing targets for anti-tumour immune responses.

3.1.1. MELANOMA TUMOUR-ASSOCIATED ANTIGENS

Melanoma is the most deadly type of skin cancer due to its tendency to rapidly spread in systemic metastases and its resistance to therapy. While melanoma represents only 5% of diagnosed skin cancers, more than 75% of skin cancer deaths are caused by it (Hanahan and Weinberg 2000; Yan, Tingey et al. 2014). Furthermore, the incidence of melanoma is still increasing (Jemal, Devesa et al. 2001; Jemal, Siegel et al. 2010) and treatment options are limited when disease has progressed to the metastatic stage. Nevertheless, several melanoma-associated tumour antigens have been identified (Novellino, Castelli et al. 2004; Boon, Coulié et al. 2006; Cipponi, Wieers et al. 2011) and patients can raise autologous anti-melanoma and tumour-infiltrating T cell responses (Boon, Coulié et al. 2006; Cipponi, Wieers et al. 2011). Melanoma-associated antigens can be grouped into three categories; early melanocyte differentiation antigens, late melanocyte differentiation antigens, and a group of cancer-testis antigens. Early
melanocyte differentiation antigens are represented by gp100 (Bakker, Schreurs et al. 1994; Kawakami, Eliyahu et al. 1994) and Melan-A/MART1 (Kawakami, Eliyahu et al. 1994), while late melanocyte differentiation antigens include tyrosinase (Brichard, Van Pel et al. 1993), TRP1 (Tyrosinase-related protein 1) (Wang, Johnston et al. 1998) and TRP2 (Tyrosinase-related protein 2) (Wang, Appella et al. 1996). The MAGE (Melanoma antigen gene) superfamily (van der Bruggen, Traversari et al. 1991) and NY-ESO-1 (Chen, Scanlan et al. 1997) antigens are cancer-testis antigens, also over-expressed in other cancer types (Brasseur, Marchand et al. 1992; Duffour, Chaux et al. 1999; Gnjatic, Nishikawa et al. 2006; Pollack, Jungbluth et al. 2012).

Many pre-clinical (Overwijk, Lee et al. 1999; Bronte, Apolloni et al. 2000; Guevara-Patino, Engelhorn et al. 2006; Bobisse, Rondina et al. 2009; Liu, Peng et al. 2009; Vacchelli, Martins et al. 2012) and clinical studies (Marchand, van Baren et al. 1999; Germeau, Ma et al. 2005; Ribas, Weber et al. 2011; Vacchelli, Martins et al. 2012; Aranda, Vacchelli et al. 2013; Wilgenhof, Corthals et al. 2014; Rotte, Bhandaru et al. 2015) using melanoma TAAs have been conducted. Melanoma-specific responses initiated by vector vaccination encoding TRP2 rejected established B16 tumours in mice and this effect depended on CTL responses (Bronte, Apolloni et al. 2000). Genetic TRP1 vaccines on the other hand were only effective at inducing anti-tumour responses in mice when TRP1 was mutated, suggesting that wild-type TRP1 is a poorly immunogenic TAA (Guevara-Patino, Engelhorn et al. 2006; Liu, Peng et al. 2009). In a different pre-clinical study, vaccination with a recombinant vaccinia virus encoding TRP1 led to elimination of normal and malignant melanocytes and could confer protection from B16 melanoma. Interestingly, while this response depended on TRP1-specific antibodies, no TRP1-specific CTLs could be detected (Overwijk, Lee et al. 1999). Ex vivo engineered TRP1-specific CTLs slowed-down B16 tumour progression but tumours eventually grew out. The authors suggested that T cell exhaustion could be a possible cause for tumour escape (Dougan, Dougan et al. 2013). Thus, TRP1/TRP2 antigens are of interest for human melanoma therapy (Khong and Rosenberg 2002) but TRP1 is not one of the most immunogenic melanoma TAAs (Overwijk, Lee et al. 1999; Bronte, Apolloni et al. 2000; Sierro, Donda et al. 2011; Escors 2014). Poor immunogenicity of some tumour antigens is due to the instability of the peptide-MHC complex (Yu, Theoret et al. 2004). TRP1 vaccines may not be as effective as other melanoma antigen vaccines as TRP1 had to be
mutated in several studies to increase peptide processing and avidity to mutant peptide, thus enhancing antigen presentation to T lymphocytes (Guevara-Patino, Engelhorn et al. 2006; Liu, Peng et al. 2009).

Gp100, MAGE, Melan-A/MART-1, and NY-ESO on the other hand are commonly used for the preparation of melanoma vaccines for use in clinical practice (Rotte, Bhandaru et al. 2015). Clinical studies have shown an increase in TAA-specific CTL numbers but responses have been rather limited (Ribas, Weber et al. 2011). Vaccination of melanoma patients with MAGE, Melan-A, or tyrosinase antigens can raise tumour-infiltrating T cell responses against various melanoma TAAs (including TAAs not included in the tumour vaccines) (Germeau, Ma et al. 2005; Ribas, Weber et al. 2011) and can cause stable disease or tumour regression in about 30% of patients (Marchand, van Baren et al. 1999; Ribas, Weber et al. 2011). Further, a recent clinical trial used autologous DC transfer where monocyte-derived DCs were electroporated with mRNA encoding fusion proteins between MAGE-A1, A-3, -C2, tyrosinase, MelanA/MART-1, or gp100 and an HLA class II-targeting sequence. In combination with IFNα-2b this resulted in encouraging long-term overall survival rates (Wilgenhof, Corthals et al. 2014). Thus, this data confirms that natural melanoma TAA-specific T cell responses exist but that they eventually lose efficacy. Thus, while tumour vaccines have been able to raise high numbers of TAA-specific CTLs in immunized patients, clinical results have been rather disappointing. Delivery of TAAs alone is therefore not enough to raise effective CTL responses. Tumour-infiltrating TAA-specific CTLs are defective as they fail to produce cytokines, exhibit reduced cytotoxicity, and express high levels of co-inhibitory molecule receptors, such as PD-1 (Programmed Death-1) (Zhou, Xiao et al. 2010; Sierro, Donda et al. 2011). Natural immunological tolerance to TAAs and the immunosuppressive tumour environment prevent TAA-specific effector functions. Thus, TAAs should be presented to TAA-specific T cells in an immunostimulatory context that can override tumour-derived suppressive signals.
3.1.2. THE THREE-SIGNAL MODEL OF ANTIGEN PRESENTATION

To modulate T cell activation, it is first necessary to understand the highly regulated process of antigen presentation and T cell activation. DCs are the main professional APCs and when they encounter pathogenic or tumour-associated antigens, DCs mature, and migrate to secondary lymphoid organs (Rescigno, Martino et al. 1998; Gallucci, Lolkema et al. 1999; Ardesna, Pizzey et al. 2000; Fong and Engleman 2000; Lipscomb and Masten 2002; Breckpot, Escors et al. 2010; Goold, Escors et al. 2011). In secondary lymphoid organs DCs present antigen to CD4 or CD8 T cells, depending on whether the antigens are complexed to MHC II or MHC I molecules, respectively. MHC I molecules are complexed to intracellular antigens, such as TAAs or viral antigens. Intracellular antigens are degraded by the proteasome and loaded onto MHC I in the endoplasmic reticulum (ER) before transport to the cell membrane (Fong and Engleman 2000). MHC I molecules are recognized by CD8 T cells, which can be activated to acquire CTL effector functions upon antigen encounter. MHC II molecules are complexed to antigens that are usually (but not always) phagocytosed by APCs and degraded in endosomes, thus generally representing peptides from extracellular pathogens. MHC II molecules are recognized by CD4 T cells and various cytokine signals promote their polarization into various subtypes. Th1 and Th17 cells can stimulate CTL responses, Th2 cells aid in the initiation of humoral (B cell) immune responses, and Treg cells regulate immune responses (Liechtenstein, Dufait et al. 2012). Upon TCR recognition and binding of the peptide-MHC complex an immunological synapse is formed between the two cell types. The immunological synapse allows the APC to deliver signals to the interacting T cell and thus regulate its activation, proliferation, and differentiation (Boisvert, Edmondson et al. 2004; Rothoeft, Balkow et al. 2006; Fooksman, Vardhana et al. 2010; Huppa, Axmann et al. 2010). T cell activation is regulated at multiple levels and can be categorized into three types of signals.

Signal 1 is initiated by the specific binding of the TCR to the peptide-MHC (p-MHC) complex but this is not sufficient for T cell activation. Quite the contrary, TCR triggering alone will lead to T cell anergy, characterized by limited expansion and unresponsiveness upon antigen reencounter (Bachmaier, Krawczyk et al. 2000; Chiang, Kole et al. 2000). Further signals have to be provided in the immunological synapse to
activate T cells. Co-stimulatory signals (Signal 2) are induced by the binding of DC surface ligands with receptors on the T cell surface. Positive co-stimulatory signals are generated by the binding of such surface receptors such as CD80/CD86 on DCs with CD28/CD27 on T cells, leading to proliferation and acquisition of cytotoxic activities (Nurieva, Thomas et al. 2006). But CD80/CD86 can also bind to inhibitory receptor CTLA-4 on T cells, leading to inhibitory signals initiating T cell anergy or Treg differentiation (Fooksman, Vardhana et al. 2010). Another important negative co-stimulatory interaction is initiated by PD-L1 binding to PD-1, which inhibits T cell activities in multiple ways (Latchman, Liang et al. 2004; Liang, Greenwald et al. 2006; Butte, Keir et al. 2007; Karwacz, Bricogne et al. 2011). Thus, the overall activation status of T cells depends on the integration of positive and negative signals induced by surface receptor binding between DC and T cells. Immature and tolerogenic DCs express few co-stimulatory molecules but high levels of inhibitory molecules. Thus TCR recognition of antigens presented by tolerogenic DCs will not lead to T cell activation. Pathogens trigger pathogen pattern recognition receptors such as toll-like receptors (TLRs) on DCs, leading to up-regulation of co-stimulatory molecules and p-MHC complexes (Nurieva, Thomas et al. 2006; Escors, Lopes et al. 2008; Arce, Breckpot et al. 2011).

At this stage, the T cell has recognized the antigen on DCs and has received sufficient positive co-stimulatory signals to be activated. However, T cell activation alone is not sufficient for the T cell to acquire appropriate effector capacities. Additional signals have to be provided in the immunological synapse to polarize T cell differentiation. Signal 3 thus determines the differentiation of the activated T cell through cytokine release, also called cytokine priming (Fig. 3.1.A). Depending on the context in which DCs encounter the pathogenic molecules they secrete different cytokines into the immunological synapse. (Kapsenberg, Hilkens et al. 1999; Curtsinger, Johnson et al. 2003; Curtsinger, Lins et al. 2003). CD4 T cells thereby acquire effector phenotypes and functions (Th1, Th2, Th17, Treg)(Fig. 3.1.A). For Th1 polarization DCs are required to secrete IFNy and IL12 into the immunological synapse. CD4 Th1 cells are crucial for the effective activation of anti-tumour CD8 CTL responses and effective anti-tumour responses therefore need to polarize CD4 T cells to a Th1 type (Macatonia, Hosken et al. 1995; Curtsinger, Schmidt et al. 1999; Schmidt 1999; Albert, Jegathesan et al. 2001; Hernandez, Aung et al. 2002; Schmidt 2002; Curtsinger, Lins et al. 2003).
presence of IL4 and IL10 in the immunological synapse, on the other hand, will lead to Th2 differentiation, polarizing the immune response to a humoral response. IL23, TGFβ, IL17, IL1β, and IL6 may induce Th17 differentiation. Th17 cells express IL17 and trigger strong and rapid pro-inflammatory reactions (Bettelli, Carrier et al. 2006; Sutton, Brereton et al. 2006; McGeachy, Bak-Jensen et al. 2007; Lewkowich, Lajoie et al. 2008; Larsen, Bonefeld et al. 2009; Ortega, Fernandez et al. 2009). Further, Tregs can be induced by secretion of IL10 and TGFβ (O’Garra and Vieira 2004; O’Garra, Vieira et al. 2004; Rutella, Danese et al. 2006; Escors, Lopes et al. 2008; Saraiva and O’Garra 2010; Arce, Breckpot et al. 2011). Appropriate cytokine secretion by APCs is thus crucial for the acquisition of cytolytic effector functions. In the absence of signal 3 T cells acquire a tolerogenic phenotype, unless antigen levels are high (Curtsinger, Lins et al. 2003; Ramanathan, Dubois et al. 2011). Therefore, signal 3 is especially important when antigen levels are low.

It is crucial that cytokine priming is provided in cis in the immunological synapse. To achieve this, direct DC activation is essential (Fig. 3.1.B). Direct DC activation entails the engagement of pattern recognition receptors (such as TLRs) on their surface. TLR ligation ensures the strong activation of DCs and determines the type of cytokines that are secreted into the immunological synapse. Different TLR ligands will result in activation of differential signalling pathways in DCs, thus determining their activation status and cytokine production. TLR4 ligation for example induces DC maturation and IL12 secretion, leading to stimulation of anti-tumour immune responses (Cisco, Abdel-Wahab et al. 2004; Bekeredjian-Ding, Roth et al. 2006; Apetoh, Ghiringhelli et al. 2007; Breckpot and Escors 2009). On the contrary, TLR2 stimulation mainly activates ERK (Extracellularly regulated protein kinase) signalling, prevents DC maturation and stimulates IL10 secretion. TLR2 stimulation will therefore lead to immune suppression rather than activation (Dillon, Agrawal et al. 2006; Manicassamy, Ravindran et al. 2009). DCs can indirectly receive inflammatory signals from other immune cells within the inflammatory tissue. There is increasing evidence suggesting that directly and indirectly activated DCs behave rather differently. While indirectly activated DCs up-regulate surface expression of MHC and co-stimulatory molecules, they can only stimulate T cell proliferation and not acquisition of effector cell functions. Lack of signal 3 provision by the DC in cis prevents effector T cell differentiation (Santini, Lapenta et al. 2000; Sporri

Modulation of the immunological synapse thus represents an attractive strategy to induce desired immune responses. Modulating co-stimulation and cytokine provision can expand CTL responses for cancer or infectious disease treatments or Treg responses for use in patients suffering from autoimmune diseases (Liechtenstein, Dufait et al. 2012).
A

Cytochrome priming
Th1

Signal 3
IFNγ
IL12
IL1β

CD28
CD80/86

MHC II
TCR

Positive co-stimulation
IL4
IL10

Negative co-stimulation
IL23
TGFβ
IL17
IL6

Th2

Th17

Treg

B

CIS CYTOKINE PRIMING

Proliferating Effector T cell

DC

PATHOGEN, DANGER
MOLECULES
TLR

TRANSPORT CYTOKINE
PRIMING

Proliferating Non-effector T cell

DC

CD28
CD80/86

MHC II
TCR
**Fig. 3.1. Three-signal model of antigen presentation in the immunological synapse.** Schemes depict antigen presentation by DCs (left) to CD4 T cells (right). Peptide-MHC complexes interact with TCR of T cell to initiate signal 1. Co-stimulatory (CD80/CD86) or co-inhibitory (CD80/PD-L1) ligands on DCs bind to their receptors on T cells (CD28, CTLA.4, PD-1), representing signal 2. Furthermore, cytokine secretion (Signal 3) by the DC induces T cell differentiation. (A) Cytokine combinations leading to differentiation of several CD4 T helper subtypes are depicted. (B) Scheme depicts indirectly versus directly activated DCs. On top, the directly activated DC provides all 3 signals to the interacting T cell, thus leading to expansion of effector T cells. Below, indirectly activated DC fails to polarize T cells due to a lack of cytokine production within the immunological synapse. Figures reproduced from Liechtenstein et al., *Immunol Endocr Metab Agents Med Chem*, Vol. 12(3), 224-235, 2012.

### 3.1.3. PD-L1 SILENCING FOR CANCER IMMUNOTHERAPY

Considering the fact that spontaneous TAA-specific CTL responses exist and can be re-activated in patients (Novellino, Castelli et al. 2004; Germeau, Ma et al. 2005; Boon, Coulie et al. 2006; Cipponi, Wieers et al. 2011; Mellman, Coukos et al. 2011), targeting T cell re-activating mechanisms represents an attractive strategy for cancer immunotherapy. One way to achieve this would be through modulation of co-stimulation during TAA presentation. Modulation of co-stimulation can be achieved through activation of signalling pathways in DCs (Escors, Lopes et al. 2007; Breckpot and Escors 2009; Arce, Breckpot et al. 2011; Arce, Kochan et al. 2012; Franks, Wang et al. 2013). Activating the appropriate intracellular pathways induces up-regulation of MHC, co-stimulatory, adhesion, and cytokine molecules, thus assuring that T cells receive the appropriate signals in the immunological synapse (Liechtenstein, Dufait et al. 2012). Another way to modulate co-stimulation is to block negative co-stimulatory interactions between tolerogenic DCs and naïve or antigen-experienced T cells. This approach has recently become rather popular under the term immune checkpoint modulators. Blocking of B7 co-stimulatory family molecules such as PD-L1, on DCs or other cells of the tumour microenvironment is of particular interest as their ligation to T cell surface receptors induces their inhibition (Greenwald, Freeman et al. 2005; Hirano, Kaneko et al.
Blocking antibodies against CTLA-4, PD-1, or both on T cells is similarly extensively explored (Hirano, Kaneko et al. 2005; Parry, Chemnitz et al. 2005; Curran, Montalvo et al. 2010).

T cells up-regulate PD-1 upon activation during antigen presentation. PD-L1 (also known as B7-H1) on DCs and other cell types is the ligand of PD-1. PD-L1 is part of the B7 family of co-accessory molecules and is constitutively expressed by various immune cell types including DCs, T cells, B cells, and macrophages (Latchman, Liang et al. 2004). In addition, PD-L1 is expressed by various non-haematopoietic cell types and can be up-regulated in many haematopoietic and non-haematopoietic cells upon IFNγ stimulation (Latchman, Wood et al. 2001; Rodig, Ryan et al. 2003; Blank, Gajewski et al. 2005; Yang, Chen et al. 2008; Yang, Li et al. 2009). Upon PD-L1/PD-1 ligation, Src homology 2 domain (SH2)-containing SHP1 and SHP2 phosphatases are recruited to the cytoplasmic domain of PD-1, thus inhibiting TCR-mediated signalling (Freeman, Long et al. 2000; Chemnitz, Parry et al. 2004; Sheppard, Fitz et al. 2004). PD-L1/PD-1 signalling does not only inhibit T cells but also induces Treg differentiation (Wang, Pino-Lagos et al. 2008; Fife, Pauken et al. 2009; Francisco, Sage et al. 2010). While PD-L1/PD-1 signalling plays an important role during antigen presentation by professional APCs, it can also induce T cell anergy or immune unresponsiveness when expressed on target cells (Keir, Butte et al. 2008; Fife, Pauken et al. 2009). Thus PD-L1/PD-1 is critical for peripheral immune tolerance (Wang, Pino-Lagos et al. 2008; Fife, Pauken et al. 2009; Francisco, Sage et al. 2010). PD-L1 can control peripheral tolerance at two stages of the adaptive immune response. First, PD-L1 can inhibit the activation, expansion, and differentiation of naive self-specific T cells. Second, PD-L1 interaction can inhibit the reactivation, expansion, and function of effector and antigen-experienced T cells (Ansari, Salama et al. 2003; Keir, Butte et al. 2008; Francisco, Sage et al. 2010).

Importantly, PD-L1 is highly expressed in many human and mouse cancers (Latchman, Wood et al. 2001; Blank, Gajewski et al. 2005), including melanoma (Gadiot, Hooijkaas et al. 2011), hepatocellular carcinoma (Gao, Wang et al. 2009), and lymphoma (Andorsky, Yamada et al. 2011; Chen, Chapuy et al. 2013). In certain studies, PD-L1 expression was shown to correlate with poor prognosis, although this remains somewhat controversial and varies between tumour types (Gao, Wang et al. 2009; Gadiot, Hooijkaas et al. 2011). It is clear though that tumours can use PD-L1 to
effectively inhibit anti-tumour immune responses (Blank, Gajewski et al. 2005; Zhang, Gajewski et al. 2009; Samimi, Benoit et al. 2010; Zhou, Munger et al. 2010; Andorsky, Yamada et al. 2011). Interference with PD-L1/PD-1 signalling can therefore be used to enhance co-stimulation and thus improve tumour vaccines. Several approaches can be used to target this signalling pathway. The simplest approach is to systemically administer blocking antibodies to PD-L1 or PD-1 (Hirano, Kaneko et al. 2005; Zhang, Gajewski et al. 2009; Zhou, Xiao et al. 2010). Blocking of PD-L1/PD-1 leads to acquisition of cytotoxic effector T cell functions in vitro (Blank, Kuball et al. 2006; Pilon-Thomas, Mackay et al. 2010) and can lead to a reduction in murine tumour load and prolonged survival (Zhang, Gajewski et al. 2009; Zhou, Munger et al. 2010). Alternatively, silencing PD-L1 by delivery of microRNAs is an approach that leads to more targeted PD-L1/PD-1 blockade, expansion of effector CD8 T cells from patients, and anti-tumour activities in vivo in tumour-bearing mice (Hobo, Maas et al. 2010; Karwacz, Bricogne et al. 2011). PD-L1 blockade or silencing has been shown to prolong the interaction between CD8 T cells and DCs in vitro and in vivo and enhances anti-tumour activities in vivo (Fife, Pauken et al. 2009; Karwacz, Bricogne et al. 2011; Sierro, Donda et al. 2011). Of note, inhibition of PD-L1/PD-1 signalling alone may lead to limited efficacy and is therefore mostly combined with tumour vaccines (Curran, Montalvo et al. 2010; Zhou, Xiao et al. 2010; Karwacz, Bricogne et al. 2011; Sierro, Donda et al. 2011), anti-CTLA4 antibodies (Curran, Montalvo et al. 2010; Mangsbo, Sandin et al. 2010), cytokines (Yu, Steel et al. 2012), chemotherapy (Sierro, Donda et al. 2011), and DC intracellular signalling pathway activators/modulators (Karwacz, Bricogne et al. 2011; Liechtenstein, Dufait et al. 2012).

Nevertheless, clinical trials have been performed using systemic anti-PD-L1 (Brahmer, Tykodi et al. 2012) or PD-1 (Brahmer, Drake et al. 2010; Topalian, Hodi et al. 2012; Ansell, Lesokhin et al. 2015) antibodies as monotherapy. Patients in these clinical trials had a wide range of advanced cancer types, including melanoma, lymphoma, breast, lung, pancreatic, colon, renal, and gastric cancers. But objective responses could only be achieved in patients with non-small-cell lung cancer, melanoma, renal-cell cancer, colorectal cancer, and ovarian cancer. Although these clinical trials showed objective responses with durable tumour regression (between 6-20% of patients) serious immune-related adverse events were observed (in 9-14% of patients) (Brahmer, Tykodi et al. 2012; Topalian, Hodi et al. 2012). A recent and still ongoing clinical trial
using anti-PD-1 antibodies in patients with refractory Hodgkin’s lymphoma reported complete response in 17% of patients while 70% exhibited partial responses. Again, adverse events in patients remain an issue (Ansell, Lesokhin et al. 2015). While these clinical trials achieved promising results in a wide range of unresponsive tumours, they also indicate that localized PD-L1/PD-1 blockade will likely be a safer approach.

Previously, our laboratory has shown that microRNA-mediated PD-L1 silencing in mouse DCs leads to decreased TCR down-regulation in activated T cells upon antigen presentation. This suggests that PD-L1 silencing enhances signal 1 and thus leads to proliferation and hyperactivation of TCR$^{\text{high}}$ CD8 T cells. These hyperactivated CD8 T cells expressed high levels of IFNγ and IL17. Vaccinating mice with PD-L1-silenced DCs inhibited tumour growth and prolonged survival (Karwacz, Bricogne et al. 2011; Escors, Bricogne et al. 2012; Karwacz, Arce et al. 2012). Of note, overall cure rates were not improved in a thymoma mouse model and PD-L1 silencing had to be combined with ERK inhibition or p38 activation in DCs to improve cure rates (Karwacz, Bricogne et al. 2011). Thus, PD-L1 silencing can enhance activation, expansion, and cytokine secretion of tumour-specific T cells but other signals are needed to enhance their cytotoxicity and effector activities. To efficiently induce effector activities in activated CD8 T cells, we hypothesized that a signal 3 (cytokine signal) needs to be supplied. Importantly, this signal has to be provided in cis by the APC itself (Liechtenstein, Dufait et al. 2012).

3.1.4. CYTOKINES USED IN THIS STUDY

The cytokines provided during antigen presentation by DCs determine T cell polarization. Different combinations induce differentiation of various CD4 T helper subtypes. In addition, cytokines are pleiotropic molecules and therefore induce various functions, depending on context and cell target. Thus we selected a wide range of cytokines for this study. These included pro-inflammatory cytokines IFNγ, IL12, IL15, and IL6; anti-inflammatory and Th2 cytokines IL10 and IL4; Treg inducers IL10 and TGFβ; and Th17 inducers IL23, IL17A, and IL6. It is hard to categorize cytokines into groups as they not only induce various functions but can even initiate opposing functions in certain conditions. Thus, it is particularly difficult to use cytokines as monotherapy through
systemic administration. Local delivery in cis to T cells will avoid toxicities and enable targeted application for the enhancement of antigen presentation.

IFNγ, IL12, and IL15 are among the most effective stimulators of anti-tumour immune responses in pre-clinical studies (Waldmann, Dubois et al. 2001; Kishida, Asada et al. 2003; Weiss, Subleski et al. 2007; Zaidi and Merlino 2011). The crucial role of IFNγ in anti-tumour immune responses and in cancer immunosurveillance was already discussed in chapter 1 (Dighe, Richards et al. 1994; Dunn, Old et al. 2004; Schreiber, Old et al. 2011). Briefly, IFNγ is vital for the maturation and function of most immune cell types, activates innate immune cells to acquire cytotoxic activities, polarizes Th1 differentiation, and suppresses Th2 differentiation (Miller, Maher et al. 2009; Zaidi and Merlino 2011). Thus IFNγ represents an attractive candidate to polarize T cell responses with anti-tumour activities. In addition, it displays direct anti-angiogenic, anti-proliferative, and pro-apoptotic effects on tumour cells and enhances tumour cell immunogenicity, detection, and elimination (Miller, Maher et al. 2009; Zaidi and Merlino 2011). Recombinant IFNγ has been used in the clinic but due to its limited efficacy and high toxicity it is not approved as monotherapy (Laszlo, Goldstein et al. 1990; Miller, Maher et al. 2009; Bekisz, Sato et al. 2013). Use of IFNγ in melanoma patients exhibited limited efficacy combined with severe side effects (Zaidi and Merlino 2011). The limited efficacy of IFNγ treatment may seem surprising as anti-tumour effects of IL12 depend on IFNγ and endogenous IFNγ production seems to be necessary for anti-tumour efficacy of cancer immunotherapeutic strategies (Wigginton, Gruys et al. 2001; Weiss, Subleski et al. 2007). But IFNγ does not only play a role in immunoediting/elimination but also in the equilibrium/subversion stages of tumour development (Zaidi and Merlino 2011). Thus, it may not be surprising that systemic administration of IFNγ leads to limited efficacy. Local IFNγ secretion during antigen presentation may have other effects.

IL6 has also been associated with anti-tumour effects in vitro and in pre-clinical models. This cytokine would thus also be a promising candidate for cancer immunotherapy (Revel 1992; Veldhuis, Willemse et al. 1996). IL6 possesses pleiotropic activities and is secreted mainly by DCs, macrophages, B cells, and T cells during immune responses (Kopf, Baumann et al. 1994). IL6 promotes Th2 differentiation and inhibits Th1 differentiation (Rincon, Anguita et al. 1997; Diehl and Rincon 2002) but also plays a role in Th17 differentiation (McGeachy, Bak-Jensen et al. 2007; Zhou, Ivanov et al. 2007).
However, response rates of recombinant human IL6 in cancer patients have been rather low (Veldhuis, Willemse et al. 1996). IL6 is involved in haematopoiesis and thus it may not be surprising that increased MDSC accumulation is associated with IL6 levels in tumour models and patients (Kopf, Baumann et al. 1994; Chen, Kuan et al. 2014). IL6 also inhibits DC differentiation through STAT3 signalling (Park, Nakagawa et al. 2004).

Another attractive cytokine for this study is IL15. IL15 induces NK and activated T cell proliferation, aids in the expansion of effector T cells, and induces expansion of memory CD8 T cells (Fehniger, Cooper et al. 2002; Waldmann 2006). IL15 administration was shown to enhance CD8 T cell anti-tumour activities in pre-clinical models, when combined with other cytokines (such as IL21) (Kishida, Asada et al. 2003), secreted by tumour cells (Hazama, Noma et al. 1999), or in combination with other immunostimulatory treatments (Zhang, Yao et al. 2009). Clinical trials using recombinant IL15 in cancer patients are underway (https://clinicaltrials.gov).

IL23 is secreted by activated DCs (Oppmann, Lesley et al. 2000) and induces the development of Th17 cells (Katstelein, Hunter et al. 2007). In addition, IL23 can stimulate T cell and memory T cell proliferation, IFNγ secretion from activated T cells, and CTL activity. IL23 secreted by tumour cells was shown to enhance splenocyte proliferation, CTL activity, DC numbers, and production of IFNγ, IL12, and TNFα (Shan, Hao et al. 2006). Tumour-secreted IL23 also exhibits anti-tumour effects in several mouse tumour models, including poorly immunogenic B16 melanoma (Oniki, Nagai et al. 2006; Shan, Hao et al. 2006). On the other hand, IL23 had to be combined with a gp100 peptide vaccine to elicit anti-tumour responses (Overwijk, de Visser et al. 2006). Thus, IL23 supplied in cis by activated DCs may enhance anti-tumour immune responses, making it an attractive cytokine for this study.

IL12 is structurally related to IL23 as they share the p40 subunit. IL23 binds to IL12R beta 1 but not to IL12R beta 2 (Oppmann, Lesley et al. 2000). IL12 is a heterodimer of p40 and p35, connected through a disulfide bond. IL12 was initially purified by two different groups (Kobayashi, Fitz et al. 1989) (Stern, Podlaski et al. 1990). As IL12 was found to synergize with IL2 to induce CTLs and augment NK cytotoxicity, it was first called cytotoxic lymphocyte maturation factor and natural killer cell stimulatory factor, respectively (Kobayashi, Fitz et al. 1989; Stern, Podlaski et al. 1990). IL12 is generally viewed as a hallmark pro-inflammatory cytokine and it is mainly produced by activated
DCs, macrophages, and B cells (Hendrzak and Brunda 1996; Trinchieri 1998; Trinchieri 2003). The IL12-R is expressed by tumour and various immune cells including activated T cells, NK cells, DCs, and other myeloid cells such as MDSCs (Airoldi, Di Carlo et al. 2007) (Wu, Warrier et al. 1996; Trinchieri 2003; Pistoia, Cocco et al. 2009; Steding, Wu et al. 2011; Kerkar, Leonardi et al. 2013). IL12 secretion elicits potent innate and adaptive immune responses and it is an important connector of the two responses (Weiss, Subleski et al. 2007; Vignali and Kuchroo 2012). IL12 further augments effector cell functions of NK, CD4, CD8, and NKT cells, thus enhancing their capacity to eliminate tumour cells (Trinchieri 2003). In the context of a strong antigen signal, IL12 secretion leads to T cell proliferation and thus amplifies antigen-specific T cell responses (Bertagnolli, Lin et al. 1992). Recently it has been suggested that IL12 plays a role in the recovery of APC functionality within the tumour microenvironment (Kerkar, Muranski et al. 2010; Kerkar, Goldszmid et al. 2011; Kerkar, Leonardi et al. 2013). Apart from its immunostimulatory effects, IL12 is also involved in direct tumour cytotoxicity and has anti-metastatic and anti-angiogenic effects in various tumour mouse models (Hendrzak and Brunda 1996; Trinchieri 1998; Colombo and Trinchieri 2002; Trinchieri 2003; Airoldi, Di Carlo et al. 2007; Chinnasamy, Yu et al. 2012). It is evident that IL12 administration can have significant adjuvant activities for cancer immunotherapy treatments. Clinical application has been limited as systemic administration is highly toxic (Cohen 1995; Leonard, Sherman et al. 1997; Car, Eng et al. 1999), possibly due to enhanced IFNγ production and toxicity (Leonard, Sherman et al. 1997). Thus, more targeted and local delivery of IL12 is necessary. Several studies have been conducted using either engineered DCs or fibroblasts to locally deliver IL12 to the tumour, delaying tumour growth in vivo through enhanced priming of CD8 T cell responses (Zitvogel, Tahara et al. 1995; Zhao, Bose et al. 2011). In addition, there are several pre-clinical studies using TAA-specific or CAR-engineered T cells with enhanced IL12 expression that show efficacy in tumour mouse models (Kerkar, Muranski et al. 2010; Chmielewski, Kopecky et al. 2011; Chinnasamy, Yu et al. 2012; Pegram, Lee et al. 2012).

IL17A is the hallmark pro-inflammatory cytokine of Th17 cells. IL17A is linked to the development of autoimmunity, inflammation, and cancer but it is also important in anti-bacterial or fungal responses (Iwakura, Ishigame et al. 2011). Even though IL17A is a pro-inflammatory cytokine it exhibits significant pro-tumourigenic activities. In
colorectal cancer IL17A is associated with rapid tumour progression as well as resistance to therapy (Wang, Kim et al. 2014). IL17A exhibits significant tumour-promoting functions in a wide variety of cancer types and promotes angiogenesis and granulopoiesis in pre-clinical tumour models (Zhang, Weng et al. 2014).

While the previously listed cytokines are generally viewed as pro-inflammatory cytokines, we also included IL4, TGFβ, and IL10 in this study as comparative controls. While IFNγ and IL12 are generally viewed as Th1 polarizing cytokines, IL6 and IL4 induce Th2 differentiation (Rincon, Anguita et al. 1997). But IL4 is not only involved in Th2 differentiation (Serre, Mohr et al. 2010). IL4 also exhibits pleiotropic effects on various immune cell types. In addition, it can enhance the immunogenicity of cancer cells and possesses anti-tumour effects in vitro and in pre-clinical mouse models (Hoon, Banez et al. 1991). TGFβ is a highly regulated and pleiotropic cytokine. Its regulatory effects affect many different immune cell types, although T cells seem to be crucial targets. T cell differentiation status plays a role in determining whether TGFβ acts in an immunostimulatory or -suppressive way. TGFβ is involved in the regulation of T cell development, homeostasis, differentiation, and tolerance. Differentiation of Th1 and CTLs can be inhibited by TGFβ, and in combination with other factors TGFβ induces Th17 and inducible Treg responses (Oh and Li 2013). Apart from Treg induction, TGFβ is known to have various effects in cancer progression. Depending on the stage of tumour progression TGFβ plays differential roles. Thus, TGFβ can inhibit cancer cell proliferation and induce apoptosis before tumours are established. Once tumours have developed further, TGFβ promotes cancer cell motility, invasion, and metastasis. (Inman 2011). Thus, TGFβ is generally viewed to contribute to tumour progression and inhibitors are being used in pre-clinical and clinical studies (Rodon, Carducci et al. 2015).

IL10 is another pleiotropic cytokine that is generally viewed to contribute to the immunosuppressive tumour microenvironment, by regulating and terminating inflammation. IL10 is produced by Tregs, MDSCs, and tumour cells, affects growth and differentiation of many immune cell types, and exerts various other effects on most haematopoietic cell types. IL10 affects APCs by inhibiting expression of MHC II molecules, co-stimulatory molecules, and pro-inflammatory cytokines. Further, IL10 can inhibit the in vitro activation of CD4 T cells and macrophages (Moore, de Waal-Malefyt et al. 2001). Depending on their activation status, IL10 exhibits differential effects on
CD8 T cells and can enhance their cytotoxic potential (Santin, Hermonat et al. 2000). Similar to TGFβ, IL10 exhibits both tumour-promoting (Fuji, Shimizu et al. 2001) and tumour growth-inhibiting (Dorsey, Kundu et al. 2002) activities in mouse tumour models. In addition, the timing of IL10 administration is highly important as it can impair vaccination when simultaneously administered or enhance vaccination when given at a later time-point (Fuji, Shimizu et al. 2001). Although IL10 is normally viewed as an immunosuppressive cytokine, it has recently been shown to induce IFNγ-mediated CD8 T cell cytotoxicity in tumour-specific and tumour-resident CD8 T cells (Emmerich, Mumm et al. 2012), thus making it an intriguing cytokine to include in this study.

In summary, we included in this study a wide range of cytokines, including Th1, Th2, Th17, and Treg-promoting cytokines. As cytokines are highly pleiotropic their activity is context-dependent and thus of interest to study in the context of tumour vaccination and therapy. As all three signals need to be provided in cis for optimal T cell activation and differentiation, I hypothesized that lentivector transduction providing all three signals at the same time could enhance tumour-specific T cell responses. Furthermore, I propose that local PD-L1 silencing by direct lentivector transduction only occurs in a limited number of target cells and thus reduces toxicities observed following systemic PD-L1 blockade. In this way, PD-L1/PD-1 signalling is inhibited during antigen presentation and thereby enhances the potential of cytokines to steer TAA-specific T cell differentiation. Secondly, local PD-L1 silencing in the tumour environment could avoid expansion of auto-reactive regulatory T cells and overcome CTL unresponsiveness and exhaustion (Liechtenstein, Dufait et al. 2012; Liechtenstein, Dufait et al. 2012).
<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Inflammatory effect</th>
<th>Effect on immune cells</th>
<th>Effect on cancer cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNγ</td>
<td>Pro-inflammatory</td>
<td>Th1, CTL, MDSC, NK, Macrophages, Granulocytes</td>
<td>Pro-apoptotic Anti-proliferative Anti-angiogenic Pro-immunogenic</td>
</tr>
<tr>
<td>IL12</td>
<td>Pro-inflammatory</td>
<td>NK, NKT, Th1, CTL, DC, macrophages</td>
<td>Cytotoxic Anti-angiogenic Anti-metastatic</td>
</tr>
<tr>
<td>IL15</td>
<td>Pro-inflammatory</td>
<td>NK, effector T, memory T</td>
<td>Anti-tumourigenic</td>
</tr>
<tr>
<td>IL23</td>
<td>Pro-inflammatory</td>
<td>Th17, effector T, memory T</td>
<td>Anti-tumourigenic</td>
</tr>
<tr>
<td>IL17A</td>
<td>Pro-inflammatory</td>
<td>Th17, granulopoiesis</td>
<td>Pro-angiogenic Pro-tumourigenic</td>
</tr>
<tr>
<td>IL6</td>
<td>Pro-inflammatory</td>
<td>Th2, Th17, haematopoiesis</td>
<td>Cytotoxic</td>
</tr>
<tr>
<td>IL4</td>
<td>Pleiotropic</td>
<td>Th2, various</td>
<td>Pro-immunogenic Anti-proliferative</td>
</tr>
<tr>
<td>IL10</td>
<td>Pleiotropic</td>
<td>DCs, macrophages, CD4 and CD8 T cell</td>
<td>Pro-tumourigenic Anti-tumourigenic</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Pleiotropic</td>
<td>Mainly T cells</td>
<td>Pro-apoptotic Anti-proliferative Pro-metastatic Pro-angiogenic</td>
</tr>
</tbody>
</table>

**Table 3.1. Summary of Effects of cytokines used in this study.** Of note, these cytokines have many more effects on immune cells and cancer cells and this should be viewed as a rough guide to their functions.
3.1.5. AIMS OF CHAPTER

In this chapter lentivectors for the use in melanoma immunotherapy were designed and tested. These lentivectors co-expressed an antigen, a T cell-polarizing cytokine array, as well as a PD-L1-silencing short hairpin RNA (shRNA) within a microRNA 30 context. OVA-specific T cell responses were first evaluated in vitro by modifying DCs using lentivectors containing the model antigen OVA. These lentivectors were also used to assess OVA-specific T cell responses in vivo in healthy mice and anti-tumour activities in a B16 mouse model, which expressed the xenoantigen OVA.

To work in a more realistic setting, the model antigen OVA was replaced with a poorly immunogenic endogenous melanoma TAA, TRP1. Anti-TRP1 T cell responses were tested in healthy mice as well as tumour growth in prophylactic and therapeutic settings. For these tumour experiments autologous syngeneic B16 melanoma cells were used, which did not express model antigens. Further, mechanistic studies assessing the effect of the cytokines versus PD-L1 silencing and the role of transduction at the vaccination site were performed.
3.2. RESULTS

3.2.1. ENGINEERING OF LENTIVECTORS TO SIMULTANEOUSLY DELIVER AN ANTIGEN, A PD-L1 SILENCING microRNA, AND T CELL POLARIZING CYTOKINES

Previous results of our laboratory demonstrated that PD-L1 silencing in transferred OVA-specific DCs was not enough to confer potent anti-tumour activities in a thymoma mouse model (Karwacz, Bricogne et al. 2011; Karwacz, Arce et al. 2012). Since PD-L1 silencing hyperactivated OVA-specific CD8 T cell responses, we reasoned that further signals such as adequate cytokine priming, would improve anti-tumour effector activities of these CD8 T cells (Karwacz, Arce et al. 2012; Liechtenstein, Dufait et al. 2012; Liechtenstein, Dufait et al. 2012). Therefore engineered lentivectors that would not only silence PD-L1 by including a shRNA within a microRNA30 context sequence (termed p1 henceforth), but also induce expression of an antigen of interest and a selected collection of cytokines in target cells. The array of mouse cytokine genes and an enhanced p1 version were expressed under the control of the spleen focus-forming virus (SFFV) promoter. IL12 and IL23 are heterodimeric proteins and hence a linker sequence was introduced in the coding sequence between their two constituent chains, as described in chapter 2. Dual lentivector plasmids used to deliver the constructs were previously used in our laboratory (Arce, Breckpot et al. 2011; Karwacz, Bricogne et al. 2011). The second transgene was expressed under the control of the ubiquitin (UBI) promoter (Fig.3.2.A).

To elucidate which cytokine induced the most potent anti-tumour activities in combination with PD-L1 silencing, we engineered plasmids containing cytokines with a wide range of properties and functions (Table 3.1). To test whether the inserted genes were indeed expressed from the lentivector constructs I first included the cytokines, p1, as well as the reporter gene green fluorescent protein (GFP) into the lentivector backbone (Fig. 3.2.A). I tested PD-L1 silencing activity of p1 in B16 mouse melanoma and in bone marrow-derived DCs (BM-DCs). To ensure high baseline levels of PD-L1 expression, B16 cells and BM-DCs were treated with IFNγ and LPS, respectively, as these treatments enhance PD-L1 expression. Transduction with p1-containing lentivectors decreased PD-L1 expression in both cell types (Fig. 3.2.A). Furthermore, cytokine expression was measured by transducing 293T cells with the lentivectors and
performing flow cytometric analyses after intracellular staining using cytokine-specific antibodies conjugated to fluorochromes. In the case of IL15, a western blot of transduced 293T cells was performed (Fig. 3.2.B-C).

![Diagram](image)

**Fig. 3.2. Assessment of lentivector constructs expressing a PD-L1-targeting microRNA, cytokines, and an antigen of interest.** (A) Above: Schematic representation of the lentivector constructs co-expressing the reporter gene green fluorescent protein (GFP), cytokine genes, and a PD-L1-targeting microRNA (p1). Below: Flow cytometry histograms representing PD-L1 expression in B16 cells (left) and bone marrow-derived DCs (BM DC, right). Cells were transduced with lentivectors co-expressing p1 and GFP or GFP alone as a control. PD-L1 expression was ensured by previously treating B16 cells with IFNγ and BM-DCs with LPS. Percentages and mean fluorescent intensities (MFI) are indicated.
Horizontal lines within the histograms were drawn to include no more than 5% of non-transduced (GFP-) cells. LTR, long-terminal repeat; SFFVp, spleen focus-forming virus promoter; UBIp, ubiquitin promoter; SIN, self-inactivating LTR. (B) Flow cytometry density plots of 293T cells transduced with the indicated cytokine-containing lentivectors. Cytokine-specific antibodies were used for intracellular staining. Horizontal lines exclude 95% of non-transduced cells and percentages of cytokine-expressing cells are shown. (C) Western blot of 293T cells transduced with a lentivector encoding IL15. Golgi Plug was added overnight to ensure accumulation of IL15 prior to protein extraction. UT, untransduced 293T cells.

3.2.2. TRANSDUCTION OF BM-DCs IN VITRO WITH LENTIVECTORS MODULATING ANTIGEN PRESENTATION DOES NOT SIGNIFICANTLY UP-REGULATE MATURATION MARKERS.

The purpose of the engineered lentivector constructs was to modulate antigen presentation to T cells, polarizing T cell differentiation towards a cytotoxic effector type. Initially the main target cell types for the lentivector vaccines were APCs, especially DCs. Hence, before analysing T cell responses to transduced APCs, I investigated whether lentivector transduction with the constructs would affect the phenotype of immature BM-DCs themselves. For these experiments the GFP-containing constructs were used, which would allow the assessment of marker expression in transduced cells by flow cytometry in GFP+ DCs. Previous data from our laboratory indicated that PD-L1 silencing slightly increased maturation of BM-DCs (experiments previously performed by Dr. Kasia Karwacz, data not shown). The p1-GFP lentivector constructs containing the pro-inflammatory cytokines IFNγ, IL6, IL12, IL17, and IL23 as well as the immunosuppressive cytokine IL10 were selected for testing. To assess maturation, the expression of markers CD80, ICAM-I, MHC I, MHC II, and CD86 were tested. With the exception of the IFNγ-p1-GFP lentivector, which up-regulated ICAM-I and MHC II expression, none of the tested lentivector constructs significantly affected the expression of the markers analysed (Fig. 3.3.). These results are consistent with the well-known capacities of IFNγ to up-regulate MHC II.
The ultimate goal in this study is to modulate antigen presentation to T cells using lentivectors expressing cytokines in combination with PD-L1 silencing. It may therefore not be crucial to induce full DC maturation by transduction itself. The effect the lentivectors convey on T cell polarization activities will therefore be primarily caused by cytokine priming and PD-L1 silencing.

**Fig. 3.3. Assessment of maturation phenotype of BM-DCs transduced with lentivectors expressing different cytokines, p1, and GFP.** DCs differentiated ex vivo from BM were transduced with lentivectors at day 5 of culture. The lentivector containing only GFP served as a control. After 72 hours transduced cells were stained and analyzed by flow cytometry. Bar graphs represent percentages of marker-expressing cells within the transduced (GFP+) population, normalized with the percentages from GFP-transduced control cells. Pooled data from two independent experiments. P-values were calculated by an unpaired student t-test between cyt-p1-GFP values and GFP only control values.
3.2.3. TRANSDUCTION OF BM-DCs WITH CONSTRUCTED LENTIVECTORS INDUCES OVA-SPECIFIC CD8 T CELL RESPONSES IN VITRO

As stimulation of antigen-specific effector T cell responses is crucial in inducing anti-tumour immune responses, we further tested whether transduced BM-DCs would efficiently stimulate antigen-specific T cell responses. To measure antigen-specific responses in vitro I used the model antigen ovalbumin (OVA). The reporter gene GFP was replaced in the lentivector plasmids with liOVA. To facilitate MHC class II OVA peptide epitope presentation the fusion gene between the N-terminal half of the invariant chain (ii) with HA-tagged OVA (liOVA) was used (Karwacz, Bricogne et al. 2011) (Fig. 3.4.A). In addition, a lentivector containing only GFP and liOVA was constructed to serve as a control for the following experiments (Fig. 3.4.A). Using HA-specific antibodies the expression of OVA in transduced cells could be easily tested by western blot (Fig. 3.4.B). After transduction of BM-DCs with the liOVA-expressing constructs, these cells were co-cultured with CD8 T cells from OVA-specific transgenic OT-I mice. Interestingly, only the combination of IL12 expression and PD-L1 silencing elicited a strong OVA-specific CD8 T cell response, in comparison with IFNγ levels elicited by the transduction of GFP-liOVA-expressing lentivectors (Fig. 3.4.C). Of note, transduction with IFNγ- and IL17A-containing lentivectors was toxic to target cells (data not shown).
Fig. 3.4. **BM-DCs transduced with lentivectors expressing the model antigen liOVAha induce OVA-specific T cell responses in vitro.** (A) Above: Schematic representation of the lentivector construct, containing the model antigen OVA fused to the invariant chain (liOVA). Below: Western Blot of 293T cells transduced with the liOVA-containing lentivectors, using an antibody against the haemagglutinin (HA)-tag of liOVA. Anti-GADPH antibody was used as loading control. LTR, long-terminal repeat; SFFVp, spleen focus-forming virus promoter; UBIp, ubiquitin promoter; SIN, self-inactivating LTR. (B) Transduced BM-DCs were co-cultured with purified CD8 T cells from transgenic OT-I mice for 72 hours. OVA-specific responses were assessed by IFNγ expression in CD8 T cells via flow cytometry.
3.2.4. DIRECT LENTIVECTOR VACCINATION OF HEALTHY MICE INDUCES OVA-SPECIFIC T CELL RESPONSES IN VIVO.

Following in vitro studies, we assessed the effect of our lentivector constructs in vivo. Mice were subcutaneously (s.c.) vaccinated at the base of the tail with the lentivectors containing the different cytokines, the microRNA p1, as well as the model antigen liOVA (Fig. 3.4.A). Two weeks later splenocytes were isolated and stimulated overnight with OVA peptides before analysis by flow cytometry. Compared to vaccination with a non-cytokine containing GFP-control, increased OVA-specific CD8 T cell responses were observed using various lentivector vaccines (Fig. 3.5.A). CD8 T cells were induced to produce IFNγ by the lentivector constructs containing the IFNγ, IL23, IL12, and IL15 genes. IL17 production by CD8 T cells was enhanced by IFNγ- and IL23-containing lentivectors. The most effective lentivector vaccine was the IFNγ-expressing lentivector, as vaccinated mice showed splenocytes containing CD8 T cells with enhanced IFNγ and granzyme B production. Interestingly, all lentivector vaccines exhibited low Foxp3+ regulatory T cell (Treg) numbers in splenocytes after OVA restimulation compared to the GFP-liOVA vaccination control. Importantly, Treg numbers also decreased when cytokines were included in the vectors that are known Treg inducers, such as TGFβ (Fig. 3.5.A). Thus, PD-L1 silencing in healthy mice may override TGFβ- and IL10-mediated signals. Although IFNγ-based lentivectors were the most effective in raising IFNγ and granzyme B responses, they did not decrease Treg responses.

Further, the lentivector vaccines expressing IFNγ and IL10, with and without p1, were selected for vaccination of healthy mice. In this way the effect that PD-L1 silencing exerts in the context of pro-inflammatory and anti-inflammatory cytokine induction could be studied. Interestingly, in both contexts PD-L1 silencing enhanced the percentage of CD8 T cells expressing IFNγ and GzmB (Fig. 3.5.B).
Fig. 3.5. Subcutaneous vaccination with lentivectors expressing cytokines, a PD-L1-targeted microRNA, and the model antigen iIOVA induces OVA-specific T cell responses in mice. (A) Mice were injected subcutaneously (s.c.) at the base of the tail with indicated lentivectors. Two weeks later splenocytes were isolated and stimulated overnight with OVA peptides. Analysis was performed by flow cytometry. 5 mice per group were used and pooled into two groups for in vitro analyses. Bar graphs represent number of marker positive CD8 splenocytes. Pooled data from two independent experiments. NP, no OVA.
peptide negative control. (B) Same as in A, but experiment performed only once. Bar graphs represent percentage of marker positive CD8 splenocytes. no, no OVA peptide as negative control.

3.2.5. PROPHYLACTIC VACCINATION WITH LENTIVECTORS CO-DELIVERING CYTOKINES, p1 AND THE MODEL ANTIGEN iIova DELAYS TUMOUR GROWTH IN A B16-iIova MOUSE MELANOMA MODEL

To further assess the lentivector constructs co-expressing cytokines, p1, and an antigen, their efficacy in a mouse melanoma model was tested. For preliminary analyses I used the lentivectors containing the model antigen liOVA and non-commercial B16-liOVA cells that express OVA as a surrogate tumour antigen. For this purpose, B16 cells were transduced with a lentivector encoding the OVA gene fused to li (liOVA) and the puromycin resistance cassette. Following transduction B16-liOVA cells were selected by puromycin treatment (Fig. 3.6.A). A similar approach with OVA-B16 tumour cell lines is frequently used to assess cancer immunotherapy treatments as it ensures strong antigen recognition in vivo (Escors, Lopes et al. 2008; Karwacz, Mukherjee et al. 2009; Fraser, Lousberg et al. 2010; Aranda, Llopiz et al. 2011; Arce, Breckpot et al. 2011; Karwacz, Bricogne et al. 2011; Bouwer, Saunderson et al. 2014). Mice were s.c. injected at the base of the tail with a selection of lentivector constructs, including IL12-p1-liOVA and IFNγ-p1-liOVA. Two weeks after prophylactic vaccination with lentivectors, mice were s.c. challenged with B16-liOVA melanoma cells. In this experimental setting delayed tumour growth and prolonged survival was achieved in all vaccinated groups, compared to unvaccinated control. Even GFP-liOVA-vaccinated mice displayed delayed tumour growth (Fig. 3.6.B). Of note, both GFP and liOVA are not endogenous proteins and could therefore have synergistic adjuvant effects on the host immune system. Nonetheless tumours from vaccinated mice eventually escaped. After sacrificing mice with large tumour burden, tumours were harvested and it was found that tumour tissue from certain vaccination groups had lost melanine expression (Fig. 3.6.C). Mice that retained melanine-positive tumours were unvaccinated mice as well as the IFNγ- and IL10-containing lentivector-vaccinated mice (indicated with 1 in Fig. 3.6.B). On the other hand, tumours from mice vaccinated with the lentivectors expressing GFP, IL12 and IL15,
had lost melanine expression (indicated with 2 in Fig. 3.6.B). To further analyse tumour tissue, protein was extracted and liOVA and common endogenous melanoma TAA expression was analysed. liOVA expression was lost in almost all tumours from vaccinated mice while it was still expressed in tumours from unvaccinated mice (Fig. 3.6.D). As B16-liOVA cells were selected by puromycin treatment without further clonogenic selection, B16-liOVA cells expressed various levels of liOVA. Thus, B16 cells expressing no or only low liOVA were selected within the tumour. Importantly, some of the tumours from IL12-p1-liOVA-vaccinated mice had lost Trp2 and gp100 expression, while Trp1 was still expressed in all of these tumours (Fig. 3.6.E). This data is relevant as Trp1, Trp2 and gp100 are endogenous melanoma TAAs.
A

![Image of Western blot](image)

- B16
- B16-iiOVA

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B

**Tumour size on day 18**

- Unv
- GFP
- IL12
- IFN
- IL10
- IL15

Lentivector combinations-iiOVA

- p1
- p1
- p1
- p1

![Bar graph of tumour size](image)

- Mean ± SD
- P<0.001***

**Survival from prophylactic treatment**

- Unv
- GFP
- IL12
- IFN
- IL10
- IL15

Lentivector combinations-iiOVA

- p1
- p1
- p1
- p1

![Survival graph](image)

- Day of death after challenge

C

**Unvaccinated**

- T1
- T2
- T3
- T4
- T5

**IL12-p1-iiOVA**

- T1
- T2
- T3
- T4
- T5

![Images of tumours](image)
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**HA**

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**HA**

**GADPH**

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**Tyrosinase**

**TRP1**

**TRP2**

**GP100**
**Fig. 3.6. Prophylactic vaccination of mice with liOVA-containing lentivectors in a B16-liOVA mouse melanoma model.** (A) Western Blot analysis of B16 cells transduced to express liOVA protein, using anti-HA antibody. Anti-GADPH antibody was used as loading control. (B-D) Mice were injected s.c. at the base of the tail with lentivectors. Two weeks later B16-liOVA cells were transferred and tumour growth and survival were monitored. (B) Graphs representing tumour size on day 18 (when the first unvaccinated mouse had to be sacrificed due to high tumour burden) (left) and time of death (right) after B16-liOVA challenge. Experiments were performed with 5 mice per group. 1 indicates melanine rich tumours, 2 indicates melanine poor tumours. (C) Tumour tissue was harvested when a volume of 150\(\text{mm}^2\) was reached. Only photographed tumour samples from unvaccinated and IL12-p1-liOVA vaccinated mice are represented here as examples. Samples from escaping tumours from each mouse are labelled T1-T5. (D) Western Blot analyses of liOVA and GADPH expression in tumour tissue samples from each mouse. Each tumour was analyzed separately (T1-T5). B16-liOVA cells and tumour tissue from an unvaccinated mouse (Unv T1) were used as OVA-positive controls. (E) Same as in D, but protein extracts were analysed with antibodies against indicated endogenous melanoma TAA's. Shown are the tumour samples analysed from unvaccinated and IL12-p1-liOVA vaccinated mice.
3.2.6. PROPHYLACTIC VACCINATION WITH LENTIVECTORS CO-DELIVERING IL12, p1 AND ENDOGENOUS MELANOMA ANTIGEN TRP1 ENHANCES SURVIVAL IN A B16 MELANOMA MODEL WHICH DOES NOT EXPRESS THE XENOANTIGEN OVA

As all liOVA-containing lentivector vaccines delayed B16-liOVA tumour growth, we sought to establish a more realistic setting in which to test the lentivector vaccines. Using OVA, which is an immunogenic xenoantigen, might have masked the anti-tumour activities of cytokine induction and PD-L1 silencing. liOVA was therefore replaced in selected lentivector constructs with the endogenous melanoma TAA TRP1. Lentivector vaccination with wildtype TRP1 antigen does not lead to protective or therapeutic activities (Guevara-Patino, Engelhorn et al. 2006; Liu, Peng et al. 2009) and would therefore not mask adjuvant activities as I suspected OVA had (Fig. 3.6.B). In addition, TRP1 expression was still intact in escaping tumours from mice vaccinated with our lentivectors. This indicated that TRP1 is not an immunogenic TAA in the B16 melanoma mouse model for which non-expressing variants can easily escape (Fig. 3.6.E). The TRP1-containing lentivectors were tested using unmodified B16 mouse melanoma cells, which did not express the xenoantigen OVA. To be consistent with our previous experiments, full-length HA-tagged TRP1 was fused to the N-terminal half of the MHC II invariant chain (II) (Fig. 3.7.A). Mice were s.c. vaccinated with the liTRP1-containing lentivectors, and B16 melanoma cells were transferred two weeks later to vaccinated mice. In this experimental setting only vaccination with IL12-p1-liTRP1 strongly delayed tumour growth and significantly increased survival (Figs. 3.7.B-D). In the first experiment I vaccinated mice with the liTRP1-lentivectors containing IFNy-p1, IL15-p1, IL12-p1, IL10p1, and TGFβ-p1 (Figs. 3.7.B-D). The combination of IL12 expression and PD-L1 silencing showed the best anti-tumour responses, in which one mouse never developed a tumour after regression (Fig. 3.7.B,C). Therefore a second experiment was performed where only liTRP1-lentivectors containing IL12-p1, IFNy-p1, and IL10-p1 were used for vaccination. In addition, IL12-liTRP1 (without the PD-L1 silencing microRNA) was included in this experiment. Similar results were achieved as in the first experiment, confirming the enhanced anti-tumour activity of the IL12-p1 lentivectors. Vaccination with IL12-liTRP1 delayed tumour growth, albeit not to the same extent as IL12-p1-
IiTRP1. This data suggested synergistic effects when combining IL12 expression and PD-L1 silencing (Fig. 3.7.E).

Recall responses were tested of the survivor mouse vaccinated with IL12-p1-IiTRP1 (Fig. 3.7.B,C). The survivor and a control mouse were inoculated with B16-IiOVA cells five months after lentivector vaccination. No tumour growth occurred (Fig. 3.7.F). This data suggested that the survivor mouse was protected.
A

Cytokine-p1-liTRP1

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Cytokine-p1-liTRP1

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B

Number of mice

P=0.001**

Days after B16 subcutaneous injection

C

Tumour size on day 20

Survival after prophylactic treatment

P=0.001 * *

Lentivirus combinations-liTRP1

Lentivirus combinations-liTRP1
**Fig. 3.7. Prophylactic vaccination of mice with iTRP1-containing lentivectors increases survival in a B16 mouse melanoma model.** (A) Western Blot analysis of 293T cells transduced with indicated iTRP1-containing lentivectors, using anti-HA antibody and anti-GADPH antibody as loading control. (B-D) C57/Bl6 mice were injected s.c. at the base of the tail with lentivectors. Two weeks later B16 cells were transferred and tumour growth and survival were monitored. 5 mice per group. (B) Kaplan-Meier survival plot of mice vaccinated with indicated lentivectors. (C) Left: Graphs represent tumor size on day 20 after B16 inoculation. Right: Time of death after B16 transfer. (D) Graph represents growth of tumours in mice vaccinated with the indicated lentivectors. (E) Repetition of previous experiment (B-D) but with inclusion of IL12-iTRP1 (without p1) as control. Graphs represent tumour sizes on day 13 after B16 inoculation and survival of mice vaccinated with indicated lentivectors. (F) Inoculation of survivor mouse from B and a control mouse with B16-liOVA melanoma cells 5 months after lentivector vaccination of survivor mouse. Tumour size on day 10 after s.c. transfer of \(5 \times 10^6\) B16 cells.
3.2.7. THERAPEUTIC INTRA-TUMOUR VACCINATION WITH IL12-p1-IiTRP1 CONTROLS TUMOUR GROWTH IN A B16 MELANOMA MODEL

IL12-p1-containing lentivectors elicited promising results in prophylactic therapy, particularly when considering that animals were only vaccinated once prior to tumour inoculation. I further assessed whether these lentivector vaccines would have therapeutic efficacy, in which tumours are first allowed to grow followed by lentivector inoculation. Mice were intra-tumourally injected with lentivectors GFP-IiTrp1, IL12-IiTrp1, IL12-p1-IiTrp1 or PBS (12 days after B16 transfer). Single lentivector injections were performed in rapidly growing tumours when a mean tumour volume between 50 and 200 mm$^3$ was reached. As in the prophylactic experiments, IL12-IiTrp1 or IL12-p1-IiTrp1 lentivector injection significantly delayed tumour growth and prolonged survival (Fig. 3.8.A-D). These results suggested that intra-tumour injection with IL12-expressing lentivectors increased survival compared to control PBS or GFP-IiTRP1 injections.
Fig. 3.8. Intra-tumour vaccination of mice with IL12-p1-IiTRP1 lentivectors increases survival in a B16 mouse melanoma model. (A-D) $3 \times 10^5$ B16 cells were subcutaneously injected into C57Bl/6 mice ($n = \min 5$ mice per group). When tumours reached mean tumour volume between 50-200mm$^3$ (12 days later), mice were injected intra-tumourally with the indicated lentivectors (1*10$^7$ lentivector particles/tumour) or PBS as a control. (A) Kaplan-Meier survival plot representing pooled data from two independent experiments. (B) Time of death after B16 inoculation. Pooled data from 2 independent experiments are plotted. (C) Tumour volumes are represented on the day of intra-tumour lentivector injection (day 12) and 5 days later (day 17). One of two similar experiments represented. (D) Tumour growth of individual mice, vaccinated with control lentivector GFP-IiTRP1 or IL12-IiTRP1 (above) and IL12-p1-IiTRP1 (below) lentivectors. One of two similar experiments represented.
3.2.8. **IL12-(p1)-lLTRP1 LENTIVECTORS INDUCE TRP1-SPECIFIC CD4 AND CD8 T CELL RESPONSES *IN VIVO* IN HEALTHY MICE.**

My results suggested that the inclusion of the PD-L1-silencing microRNA p1 in the IL12-lLTRP1 lentivector vaccines played a more important role in the prophylactic setting than in therapeutic experiments. Thus, further experiments to elucidate the mechanism exerted by the IL12- and p1-containing lentivector vaccines were performed. First, I tested whether the IL12-lLTRP1-based lentivector vaccines could indeed elicit *in vivo* expansion of IFNγ+ TRP1-specific T cells in healthy mice and whether inclusion of p1 would enhance T cell activation. Breaking natural tolerance to the endogenous TAA TRP1 is particularly challenging (Guevara-Patino, Engelhorn et al. 2006; Liu, Peng et al. 2009)(Fig. 3.6.E). It would therefore be an important achievement to raise TRP1-specific Th1 and CTL responses with a single lentivector vaccination of healthy mice. To this purpose mice were s.c. injected with IL12-lLTRP1 and IL-12-p1-lLTRP1 lentivectors. Two weeks later, isolated splenocytes were co-cultured overnight with TRP1-presenting BM-DCs (by lentivector transduction with GFP-lLTRP1) and the expression of IFNγ in T cells was assessed by flow cytometry. To ensure that TRP1-presenting BM-DCs were activated for this assay, immature transduced BM-DCs were incubated with lipopolysaccharide (LPS) prior to co-culture. A single vaccination with the IL12-containing lentivectors induced systemic TRP1-specific CD8 and CD4 T cell responses (Fig. 3.9). Inclusion of the microRNA p1 in the IL12-lLTRP1 lentivector vaccines did not increase TRP1-specific T cell responses in vaccinated mice. This suggested that in combination with IL12, PD-L1 silencing may not play a crucial role in enhancing MHC I and MHC II TRP1-specific T cell responses at the priming stage. Alternatively, our assay may not have sufficient sensitivity to discriminate any enhancing effect by p1. Mice were also vaccinated with control GFP and GFP-lLTRP1 lentivectors, which did not elicit significant TRP1-specific CD8 or CD4 T cell responses (Fig. 3.9). In addition, vaccination with IL10-p1-lLTRP1 did not induce TRP1-specific responses.
Fig. 3.9. Single injection of healthy mice with IL-12-(p1-)iTRP1 lentivectors induces TRP1-specific CD8 and CD4 T cell responses. C57Bl/6 mice were s.c. injected with lentivectors GFP, GFP-iTRP1, IL12-iTRP1, IL12-p1-iTRP1, and IL10-p1-iTRP1 (1*10^7 lentivector particles per mouse, n = 2-3 mice per group). Two weeks later splenocytes were isolated and cultured overnight with DCs that had been previously transduced with GFP-iTRP1 lentivectors and activated with LPS. Splenocytes were stained with anti-CD4, CD8, and IFNy antibodies for flow cytometric analyses. Bar graphs represent pooled data from two independent experiments.
3.2.9. PD-L1 SILENCING IN NON-HAEMATOPOIETIC CELLS AT THE INJECTION SITE AMPLIFIES T CELL NUMBERS AND COOPERATES WITH CYTOKINE PRIMING

Subcutaneous delivery of several cytokines (namely IFNγ, IL23, IL12, and IL15) and the PD-L1 silencing microRNA p1 enhanced OVA-specific CD8 T cell responses in vivo in healthy mice (Fig. 3.5). Additionally, expression of IL12 alone was sufficient to break natural tolerance to TRP1 (Fig. 3.9). Subcutaneous vaccination with lentivectors transduces conventional DCs at the injection site. Transduced DCs then migrate to lymph nodes where they induce T cell responses (Goold, Escors et al. 2011). DCs are however not the only cell type transduced by lentivectors at the injection site (Esslinger, Chapatte et al. 2003; Breckpot, Escors et al. 2010; Arce, Breckpot et al. 2011). I therefore characterized the role cytokine expression and PD-L1 silencing in non-professional APCs would play in the overall induction of T cell responses. To specifically exclude cells derived from the haematopoietic system from the target cell population, the target sequence for the haematopoietic endogenous microRNA 142 3p was included in the lentivector constructs (Fig. 3.10.A). By including the microRNA 142 3p target sequence, transgene expression is abrogated in cells from the haematopoietic lineage due to their expression of microRNA 142 3p. Thus, myeloid cells such as DCs, Langerhans cells, and macrophages as well as lymphocytes will not express transgenes that contain the microRNA 142 3p target sequence (Brown, Sitia et al. 2006; Brown, Gentner et al. 2007; Annoni, Brown et al. 2009; Gentner, Visigalli et al. 2010; Goudy, Annoni et al. 2011; Matsui, Hegadorn et al. 2011).

To test its efficacy, the microRNA 142 3p target was introduced into the GFP-liOVA lentivector backbone (GFP-liOVA142 3p , Fig. 3.10.A). After transduction of BM-DCs, GFP as well as OVA expression was abrogated (Fig. 3.10.A). S.c. vaccination of mice with GFP-liOVA142 3p lentivectors showed that OVA-specific CD8 T cell responses were strongly reduced while CD4 T cell responses were completely abrogated (Fig. 3.10.A). In this context, and as previously shown by others, liOVA expression from haematopoietic cells was needed to induce antigen-specific T cell responses (Brown, Venneri et al. 2006). We further introduced the microRNA 142 3p target into the lentivector construct containing the cytokine IL12, p1 and liOVA (IL12-p1-liOVA142 3p) (Fig. 3.10.B-C). The lentivector construct containing IL12 was selected for this experiment as the IL12-p1-based
lentivector was the highest inducer of OVA-specific CD8 T cell responses \textit{in vitro} and exhibited potent anti-tumour efficacy \textit{in vivo}. In addition to the constructs containing the microRNA 142 3p target, control constructs without the microRNA p1 (IL12-liOVA and IL12-Δp1-liOVA\textsubscript{142 3p}) were s.c. injected into healthy mice. IL12-liOVA would allow expression of IL12 and liOVA in all target cells while IL12-Δp1-liOVA\textsubscript{142 3p} would allow IL12 and liOVA expression only in non-haematopoietic cells (Table 3.2). Two weeks later, splenocytes were isolated and re-stimulated with OVA peptide before analysis. OVA-specific responses were assessed by IFNγ expression of CD8 T cells.

Vaccination with IL12-liOVA and IL12-p1-liOVA constructs induced similar percentages of OVA-specific CD8 T cells, representing similar “specificity” of the response. The introduction of the 142 3p target in IL-12-p1-liOVA, abrogating transgene expression in cells of the haematopoietic lineage at the injection site, led to significantly lower percentages of OVA-specific CD8 T cell responses (Fig. 3.10.B). Removal of p1 (Δp1) from this 142 3p-containing vector ensures normal, unmodified PD-L1 expression at the injection site and vaccination with this construct decreased CD8 T cell responses close to background levels (Fig. 3.10.B). Surprisingly, the results were quite different when total numbers of OVA-specific CD8 T cells were quantified, representing the “strength” of the response. In this context the lentivector construct IL12-p1-liOVA elicited a stronger response than IL12-liOVA. Removal of transgene expression from the haematopoietic lineage by vaccination with IL12-p1-liOVA\textsubscript{142 3p} induced the same total number of OVA-specific CD8 T cells as vaccination with IL12-liOVA. Unmodified, normal PD-L1 expression at the injection site through vaccination with IL12-Δp1-liOVA\textsubscript{142 3p} reduced total number of OVA-specific CD8 T cells close to background levels (Fig. 3.10.B).

This data suggested that PD-L1 silencing at the injection site increased the strength of T cell responses. In vaccinations where IL12 expression was combined with PD-L1 silencing in cells from haematopoietic and non-haematopoietic origin the highest number of OVA-specific CD8 T cells was induced (Fig. 3.10.B).
<table>
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Table 3.2. Expression of lentivector transgenes in haematopoietic (haematop.) and non-haematopoietic (non-haematop.) cells at the injection site.

A

[Image of a figure showing results of experiments with GFP-liOVA and GFP-liOVA 142 3p in 293T and DCs cells, with bar graphs showing CD8 and CD4 responses with statistical significance marked as P=10^{-***}.]
Fig. 3.10. Subcutaneous vaccination of mice using lentivector constructs containing the microRNA target 142 3p. (A-B) C57Bl/6 mice were injected s.c. at the base of the tail with indicated lentivectors. Two weeks later splenocytes were isolated and stimulated overnight with OVA peptides. Analysis was performed via flow cytometry. 5 mice per group were used and pooled into two groups for in vitro analysis. (A) Above, schematic representation of the lentivector constructs containing GFP, liOVA, and the microRNA 142 3p target sequence. Below left, western blot of transduced 293T and BM-DCs, using the anti-HA antibody. On the right, bar graphs represent OVA-specific CD4 and CD8 T cell responses in vaccinated mice, as assessed by ELISpot. (B) Same as in A but using lentivector constructs containing the IL-12 cytokine. OVA-specific responses were assessed via IFNγ expression by flow cytometry.
3.2.10. PD-L1 SILENCING IN TUMOUR CELLS DELAYS TUMOUR GROWTH

The previous two mechanistic experiments (Fig. 3.9 and 3.10) were performed in healthy mice and thus gave us insight into the prophylactic mechanisms of the lentivectors in priming immune responses. I further investigated the mechanisms underlying the therapeutic activity of the lentivectors after intra-tumour injection. During intra-tumour lentivector injection the majority of transduced cells are cancer cells themselves (Emeagi, Van Lint et al. 2012). To evaluate the effects over cancer cells only, lentivector transduction was limited to B16 tumour cells by transducing them ex vivo prior to in vivo transfer. B16 cells were transduced ex vivo with GFP-liOVA, IL12-p1-liOVA, IFNγ-p1-liOVA, and IL10-p1-liOVA. Lentivector transduction did not affect B16 growth in vitro, with the exception of IFNγ-p1-liOVA, which strongly inhibited B16 growth in vitro (data not shown). While B16-GFP-liOVA inoculated mice exhibited fast tumour growth, it was significantly delayed in mice inoculated with B16-cytokine-p1-liOVA cells. The most effective at delaying tumour growth was IL12-p1-liOVA (Fig. 3.11.A). In addition, IL12-p1-liOVA significantly increased survival (Fig. 3.11.B).

As transduction with GFP-liOVA led to fast tumour growth, GFP-liOVA expression alone did not seem to prevent tumour engraftment in vivo, or raise strong anti-tumour immune responses. As melanoma cells use PD-L1 to escape from immune responses (Blank, Gajewski et al. 2005) I further assessed the contribution of PD-L1 silencing on cancer cell growth in the absence of any other immunostimulating agent. B16 cells were transduced ex vivo with GFP-liOVA or GFP-p1-liOVA before transfer to mice. To test transduction efficiency, transduced B16 cells were analysed by flow cytometry. B16-GFP-liOVA cells expressed 50% GFP while B16-GFP-p1-liOVA cells expressed only 29% of GFP (data not shown). Interestingly, silencing of PD-L1 in only 29% of transferred B16 cells significantly delayed tumour growth and prolonged survival. Nevertheless, IL12 induction combined with PD-L1 silencing in B16 melanoma cells was the most efficient at prolonging survival.
A

Tumour size day 13

Tumour surface (mm²)

GFP  IL12-p1  IL10-p1  IFNg-p1

*** p<0.0001

Tumour size day 18

GFP  IL12-p1

*** p<0.0001

iiOVA-lentivectors used for ex vivo B16 transduction

B

Number of mice

Days after subcutaneous B16 transfer

B16-GFP-iiOVA
B16-IL12-p1-iiOVA

p=0.0023**

C

Tumour size day 15

Tumour surface (mm²)

GFP  GFP-p1

p=0.0132*

D

Percent survival of 4-5 mice

Days after subcutaneous B16 transfer

B16-GFP-iiOVA
B16-GFP-p1-iiOVA

p=0.0187*
Fig. 3.11. Transfer of ex vivo transduced B16 cells slows tumour growth and prolongs survival. (A-D) B16 cells were transduced ex vivo with indicated lentivectors. $5 \times 10^5$ B16 cells were subcutaneously injected into C57/Bl6 mice, $n = 5$ mice per group. (A) Graphs represent tumour sizes on day 13 and 18 respectively. (B) Kaplan-Meier survival plot of mice inoculated with GFP-liOVA and IL12-p1-liOVA. (C) Graph representing tumour sizes of mice inoculated with GFP-liOVA and GFP-p1-liOVA. (D) Kaplan-Meier survival plot of mice inoculated with GFP-liOVA and GFP-p1-liOVA.
3.3. DISCUSSION

This study demonstrates that modulating co-stimulation during antigen presentation by manipulating cytokine priming and PD-L1 silencing is an effective approach to enhance anti-tumour immune responses. Assessing a wide range of cytokines we found that IL12 was the most effective at raising anti-tumour immune responses, when combined with a PD-L1 silencing microRNA (p1) and full-length TRP1 expression fused to the N-terminal half of li. Co-culture experiments with different lentivector constructs showed that IL12-p1-liOVA expression within BM-DCs induce potent OVA-specific CD8 responses in vitro (Fig. 3.4B). Interestingly, this immunostimulatory capacity was not accompanied by BM-DC maturation, at least as assessed by the expression of the maturation markers tested here (Fig. 3.3). Of utter importance for the effective activation of T cell effector responses is that the cytokine signals are delivered in cis from the APC to the T cell (Liechtenstein, Dufait et al. 2012). The strategy presented in this PhD thesis ensures that cytokines are delivered by APCs, together with TAA presentation and down-modulation of co-inhibitory PD-L1 signalling. Thus, secretion of IL12 and a decrease in PD-L1/PD-1 signalling may be sufficient to effectively stimulate antigen-specific CD8 T cell responses and thus up-regulation of other co-stimulatory, MHC, and adhesion molecules may not be necessary, at least initially.

Ex vivo lentivector transduction itself, especially at low multiplicities, does not induce sufficient DC phenotypic maturation for therapeutic efficacy. On the other hand, it has been shown that lentivectors activate and mature myeloid and plasmacytoid DCs in vivo (Breckpot, Escors et al. 2010; Rossetti, Gregori et al. 2011; Liechtenstein, Perez-Janices et al. 2013). Thus, it might not be surprising that I could not see a phenotypic change in transduced BM-DCs ex vivo, with the exception of the IFNy-containing lentivectors. In fact, this is a well-known effect of this cytokine. Nevertheless, ex vivo transduced DCs can be transferred to mice where they trigger lentivector-encoded TAA-specific T cell responses (Esslinger, Romero et al. 2002; Breckpot, Dullaers et al. 2003; Karwacz, Arce et al. 2012) and inhibit tumour growth (He, Zhang et al. 2005; Karwacz, Arce et al. 2012). Importantly, this strategy is also used in human immunotherapy. This strategy involves the isolation of patient-specific peripheral blood monocytes (PBMCs),
their differentiation and genetic modification or TAA-loading ex vivo. Finally the mature DCs are transferred back into the patient. Autologous DCs have been transduced with adenoviral vectors encoding IL12 but clinical efficacy was somewhat limited in patients with metastatic gastrointestinal carcinomas (Mazzolini, Alfaro et al. 2005). In addition, this strategy represents a rather laborious and expensive process. As lentivectors can transduce and activate DCs in vivo, direct lentivector vaccination could be an effective and more feasible alternative. Not only can direct lentivector administration efficiently transduce DCs in vivo (VandenDriessche, Thorrez et al. 2002; Goold, Escors et al. 2011), but this strategy may even be more effective than autologous DC adoptive transfer (Esslinger, Chapatte et al. 2003). Direct lentivector vaccination has been shown to initiate CTL responses that are less dependent on CD4 Th1 induction and nevertheless lead to memory T cells (Chapatte, Colombetti et al. 2006; Dullaers, Van Meirvenne et al. 2006; Goold, Escors et al. 2011). Further supporting direct lentivector vaccination strategies with broad-tropism lentivectors is recent data showing that APC-targeting lentivector vaccines are less effective than broad-tropism lentivector vaccines (Goyvaerts, Lienenklaus et al. 2015).

As direct lentivector vaccination in vivo can induce strong CD8 effector and memory T cell responses, this approach was used for my studies. In vivo vaccination of healthy mice showed that a wide range of cytokine-containing lentivectors induce strong OVA-specific CD8 and CD4 T cell responses while down-regulating Treg numbers, at least within spleen and draining lymph nodes (Fig. 3.5). As all of these vectors contained the PD-L1 silencing microRNA, our data confirmed that PD-L1/PD-1 signalling plays a role in Treg induction. Other researchers have shown that PD-L1 ligation plays a critical role in inducible antigen-specific Treg differentiation (Wang, Pino-Lagos et al. 2008; Vacchelli, Vitale et al. 2013) and data previously generated in our laboratory (experiments performed by Dr. Kasia Karwacz) confirmed that PD-L1 silencing alone in DCs reduces Treg levels (Liechtenstein, Perez-Janices et al. 2014). Interestingly, silencing PD-L1 down-regulated Treg cells in vivo also when combined with IL10 expression. While the IFNγ-containing lentivector elicited the highest OVA-specific GzmB+ IFNγ+ CD8 T cell responses, it was not as effective at inhibiting Treg responses. The IL12-containing lentivector on the other hand raised IFNγ+ CD8 T cell responses but not GzmB+ IFNγ+ CD8 responses (Fig. 3.5). Treg induction was significantly inhibited by the IL12-containing
lentivector, which might explain why IL12 in our system exhibits anti-tumour activities but not IFNγ. Of note, in these experiments the model antigen OVA was used and thus various cytokine-expressing lentivectors initiated effector CD8 T cell responses. As chicken OVA is a xenoantigen no natural tolerance exists in mice, facilitating the initiation of an immune response. Thus, it may not be surprising that all the lentivector constructs enhanced survival in a B16-liOVA model, even when containing the reporter gene GFP, anti-inflammatory or Treg-inducing cytokines (Fig. 3.6.B). B16-liOVA tumours eventually grew out and escaping tumours from vaccinated mice had lost OVA expression (Fig. 3.6.D). This data suggested that through anti-OVA immune selective pressure, tumour cell variants with reduced or without OVA expression escaped. Certain tumours from IL12-p1-liOVA-vaccinated mice also down-regulated other melanoma TAAs, such as TRP2 and gp100, while TRP1 remained expressed by all tumours from IL12-p1-liOVA-vaccinated mice (Fig. 3.6.E). The B16-OVA model is frequently used in research (He, Zhang et al. 2005; Fraser, Lousberg et al. 2010; Aranda, Llopiz et al. 2011) but I used a slightly different model, which I engineered for this thesis. In this model OVA was fused to the invariant chain (ii). As OVA is highly immunogenic its use likely overshadowed the adjuvant effects of the cytokines and PD-L1 silencing. Thus, using OVA models is not ideal for the evaluation of cancer vaccines.

In the B16 experimental system, TRP1 itself was not an immunogenic TAA (Fig. 3.6.E). In agreement with results presented in this thesis, TRP1 has been shown by other researchers not to elicit protective or therapeutic activities when administered on its own (Guevara-Patino, Engelhorn et al. 2006; Liu, Peng et al. 2009). Vaccination of mice using IL12-liTRP1-based lentivectors could break tolerance to the endogenous TAA TRP1, raising polyclonal class I and class II TRP1-specific T cell responses (Fig. 3.9). Stimulation of a CD4 Th1 response is important in cancer immunotherapy. CD4 Th1 cells can exhibit direct tumour cell cytotoxicity (through IFN secretion) and aid in generating potent CTL responses, long-lasting immunity, and epitope spreading (Knutson and Disis 2005). It is therefore important that vaccination with IL12-liTRP1-based lentivectors induces TRP1-specific CD8 and CD4 T cell responses (Fig. 3.9). CD4 Th1 induction suggested that TRP1 is presented by APCs with MHC II expression in vivo, although in vitro I could not detect significant MHC II up-regulation (Fig. 3.3.B). Vaccination of mice with liOVA-containing lentivectors did not have a high predictive value for anti-tumour efficacy. Vaccination of
mice with TRP1-containing lentivectors showed that IL12 expression in combination with TRP1 could break T cell tolerance. The data from this experiment suggested that PD-L1 silencing on its own does not enhance CTL or Th1 responses and that IL12 is the main driver for breaking tolerance (Fig. 3.9). In stark contrast to these results we found that silencing of PD-L1 during prophylactic vaccination with IL12-containing lentivectors enhanced protective immunity against melanoma (Fig. 3.7.E). As vaccinations in both experiments were performed in healthy mice, additional factors (apart from raising CTL and Th1 responses) may play a role in inducing anti-TRP1 immune responses in this experimental model.

Direct lentivector vaccination induces transduction of multiple cell types at the injection site. An experiment silencing transgene expression in haematopoietic cells at the injection site was conducted. While antigen-specific T cell responses were reduced, there was still a significant antigen-specific T cell response elicited by transduction of cells of non-haematopoietic origin (Fig. 3.10.B). Expression of only IL12 and IiOVA without p1 in cells of non-haematopoietic origin (which includes professional APCs) reduced the T cell responses to background levels, thus highlighting the importance of PD-L1 silencing in “non-immune” cells. These results were confirmed in a recently submitted study by Goyvaerts et al.. In this study, introduction of the microRNA 142 3p target into the lentivector construct containing the IL12 and IiOVA genes reduced OVA-specific CTL lytic activity in vivo close to background levels. Interestingly, they further found that lentivector vaccines specifically targeting APCs were inferior to broad tropism lentivector vaccines, further confirming the role of non-APCs in anti-tumour immune responses (Goyvaerts, Lienenklaus et al. 2015). My data correlates with data demonstrating that PD-L1 expression in endothelial cells regulates CD8 T cell activation, cytolytic activity, and extravasation (Rodig, Ryan et al. 2003). Further, PD-L1 silencing in both cells of haematopoietic and non-haematopoietic origin at the injection site enhances the strength of the CD8 T cell response as the absolute numbers of antigen-specific CD8 T cells is enhanced as compared to IL12 expression alone (Fig. 3.10.B). While local delivery of a PD-L1-silencing microRNA amplified T cell responses, it would be interesting to conduct further experiments assessing the mechanisms of IL12 induction and PD-L1 silencing in the various cell types that are present at the injection site.
Interestingly, the IFNγ-p1-liTRP1 lentivector did not induce protective capabilities in the B16 tumour model. While IFNγ-p1-liOVA was particularly successful at inducing OVA-specific CTL responses in mice, BM-DCs transduced with IFNγ-p1-liOVA did not induce potent OVA-specific CD8 T cell responses \textit{in vitro}, although this result could be due to IFNγ-mediated cytotoxicity. As IFNγ-p1-liOVA was not effective at inhibiting Treg responses in vaccinated mice (Fig. 3.5), early-elevated IFNγ expression may initiate regulatory mechanisms that reduce responses against cancer cells (Zaidi and Merlino 2011). \textit{Ex vivo} transduction of B16 melanoma cells with IFNγ-p1-liOVA was not as effective as IL12-p1-liOVA at delaying tumour growth (Fig. 3.11.A). This was observed despite direct B16 cell cytotoxicity \textit{in vitro} by IFNγ-p1-liOVA transduction, before transfer to mice. Further, IFNγ-p1-liTRP1 did not delay tumour growth or prolong survival in B16 melanoma-bearing mice, confirming the limited efficacy of IFNγ for cancer immunotherapy strategies (Meyskens, Kopecky et al. 1990; Meyskens, Kopecky et al. 1995; Schiller, Pugh et al. 1996; Wigginton, Gruys et al. 2001; Zaidi and Merlino 2011). This may be somewhat surprising as endogenous IFNγ is necessary for the anti-tumour activity of IL12 (Wigginton, Gruys et al. 2001; Weiss, Subleski et al. 2007) and maintenance of high systemic endogenous IFNγ in patients correlates with tumour regression in patients treated with IL12 (Gollob, Mier et al. 2000). Zaidi et al proposed a “dark side” for IFNγ. IFNγ does not only play a role in immunoediting/elimination but also in the equilibrium/subversion stages of tumour development (Zaidi and Merlino 2011). As IFNγ is involved in inflammatory responses it also protects tissues from inflammation. IFNγ attracts macrophages for tissue repair and induces Treg to control inflammatory processes (Zaidi and Merlino 2011). Further, IFNγ-activated myeloid cells can induce CTL and Th suppression by IDO-dependent mechanisms and control myeloid cell tumour infiltration by induction of MDSCs (Fallarino, Vacca et al. 2002; Ostrand-Rosenberg and Sinha 2009). Thus, it may not be surprising that IFNγ did not show efficacy in this study.

In prophylactic experiments using B16 melanoma cells without OVA expression, vaccination with IL12-p1-liTRP1 exhibited the strongest anti-tumour activities. Prophylactic vaccination depends on the priming of T cell responses and single injections were administered to avoid boosting primary T cell responses. Although only modest protective effects might be achieved these responses could highlight relevant activities
that would be missed otherwise. Further testing of IL12-p1-IiTrp1 lentivectors in therapeutic experiments by intra-tumour administration showed that this treatment slowed down tumour growth. Importantly, in these experiments, TAA-independent mechanisms may contribute to therapeutic activities, such as IL12-mediated cancer cell cytotoxicity. PD-L1 silencing also played a differential role between the prophylactic and therapeutic settings. PD-L1 inhibition in a prophylactic setting clearly enhanced protective immunity combined with IL12-IiTRP1-based lentivectors (Fig. 3.7.E) while it was not as pronounced in the therapeutic setting (although statistically significant) (Fig. 3.8). PD-L1 acts as a break for T cell activation at two different levels. First, during antigen presentation, blocking PD-L1 enhances T cell activation and differentiation. Second, during antigen recognition on target/tumour cells, PD-L1 acts as a T cell brake by inhibiting the activity of tumour-specific CTL responses (Liechtenstein, Dufait et al. 2012). Our group previously showed that silencing of PD-L1 alone in antigen-presenting DCs hyperactivates TCR$^{\text{high}}$ CD8 T cells with enhanced IFNγ production. Thus, in the prophylactic protocol, cells of haematopoietic and non-haematopoietic origin present at the injection site present TRP1 in the context of IL12 and reduced PD-L1. In this way they can hyperactivate T cells specific for TRP1 while at the same time inducing effector capacities by IL12 secretion. Thus, effective anti-TRP1 Th1 and CTL responses are induced. PD-L1 mediated CD4 inducible Treg differentiation, maintenance, and function would also be impaired by PD-L1 silencing (Francisco, Salinas et al. 2009; Liechtenstein, Perez-Janices et al. 2014). However, it has been shown that Treg-depleting therapies in mice are only effective if administered prior to tumour establishment (Onizuka, Tawara et al. 1999; Elpek, Lacelle et al. 2007). Thus, PD-L1-mediated Treg depletion may only be effective in the prophylactic experimental model. In the therapeutic setting, the majority of transduced cells will be tumour cells, impairing their capacity to inactivate TAA-specific CTL responses. The PD-L1-mediated T cell brake is removed within the tumour environment, thus re-activating already present infiltrating effector CD8 T cells. In addition, PD-L1 silencing in B16 tumour cells ex vivo led to delayed tumour growth in vivo, thus directly contributing to inhibition of cancer cell growth (Fig. 3.11.C,D). The underlying mechanism is still unknown. B16 tumours transduced ex vivo with IL12-p1-IiOVA exhibited further delayed tumour growth. Thus, secretion of IL12 by B16 tumours had beneficial effects that synergized with PD-L1 silencing.
The exact role of IL12 in anti-tumour therapeutic efficacy (Macgregor, Li et al. 2006; Chmielewski, Kopecky et al. 2011; Steding, Wu et al. 2011; Zhang, Kerkar et al. 2011; Zhao, Bose et al. 2011) has not been established. IL12Rb2 knockout mice spontaneously develop cancer, including B-cell and lung tumours (Airoldi, Di Carlo et al. 2005). However, IL12 knockout mice do not seem to spontaneously develop tumours but are more susceptible to tumour growth when treated with carcinogens or inoculated with tumour cells. IL12 deficiency was associated with greater risk of photocarcinogenesis and the authors suggested that this may be due to reduction in damaged DNA repair ability (Meeran, Mantena et al. 2006). Inoculating IL12 knockout mice with syngeneic bladder cancer reduced survival, as compared to wildtype mice (Riemensberger, Böhle et al. 2002). It has been suggested that IL12 appears to elicit more potent anti-tumour responses when present in the tumour micro-environment rather than systemically (Lasek, Zagozdzon et al. 2014). Nevertheless, IL12 expression affects stromal and endothelial cells in the tumour environment (Kerkar, Leonardi et al. 2013). IL12 has direct anti-tumour effects (Hendrzak and Brunda 1996; Airoldi, Di Carlo et al. 2007), inhibits metastasis (Hendrzak and Brunda 1996), and induces anti-angiogenesis (Airoldi, Di Carlo et al. 2007). Further, IL12 induces increased production of IFNγ (Nastala, Edington et al. 1994; Wigginton, Gruys et al. 2001), enhanced tumour cell lytic functions of NK cells (Smyth, Taniguchi et al. 2000), NKT cells (Smyth, Taniguchi et al. 2000) and CD8 T cells (Bhardwaj, Seder et al. 1996; Macgregor, Li et al. 2006), and reestablishment of T cell activation capacities by myeloid cells (Kerkar, Goldszmid et al. 2011). Adoptive transfer of DCs engineered to secrete IL12 has been shown to slow down B16 tumour growth in vivo in mice through priming of CD8 T cell responses (Zhao, Bose et al. 2011). The effects of IL12 may therefore mask the adjuvant effects of PD-L1 silencing in the rapidly growing B16 tumour model. Testing its efficacy in spontaneous tumour models may be beneficial to better understand the effects of the IL12-p1-IiTRP1 lentivector vaccine. Further, assessing the effect of the IL12-IiTRP1-based lentivector vaccines on the composition of tumour-infiltrating immune cells could be of value. Several studies have already evaluated the effect of IL12 on immune cells within the tumour environment. These studies used several methods to deliver IL12 to the tumour including microspheres, adenoviral vectors, or CAR-engineered T cells. IL12 delivery to the tumour induced apoptosis of Tregs and impaired CD8 memory T cells (Kilinc, Aulakh
et al. 2006; Steding, Wu et al. 2011), reactivated quiescent CD4 effector memory T cells (Broderick, Yokota et al. 2005), reactivated resident impaired effector and memory T cells (Kilinc, Aulakh et al. 2006), caused infiltration of activated and non-apoptotic CD8 effector T cells (Kilinc, Aulakh et al. 2006; Steding, Wu et al. 2011), increased NK cell numbers (Zhang, Kerkar et al. 2011), decreased percentage of MDSCs (Steding, Wu et al. 2011; Thaci, Ahmed et al. 2014), and increased DC (Thaci, Ahmed et al. 2014) and macrophage (Chmielewski, Kopecky et al. 2011) numbers in the tumour microenvironment.

Escaping B16-liOVA tumours down-regulated OVA and endogenous TAA expression (Fig. 3.6), further confirming the immune selective pressure on cancer cells. In this way, less immunogenic cancer cell variants escape the immune response. This study shows for the first time the anti-tumour effects of combining a full-length tumour antigen TRP1, a PD-L1-targeting microRNA, and IL12 in a single lentivector. This strategy ensures simultaneous delivery of all three signals during antigen presentation, leading to effective activation of TRP1-specific Th1 and CTL responses. Prophylactic vaccination with IL12-p1-liTRP1 delayed tumour growth, with IL12 expression and p1 delivery exhibiting clear synergistic effects. Clear but rather modest therapeutic effects were achieved using IL12-liTRP1-based lentivector vaccines. This may be due to the administration of only a single intra-tumour lentivector injection into already rapidly growing tumours (50-200mm³). It was hypothesized that this was a more realistic setting and that even a modest increase in therapeutic activity could be of relevance. Prime-boost, multiple intra-tumour injections, and inclusion of multiple or mutated TAAs are all strategies that will likely improve the efficacy of IL12-p1-based- lentivector vaccines. While the role of IL12 on T cells is well characterized, its role on immunosuppressive myeloid cell types within the tumour environment has only recently being studied. Several recent studies highlight effects of intra-tumoural IL12 on immunosuppressive myeloid cell types such as MDSCs, but the exact role remains to be elucidated (Kerkar, Goldszmid et al. 2011) (Steding, Wu et al. 2011; Pegram, Lee et al. 2012) (Thaci, Ahmed et al. 2014). Interestingly, IL10 produced by MDSCs decreases the production of IL12 by macrophages, skewing the CD4 T cell response towards a Th2 type rather than Th1 (Sinha, Clements et al. 2007). Thus, delivering IL12 to the tumour may compensate the loss of IL12 by macrophages. A better understanding of the biological mechanisms and
interactions that lead to immune cell accumulation and anti-tumour activity will be useful for future improvement of cancer immunotherapy strategies that include IL12 induction and PD-L1 silencing.
CHAPTER 4: DEVELOPMENT OF AN EX VIVO MYELOID-DERIVED SUPPRESSOR CELL DIFFERENTIATION SYSTEM

4.1. INTRODUCTION

Cancer immunotherapy strategies are based on the theory of immunosurveillance, claiming that the immune system can recognize and eliminate cancer cells. Stimulating endogenous anti-cancer effector cells to fight against cancer is an elegant approach to anti-cancer treatment, with the promise of low off-target toxicities. While this strategy will appeal to any immunologist, cancer immunotherapeutic strategies have been largely unsuccessful in the last decades. This was likely due to inactivation of anti-cancer effector cells by immunosuppressive cells in the tumour microenvironment. As anti-cancer treatments are initially evaluated in healthy hosts in pre-clinical studies, the effects of immunosuppressive cell types are not usually taken into account. It is therefore important to evaluate anti-cancer treatments early on for their effect on immunosuppressive cell types.

In the previous chapter the combination of IL12 induction and PD-L1 silencing was most effective at delaying tumour growth in vivo. This lentivector vaccine can induce immature BM-DCs to activate CTL responses in vitro. However, as DCs are dysfunctional in tumours, evaluation of cancer vaccines on BM-DCs from healthy mice may not be an appropriate study system. Tumour progression is accompanied by extensive MDSC infiltration and the majority of cells that express the IL-12Rb2 within B16 tumours are not only DCs but also MDSCs (Kerkar, Leonardi et al. 2013). Recently MDSCs have been recognized as one of the main cell types responsible for tumour-induced immune suppression, thus inhibiting immunotherapy approaches (Gabrilovich, Ostrand-Rosenberg et al. 2012; Escors 2014). Tumour-infiltrating MDSCs strongly inhibit CTLs and their accumulation in tumour-bearing hosts favours tumour progression and metastasis (Talmadge 2007; Ostrand-Rosenberg and Sinha 2009; Chen, Kuan et al. 2014). Counteracting MDSC activities strongly enhances anti-cancer treatments and prolongs survival (Qin, Lerman et al. 2014). The capability of generating large numbers of tumour-infiltrating MDSCs ex vivo would significantly facilitate the discovery of anti-MDSC
treatments and improve research in MDSC biology and function. I therefore set out to establish a system to assess melanoma vaccines ex vivo on MDSCs modelling tumour-infiltrating subsets.

4.1.1. MDSCs INHIBIT ANTI-CANCER IMMUNE RESPONSES AND REPRESENT A BARRIER TO CANCER IMMUNOTHERAPY TREATMENTS

Evidence for the correlation of MDSC accumulation and cancer progression, invasion, and metastasis was discussed in Chapter 1 (Ostrand-Rosenberg and Sinha 2009; Chen, Kuan et al. 2014; Condamine, Ramachandran et al. 2015). MDSCs are a heterogeneous population of immature myeloid cells, arrested at various stages of lineage development (Almand, Clark et al. 2001; Gabrilovich, Ostrand-Rosenberg et al. 2012). MDSCs accumulate in circulation, lymphoid organs, and tumours due to altered myelopoiesis and alteration of myeloid cell differentiation by tumour-secreted factors (Gabrilovich and Nagaraj 2009). MDSCs infiltrate tumours where they strongly inhibit CTL (Kusmartsev, Nefedova et al. 2004; Talmadge 2007), Th1 (Kusmartsev, Li et al. 2000; Talmadge 2007), and NK cell (Hoechst, Voigtlaender et al. 2009; Li, Han et al. 2009) responses. MDSCs strongly impair NK cell function and induce NK cell anergy. MDSCs achieve this by inhibiting cytotoxicity, activating receptor NKG2D expression, and IFNγ production by NK cells in vitro and in vivo. NK cells lost their capacity to produce IFNγ after incubation with MDSCs, and MDSC-mediated NK suppression depended on membrane-bound TGFβ (Li, Han et al. 2009). MDSCs can inhibit T cell responses in an antigen-specific way but they also inhibit T cell and other immune cell responses in antigen-independent ways. Antigen presentation in combination with secretion of immunosuppressive cytokines (or even IFNγ) induces Treg differentiation (Huang, Pan et al. 2006). Antigen presentation without positive co-stimulation on the other hand expands T cells with no or only limited effector capabilities. If these cells encounter the same antigen T cell anergy is induced. In the spleen, MDSCs associate with memory CD8 T cells, cross-presenting tumour antigens and thus inducing their tolerization (Nagaraj, Schrum et al. 2010; Ugel, Peranzoni et al. 2012). Unspecific inhibition of immune responses by MDSCs includes secretion of IL10 and TGFβ, depletion of nutrients, and release of reactive oxygen and nitrogen species (ROS and RNS).
Secretion of IL10 and TGFβ recruits and expands Tregs (Huang, Pan et al. 2006). TGFβ secreted by MDSCs inhibits T cell proliferation (Filipazzi, Valenti et al. 2007) and IL10 skews macrophages and DCs towards a M2 or tolerogenic DC function, with decreased IL12 production (Gabrilovich, Ostrand-Rosenberg et al. 2012). MDSCs also secrete CCR5 ligands, CCL3, CCL4, and CCL5, to attract Treg cells to tumour sites (Schlecker, Stojanovic et al. 2012). Lymphocyte nutrients are depleted or sequestered by MDSCs in various ways. L-arginine depletion is widely accepted to be linked to MDSC suppressive activities (Bronte and Zanovello 2005). L-arginine is depleted by Arg-1 (arginase-1) and/or iNOS (inducible nitric oxide synthase/NOS2) activity. Arg-1 and iNOS use L-arginine as a substrate, but while Arg-1 produces urea and L-ornithine, iNOS produces nitric oxide (NO). NO can suppress T cell proliferation and function by blocking the IL-2R pathway through dephosphorylation of Jak3/Stat5 (janus kinase 3/signal transducer and activator of transcription 5) (Bingisser, Tilbrook et al. 1998; Albeituni, Ding et al. 2013). In addition, NO can induce Fas-dependent T cell apoptosis (Mannick, Hausladen et al. 1999). Depletion of L-arginine leads to reduced CD3ζ chain expression in T cells (Rodriguez, Quiceno et al. 2004), which is part of the TCR complex, thus inhibiting T cell cycle progression (Rodriguez, Quiceno et al. 2004; Rodriguez, Quiceno et al. 2007; Gabrilovich, Ostrand-Rosenberg et al. 2012). IDO (Indoleamine 2,3-deoxygenase) also acts similarly to Arg-1 in tolerogenic DCs and MDSCs. IDO catalyzes tryptophan and generates cytotoxic metabolites, suppressing T cell growth and inhibiting antigen-specific T cell-mediated anti-cancer immune responses. Further, IDO plays a role in the induction of Tregs (Yu, Du et al. 2013; Yu, Wang et al. 2014).

Oxidative stress is induced through the release of ROS and RNS. MDSCs express NADPH oxidase, Arg-1, and iNOS, leading to the production of NO, peroxynitrite (ONOO⁻), and hydrogen peroxide (H₂O₂). These initiate various suppressive pathways in T cells (Talmadge 2007; Gabrilovich, Ostrand-Rosenberg et al. 2012). Production of NO, for example, decreases the responsiveness of T cells and NK cells to IFNα and IFNγ (Mundy-Bosse, Lesinski et al. 2011). The reaction of NO and superoxide anion (O₂⁻) produces peroxynitrite. Peroxynitrite nitrates TCRs thus altering the recognition of the p-MHC complex (Condamine and Gabrilovich 2011).

A mechanism by which MDSCs prevent trafficking and viability of cells is by decreasing CD62L expression on naïve T cells, thus preventing them from trafficking to
the lymph nodes (Gabrilovich, Ostrand-Rosenberg et al. 2012). Further, MDSCs enhance tumour angiogenesis (Yang, DeBusk et al. 2004; Murdoch, Muthana et al. 2008), invasion (Chen, Kuan et al. 2014), and metastasis (Steding, Wu et al. 2011; Yu, Du et al. 2013). MDSCs have a role in promoting tumour invasion, angiogenesis, and metastasis through the secretion of matrix metalloproteinases (MMPs), especially MMP9. As MMPs degrade the extracellular matrix, tumour invasion into tumour stroma is promoted. In addition, these proteolytic enzymes degrade capillary membranes, leading to intravasation of tumour cells and thus distribution to distant sites (Yang, DeBusk et al. 2004; Talmadge 2007; Du, Lu et al. 2008; Yang, Huang et al. 2008).

![Diagram](image)

*Fig. 4.1. Mechanisms used by MDSCs to suppress immune cell responses.* Scheme represents the various effects MDSCs have on the function of immune cell types. ROS, reactive oxygen species; RNS, reactive nitrogen species; IDO, Indoleamine 2,3-deoxygenase; NKG2D, natural killer group 2 member D. Figure designed according to information from Gabrilovich et al., *Nat Rev Immunol*, Vol. 12(4), 253-68, 2012.
4.1.2. CONTROVERSY AND DISCRIMINATION FROM OTHER MYELOID CELL TYPES

Mouse MDSCs are generally accepted to comprise a heterogeneous population of immature CD11b\textsuperscript{high} Gr-1\textsuperscript{-} myeloid cells. Gr-1 consists of two epitopes Ly6C and Ly6G. According to their Ly6C-Ly6G expression, mouse MDSCs can be further classified into monocytic (M-MDSC) and granulocytic (G-MDSC) subsets. M-MDSCs are Ly6C\textsuperscript{high} Ly6G\textsuperscript{-/low} while G-MDSCs are Ly6C\textsuperscript{int/low} Ly6G\textsuperscript{high} (Bronte, Apolloni et al. 2000; Youn, Nagaraj et al. 2008). Due to the heterogeneity of MDSCs, their discrimination from other immature myeloid cell types such as immature DCs, M2 macrophages, monocytes and neutrophils remains somewhat ambiguous. In addition, they comprise a range of immature myeloid cells halted at different stages of development. This leaves wide room for interpretation for researchers as what to define as MDSC. Thus some call immunosuppressive tumour-infiltrating myeloid cells with phenotype CD11b\textsuperscript{+} Ly6G\textsuperscript{+} F4/80\textsuperscript{low} expression, tumour-associated neutrophils (TANs, or N2)(Fridlender, Sun et al. 2009; Fridlender, Sun et al. 2012), while others call these cells tumour-infiltrating G-MDSCs (Youn, Collazo et al.; Liechtenstein, Perez-Janices et al. 2014). Thus, MDSC phenotype, origin, and role in cancer remain controversial (Albeituni, Ding et al. 2013).

Both M-MDSCs and tumour-associated macrophages (TAMs) can suppress immune responses, exhibit similar histological distribution, and express MCSF-R (Macrophage colony stimulating factor receptor), making it challenging to distinguish between the two cell types. TAMs are defined as mature macrophages with F4/80 expression, while M-MDSCs are immature myeloid cells. TAMs have been shown to differentiate from monocyte populations resembling tumour-infiltrating M-MDSCs (Movahedi, Laoui et al. 2010) and the hypoxic tumour microenvironment can induce MDSC differentiation into TAMs (Corzo, Condamine et al. 2010). As M-MDSCs comprise a mixture of immature myeloid cells at various stages of differentiation, it may be intuitive that they can differentiate into macrophages, be it in a suppressive or immunostimulatory form (Movahedi, Laoui et al. 2010; Talmadge and Gabrilovich 2013).

As myeloid cells are responsive to tumour-derived factors, it may be argued that circulating MDSCs differentiate into tumour-promoting macrophages, neutrophils, or DCs within the tumour microenvironment. In this line of thought, studies have claimed that MDSCs can differentiate into TAMs and TANs within the tumour (Kusmartsev and
Gabrilovich 2005; Corzo, Condamine et al. 2010). A debate remains whether tumour-promoting N2-type TAN are G-MDSCs recruited to the tumour, where they differentiate, or whether they are neutrophils that are polarized to the N2 type through secretion of tumour-derived factors, such as TGFβ (Fridlender, Sun et al. 2012). An interesting study compared the transcriptomes of TANs, splenic G-MDSCs, and neutrophils from naïve mice. It was shown that TANs exhibited lower expression of cell-cytotoxicity genes, thus exhibiting impaired anti-tumour efficacy. MDSCs and especially TANs up-regulated many immune-regulated genes and pathways as exhibited by higher expression of inflammatory cytokines (TNFα, IL1α/β) and MHC II complex genes. In addition, they up-regulated T cell, B cell, neutrophil, and macrophage chemoattractants. As TANs exhibited the same phenotype as splenic G-MDSCs, they could also be considered tumour-infiltrating G-MDSCs (Fridlender, Sun et al. 2012). Thus TAMs and TANs are hard to distinguish from M-MDSC and G-MDSC, respectively. DCs represent the third type of terminally differentiated myeloid cells. Tolerogenic DCs are present in the tumour and express CD11c. While most researchers claim that MDSCs do not express CD11c, there are studies suggesting that MDSCs may express low levels of CD11c+ (Umemura, Saio et al. 2008; Norian, Rodriguez et al. 2009; Zoso, Mazza et al. 2014).

But MDSCs do not only differentiate into suppressive myeloid cell types, they can also mature into macrophages, granulocytes, or DCs without suppressive characteristics. This highlights the effect the environment has on myeloid cells. Inhibition of C/EBPβ (CCAAT/enhancer-binding protein beta) or PIR-B (paired immunoglobulin-like receptor B), for example, can prevent MDSC establishment (Marigo, Bosio et al. 2010; Ma, Pan et al. 2011). The myeloid lineage differentiation system is not strictly hierarchical but an interconnected system (Gabrilovich, Ostrand-Rosenberg et al. 2012). Thus, tumour-infiltrating myeloid cells cannot be viewed in a rigid way but they can acquire phenotypic or functional changes depending on the signals they receive. Myeloid cells exhibit dual activities, immunostimulatory as well as –suppressive, depending on context. Further studies should be conducted to increase our understanding of the mechanisms by which myeloid cells switch to immunosuppressive activities in tumour-bearing animals and patients.
4.1.3. MDSC PHENOTYPE AND FUNCTION IS INFLUENCED BY THE TUMOUR ENVIRONMENT

As myeloid cells adapt their functions to the environment they are in, it is likely that myeloid cells with the same phenotype display different functions when present in the tumour or lymphoid organs. While MDSCs accumulate in human patients and tumour-bearing mice not only in the tumour itself, but also in peripheral blood, tumour-draining lymph nodes, and liver, these MDSCs in different locations are not equivalent. Nonetheless, increased MDSC numbers correlate with disease progression and metastasis (Diaz-Montero, Salem et al. 2009; Ilkovitch and Lopez 2009; Lechner, Liebertz et al. 2010). Several studies have shown that tumour-infiltrating and splenic MDSCs are not equivalent, neither phenotypically nor functionally (Corzo, Condamine et al. 2010; Maenhout, Van Lint et al. 2013; Liechtenstein, Perez-Janices et al. 2014). In a pancreatic adenocarcinoma mouse model splenic MDSCs were less suppressive than tumour-infiltrating MDSCs (Porembka, Mitchem et al. 2012). MDSCs from a mammary tumour model showed differential transcription levels of several genes, including Arg-1, iNOS, Flt3L (Fms-like tyrosine kinase 3 ligand), and VEGF-A (vascular endothelial growth factor A), between splenic and tumour MDSCs (Abe, Dafferner et al. 2010). Further, MDSCs from the periphery were shown to suppress T cells only by antigen-specific mechanisms while tumour-infiltrating MDSCs could also suppress T cells in unspecific ways. Interestingly, this functional change was dependent on hypoxia (Corzo, Condamine et al. 2010). Hypoxia was also shown to recruit various BM-derived myeloid cell types to tumour tissues, where they promoted tumour neovascularization and invasion (Du, Lu et al. 2008). Similar to hypoxia, inflammation also regulates MDSC function (Ostrand-Rosenberg and Sinha 2009). Prostate tumours in a prostate-specific inflammatory mouse model showed that only MDSCs found at inflammatory sites or in tumour tissues exhibited T cell suppressive function. MDSCs found in peripheral tissues acquired suppressive capacities only upon stimulation by IFNγ, thus activating iNOS (Haverkamp, Crist et al. 2011). It has become clear that peripheral and tumour MDSCs as well as immature myeloid cells from healthy mice all exhibit the same phenotype overall, but not the same immune-regulatory functions. Thus, Talmadge et al proposed the theory
that MDSCs need to be activated by certain signals, to acquire suppressive activities (Talmadge 2007).

Thus, as MDSCs within the spleen are not equivalent to MDSCs within tumours, the same applies to MDSCs from different tumour types. MDSC characteristics vary with cancer type (Peranzoni, Zilio et al. 2010; Poschke and Kiessling 2012; Youn, Kumar et al. 2013) but the same holds true for other immunosuppressive myeloid cell types. TAMs and TANs similarly differ in frequency, gene expression, and function, depending on tumour type (Elpkek, Cremasco et al. 2014). Tumours secrete various factors to alter myelopoiesis, mobilization, and accumulation of MDSCs in spleen, peripheral blood, and tumours. Various factors are known to be involved in stimulating MDSC proliferation, including VEGF (Gabrilovich, Ishida et al. 1998; Donkor, Lahue et al. 2009), GM-CSF (Bronte, Apolloni et al. 2000; Serafini, Carbley et al. 2004; Dolcetti, Peranzoni et al. 2010; Morales, Kmieciak et al. 2010), SCF (stem cell factor) (Pan, Wang et al. 2008), FLT3L (Solheim, Reber et al. 2007), and G-CSF (Donkor, Lahue et al. 2009; Dolcetti, Peranzoni et al. 2010) (Marigo, Bosio et al. 2010).

Silencing of GM-CSF in tumour cell lines impairs MDSC expansion of both MDSC subsets (Dolcetti, Peranzoni et al. 2010) and studies using GM-CSF or GMCSF-R neutralizing antibodies have confirmed that this haematopoietic growth factor is crucial for MDSC accumulation in tumour-bearing mice. In fact, it is believed that tumour-secreted GM-CSF may also be sufficient to drive MDSC development (Morales, Kmieciak et al. 2010; Bayne, Beatty et al. 2012). Under physiological circumstances GM-CSF is crucial for the recruitment of monocytes and granulocytes from the BM to sites of inflammation. GM-CSF also helps in migration and proliferation of splenic monocyte precursors, which subsequently expand to myeloid cells at the inflammation site (Cook, Turner et al. 2011; Robbins, Chudnovskiy et al. 2012; Trikha and Carson 2014). An important factor is the concentration of GM-CSF, as depending on GM-CSF levels this cytokine induces inhibitory or stimulatory immune effects (Bronte, Chappell et al. 1999; Bronte, Apolloni et al. 2000; Hansen, Hercus et al. 2008; Trikha and Carson 2014). Low GM-CSF concentrations enhance antigen presentation by DCs, while high GM-CSF concentrations induce MDSC differentiation and accumulation, and hence immune suppression (Dranoff 2003; Serafini, Carbley et al. 2004). GM-CSF is frequently used as a vaccine adjuvant in clinical trials but with mixed success. Thus, using GM-CSF as a
vaccine adjuvant has been shown to increase a highly suppressive population of CD14\(^+\) HLA-DR\(^{\text{low}}\) myeloid cells in patients. In addition, increased production of TGF\(\beta\) was observed and patients with higher MDSC numbers tended to be unresponsive to treatment (Filipazzi, Valenti et al. 2007). A study reviewing clinical trials and animal studies suggested that repeated low-dose administration of GM-CSF has immunostimulatory effects while administration of high doses presents the opposite effect, which the authors claim may be due to MDSC expansion (Parmiani, Castelli et al. 2007).

### 4.1.4. CURRENT PROTOCOLS FOR THE EX VIVO DIFFERENTIATION OF MOUSE MDSCs

For use in research, MDSCs are frequently isolated from spleens or tumour tissues of tumour-bearing mice (Youn, Collazo et al. 2012; Schouppe, Mommer et al. 2013; Aliper, Frieden-Korovkina et al. 2014). This is due to a lack of efficient ex vivo MDSC differentiation systems, unlike their immunogenic counterparts, DCs. Mouse MDSC isolation from tumour tissues is cumbersome and prone to contamination with other myeloid cell types. In addition, tumour-infiltrating MDSCs do not proliferate well ex vivo, are prone to apoptosis, and easily lose plasticity of differentiation (Youn, Nagaraj et al. 2008; Escors, Liechtenstein et al. 2013; Condamine, Kumar et al. 2014). As only low MDSC numbers can be isolated from mouse tumours (Maenhout, Van Lint et al. 2013; Thaci, Ahmed et al. 2014), MDSCs are still frequently isolated from the spleen of tumour-bearing mice (Peranzoni, Zilio et al. 2010; Alizadeh, Trad et al. 2014; Chakraborty, Das et al. 2014; Mao, Sarhan et al. 2014). While splenic MDSCs may exhibit certain suppressive activities, they are not equivalent to tumour-infiltrating MDSCs (Maenhout, Van Lint et al. 2013; Dufait, Schwarze et al. 2015).

To circumvent this drawback, several ex vivo murine MDSC differentiation systems have been developed to-date. MDSCs have been differentiated from stem cell types but with limited success (Zhou, French et al. 2010). Interestingly, a recent study has shown that NK cells can differentiate into MDSCs in the presence of GM-CSF or in tumour-bearing hosts (Park, Song et al. 2013). If these results are confirmed it would further highlight the flexibility of the haematopoietic system. Nevertheless, the most common strategy is to differentiate MDSCs from myeloid precursors present in the BM.
BM from mice is easily obtained and represents the site of myelopoiesis in vivo. As GM-CSF plays a key role in MDSC accumulation in vivo, it is applied in most ex vivo differentiation studies (Lutz, Kukutsch et al. 2000; Dolcetti, Peranzoni et al. 2010; Morales, Kmiecik et al. 2010). One of the first ex vivo differentiated MDSCs were generated by diverting BM-DC differentiation (through addition of GM-CSF) to MDSC differentiation by the addition of LPS and IFNγ. The resulting immature myeloid cells exhibited antigen specific and unspecific (iNOS) suppressive functions (Greifenberg, Ribechini et al. 2009). This study again highlighted the effect inflammatory signals have on MDSC development and function. Addition of high concentrations of GM-CSF alone or in combination with other factors frequently secreted by tumours (IL6, IL13, M-CSF, PGE₂, and TGFβ) generally leads to the generation of suppressive immature myeloid cells from BM cells (Valenti, Huber et al. 2006; Youn, Nagaraj et al. 2008; Highfill, Rodriguez et al. 2010; Marigo, Bosio et al. 2010; Morales, Kmiecik et al. 2010; Michels, Shurin et al. 2012; Kurkó, Vida et al. 2014), although the efficiency of MDSC generation generally remained low. GM-CSF in combination with IL6 alone led to accumulation of immunosuppressive myeloid cells but without proliferative capabilities (Marigo, Bosio et al. 2010). Addition of IL13 increased the suppressive capacities of immature myeloid cells generated with GM-CSF and M-CSF, but MDSCs only comprised 40-50% of culture cells (Highfill, Rodriguez et al. 2010). Addition of GM-CSF to tumour-conditioned medium rendered up to 45-60% of CD11b⁺ GR-1⁺ cells, although certain subtypes showed no suppressive capacities (Morales, Kmiecik et al. 2010; Michels, Shurin et al. 2012). Addition of GM-CSF, IL4 and cancer cell line-conditioned medium to BM cells induced immunosuppressive myeloid cells that were comparable to in vivo MDSCs, albeit without high (about 30%) proliferative capacities (Youn, Nagaraj et al. 2008). Thus, current protocols can differentiate immature myeloid cells equivalent to MDSCs in vivo in tumour-bearing mice. However, several problems remain unresolved in most strategies. Important for effective ex vivo MDSC differentiation strategies would be a high percentage of MDSCs in cultures, prolonged viability and proliferation, and plasticity of differentiation. MDSCs exhibit shorter half-lives in tumour-bearing hosts than monocytes and neutrophils (Condamine, Kumar et al. 2014), representing a barrier to culture them. Thus an effective ex vivo MDSC differentiation system should contain
myeloid cell types at various stages of development, allowing precursors to replenish apoptotic MDSCs.

4.1.5. CURRENT PROTOCOLS FOR THE EX VIVO DIFFERENTIATION OF HUMAN MDSCs

Similar to the murine system, human MDSCs are still frequently isolated from blood of patients. However, circulating MDSCs are not equivalent to tumour-infiltrating MDSCs and may skew experimental results. Researchers have used peripheral blood monocytes (PBMCs), CD34+ haematopoietic precursors, BM aspirates, and umbilical cord precursors as starting material. Several studies compared various protocols to induce MDSC differentiation from PBMCs, as they can be easily obtained. Adding various combinations of recombinant cytokines to PBMCs, the combination of GM-CSF and IL6 most effectively produced suppressive CD33+ immature myeloid cells that were consistent with human CD33+ MDSCs (Lechner, Liebertz et al. 2010). Addition of high GM-CSF concentrations was sufficient in another study to convert CD11b+ cells from healthy donors (De Santo, Salio et al. 2008), while PGE2, GM-CSF, IL4, and LPS were combined in a more recent study (Obermajer, Muthuswamy et al. 2011). Culturing human monocytes from healthy donors with IL4 differentiated a fibrocytic subset of MDSCs that was equivalent in phenotype and function to this immunosuppressive MDSC subset found in cancer patients (Zhang, Maric et al. 2013). Alternatively, PBMCs can be co-cultured with tumour cell lines. In this case, MDSC differentiation is dependent on certain cytokines including IL1β, IL6, GM-CSF, and VEGF (Lechner, Megiel et al. 2011).

Human bone marrow aspirates were treated with various cytokine combinations, of which GM-CSF and G-CSF/IL6 were most efficient at generating suppressive immature myeloid cells (Marigo, Bosio et al. 2010; Solito, Falisi et al. 2011). Differentiation of human MDSCs depended on the transcription factor C/EBPβ (Marigo, Bosio et al. 2010). A recent study defined a new subset of MDSCs, differentiated from umbilical cord blood precursors with recombinant GM-CSF and G-CSF. As the newly differentiated immature myeloid cells shared markers of DCs, MDSCs, and fibrocytes, the authors called them fibrocytic MDSCs. These cells suppressed CD4 and CD8 T cells and induced Tregs in vitro. Interestingly, MDSC suppression was not dependent on iNOS or Arg-1 but on cell-cell interactions and IDO (Zoso, Mazza et al. 2014).
Different tumours secrete varying factors, mobilizing myeloid cells from BM or other tissues. The variety of MDSC ex vivo differentiation protocols and differentiation of MDSCs with various characteristics reflects their tumour-dependency in vivo. Nonetheless, GM-CSF remains a key factor for the differentiation of human and mouse MDSCs. In addition, IL4 and IL6 seem to be additional key factors for human MDSC differentiation and suppressive function. This correlates with data showing that IL6 and MDSC levels predict prognosis of patients with squamous cell carcinoma of the esophagus (Chen, Kuan et al. 2014). Similarly, there exists a positive correlation between IL4 mRNA in PBMCs and plasma Arg-1 enhancement, which correlated with MDSC levels in PBMCs from patients with esophageal cancer (Gao, Wu et al. 2014). In addition, IL4 induces Arg-1 expression in mouse MDSCs, enhancing their T cell suppressive activities (Bronte, Serafini et al. 2003).
4.1.6. AIMS OF CHAPTER

Isolation and culture of tumour-infiltrating MDSCs remains inefficient, time-consuming, and expensive. While several \textit{ex vivo} MDSC differentiation systems have been developed, many issues remain to be solved, such as low efficiency (Escors, Liechtenstein et al. 2013). \textit{Ex vivo} differentiation systems using BM cells from healthy mice circumvent the need for tumour establishment in mice. Thus, I set out to establish a novel and simple \textit{ex vivo} mouse MDSC differentiation system. This system should generate large numbers of suppressive immature myeloid cells that are equivalent to tumour-infiltrating MDSCs rather than circulating or splenic MDSCs. In addition, MDSCs should retain viability, plasticity, and proliferative capacities \textit{ex vivo}. Extensive characterization of the newly differentiated MDSCs was undertaken to confirm that they are functionally and phenotypically equivalent to \textit{in vivo} tumour-infiltrating MDSCs from cancer-bearing mice. In addition, preliminary results for translation into the human setting are shown.
4.2. RESULTS

4.2.1. SIMULATION OF TUMOUR ENVIRONMENT FOR THE INDUCTION OF EX VIVO MYELOPOIESIS USING CONDITIONING MEDIUM GENERATED BY GM-CSF EXPRESSING CANCER CELLS.

High tumour burden and circulation of tumour-derived factors in mice and patients perturbs physiological myelopoiesis in the BM. Altered myelopoiesis prevents proper myeloid differentiation and leads to the expansion of MDSCs. MDSCs then distribute systemically and infiltrate tumours, where they actively suppress TILs (tumour-infiltrating lymphocytes) (Talmadge 2007; Gabrilovich and Nagaraj 2009; Raber, Thevenot et al. 2014). Altered myelopoiesis was recreated in vitro to differentiate MDSCs ex vivo from BM cells isolated from healthy mice.

The growth factor GM-CSF has crystallized as one of the main drivers of tumour-driven altered myelopoiesis and was shown to drive MDSC development in vitro at high concentrations (Morales, Kmiecik et al. 2010). Further tumour-derived factors influence MDSC development and function and thus I hypothesized that cancer cell line supernatants, in addition to GM-CSF, would be important in recreating the tumour environment. B16 mouse melanoma cells were genetically modified with a lentivector expressing murine GM-CSF and a puromycin resistance gene (Fig. 4.2.A). Simultaneously, 293T cells were transduced with the same lentivector as a control cell line. Culture supernatants were collected for use as MDSC-conditioning media (CM293T and CMB16) and GM-CSF concentration was assessed by ELISA. GM-CSF concentration in conditioning medium (CM) of both lentivector-modified cell lines (CM293T and CMB16) was found to be similarly high (2.9±0.2 and 2.6±0.1 µg/ml, n=6)(Fig. 4.2.B). Of note, these GM-CSF concentrations were well above the concentration of recombinant GM-CSF used for standard DC differentiation protocols (Fig. 4.2.B).
Fig. 4.2. Simulation of tumour environment for the ex vivo differentiation of MDSCs from BM cultures. (A) Above: Schematic representation of the lentivector construct co-expressing murine GM-CSF and puromycin resistance genes. Below: Schematic representation of the generation of MDSCs. Cancer cell lines are transduced with the lentivector LV-GM-CSF-puroR. Conditioning medium (CM) is collected from GM-CSF-expressing cells to simulate myelopoiesis within a tumour environment. BM cells from healthy C57/Bl6 mice were cultured in the presence of CM for minimum 5 days. (B) Bar graphs represent concentration of murine GM-CSF within CM collected from GM-CSF-expressing 293T and B16 cells. Concentration of recombinant GM-CSF (rec GMCSF) used for conventional immature DC differentiation is also represented. LTR, long terminal repeat; SFFV, spleen focus-forming virus promoter; moGM-CSF, mouse GM.CSF gene; Puromycin R, puromycin resistance gene; UBI p, ubiquitin promoter; SIN, self-inactivating LTR.
4.2.2. *EX VIVO CULTURE OF BM CELLS WITH CM DIFFERENTIATES LARGE NUMBERS OF IMMATURE MYELOID CELLS*

Initially, increasing concentrations of CM were used to culture BM cells, to assess the optimal concentration. Using increasing CM concentrations led to strong proliferation of immature myeloid cells (Fig. 4.3.A). The use of 75% of CM routinely yielded immature myeloid cell numbers that ranged from 40 to 70 million cells from the BM of a single tumour-free mouse. These numbers were comparable to immature DC numbers differentiated from BM (BM-DCs) with recombinant GM-CSF (Fig. 4.3.A). Increasing concentrations of CM correlated with decreased expression of major-histocompatibility molecule II (MHCII), co-stimulatory marker CD86, and intercellular adhesion molecule 1 (ICAM-I), indicating poor antigen presenting capacity. Myeloid marker CD11b and co-stimulatory marker CD80 remained highly expressed. Immature BM-DCs generated simultaneously with recombinant GM-CSF exhibited high ICAM-I, MHCII, and CD86 expression (Fig. 4.3.A). In addition, the conventional DC lineage marker CD11c was highly expressed by immature BM-DCs on day 8 of culture, which correlated with high MHC II expression. In contrast, immature myeloid cells differentiated with CM\textsuperscript{293T} and CM\textsuperscript{816} exhibited low surface expression of these markers (Fig. 4.3.B).
Fig. 4.3. Ex vivo differentiation using GM-CSF-containing CM yields large numbers of immature myeloid cells. (A) Left: Bar graphs represent numbers of myeloid cells on day 5 of culture with CM at indicated percentages. CM^{293T} or CM^{B16} used as indicated. The use of recombinant GM-CSF (GM-CSF) in standard protocols differentiated conventional immature DCs. Error bars correspond to standard deviations. Right: Percentage of surface expression of indicated markers. Increasing concentrations of CM^{293T} were used as indicated. (B) Flow cytometry density plots showing CD11c-MHC II expression profiles.
on day 8 of culture. Percentages of CD11c and MHC II expressing cells are indicated within the plots. (C) Pictures taken with phase-contrast microscope on day 8 of culture of myeloid cell types differentiated with conventional GM-CSF or CM.

4.2.3. IMMATURE MYELOID CELLS DIFFERENTIATED WITH CM$^{293T}$ and CM$^{B16}$ EXHIBIT CD11b$^+$ GR-1$^+$ PROFILE CONSISTENT WITH M- AND G-MDSCs

Mouse MDSCs are phenotypically immature, CD11b$^+$ GR-1$^+$ immunosuppressive myeloid cells. The GR-1 epitope consists of the two surface molecules, Ly6C and Ly6G. Hence, I first examined the Ly6C-Ly6G (GR-1) expression profile of myeloid cells differentiated with CM$^{293T}$ and CM$^{B16}$. Immature myeloid cells cultured for 5 days in CM$^{293T}$ and CM$^{B16}$ exhibited GR-1 profiles that were consistent with monocytic (M) (Ly6C$^{\text{high}}$, Ly6G$^{\text{low/neg}}$) and granulocytic (G) (Ly6C$^+$, Ly6G$^{\text{high}}$) MDSCs. In contrast, immature conventional BM-DCs were Ly6G$^{\text{low}}$ (Fig. 4.4.A).

I further tested whether the supernatant of unmodified 293T cells supplemented with recombinant GM-CSF (293T sup + GMCSF) could differentiate cells with M- and G-MDSC phenotype with the same efficiency as by using CM$^{293T}$. Interestingly, this approach differentiated cells with a similar phenotype, albeit at lower efficiency (Fig. 4.4.B). In addition, as the transduction of the 293T and B16 cell lines yielded such high concentrations of GM-CSF in the CM$^{293T}$ and CM$^{B16}$ (Fig. 4.2.B), it was tested whether a similar concentration of recombinant GM-CSF (3μg/mL) would be sufficient to differentiate GR-1$^+$ myeloid cells. Addition of 3μg/mL GM-CSF generated a high amount of CD11b$^+$ Gr-1$^+$ cells. Of note, this protocol also yielded a significant amount of CD11c$^+$ cells (Fig. 4.4.C).
Fig. 4.4. Ex vivo differentiation of myeloid cells using CM$_{293T}$ and CM$_{B16}$ results in immature myeloid cells with phenotypes consistent with M-MDSC and G-MDSC. (A) Flow cytometry density plots showing Ly6C-Ly6G expression profiles on day 5 and day 8 of culture. Percentages of Ly6C and Ly6G expressing cells are indicated within the plots. (B) Flow cytometry density plots showing GR-1 profiles of myeloid cells on day 8 of culture. BM cells were differentiated using conventional DC culture protocols (GM-CSF, recombinant GM-CSF 100 ng/mL), supernatant from unmodified 293T cells supplemented with GM-CSF 100 ng/mL (293T sup + GM-CSF), and CM$_{293T}$. (C) Flow cytometry density plots showing CD11c and Ly6G expression on day 8 of culture. BM cells were differentiated using high concentration recombinant GM-CSF (3μg/mL GM-CSF), CM$_{293T}$, and CM$_{B16}$. 
4.2.4. 293T-MDSCs and B16-MDSCs EXHIBIT A CD11b\(^+\) GR-1\(^-\) PROFILE AND ARE DISTINCT FROM IMMATURE BM-DCs AND OTHER MYELOID LINEAGE PRECURSORS

I further examined several markers of different populations of the myeloid lineage to discard that these cells were immature versions of other myeloid cell types, such as DCs and macrophages. In addition, haematopoietic progenitor markers were evaluated. Immature myeloid cells cultured for 5 days in CM\(^{293T}\) (293T-MDSCs) and CM\(^{B16}\) (B16-MDSCs) were CD11c\(^{\text{low/neg}}\), MHC I/II\(^{\text{low/neg}}\), CD34\(^{\text{neg}}\), FLT3\(^{\text{neg}}\), and F4/80\(^{\text{neg}}\), indicating that they were not immature DCs, haematopoietic progenitors, or macrophages (Fig. 4.5.A). In addition, these ex vivo MDSCs expressed low or no MHC I/II, CD86, and CD40, which are characteristics of non-efficient APCs. The surface levels of molecules involved in antigen presentation remained well below those of immature BM-DCs after 8 days of culture (Fig. 4.5.B).

An important question to answer was whether these immature myeloid cells would mature and up-regulate antigen presenting molecules upon TLR ligation, as immature DCs do. Addition of the TLR-4 ligand LPS to BM cultures on day 5 caused maturation of immature BM-DCs while the phenotype of myeloid cells differentiated in CM\(^{293T}\) and CM\(^{B16}\) remained largely unaltered (Fig. 4.5.C,D). The only significantly up-regulated marker in B16-MDSCs in response to LPS was Ly6G. Ly6G expression in BM-DCs on the other hand, was not affected (Fig. 4.5.D). Taken together, this data suggested that the immature myeloid cells differentiated in the presence of CM exhibited phenotypes and responses consistent with MDSCs and not immature DCs. In addition, 293T- and B16-MDSCs did not behave like immature DCs that become activated upon TLR-4 ligation.
Fig. 4.5. Phenotype profiling and response to LPS of ex vivo differentiated MDSCs. (A) Bar graphs represent the percentage of marker positive cells in day 5 cultures of BM-derived DCs, 293T-MDSCs, and B16-MDSCs. Cell markers are indicated within the graph. (B) Same as in A, but on days 7-8 of culture. (C) BM-DC and MDSCs were treated on day 5 of culture with 100ng/mL LPS. After 72h cells were stained with indicated surface markers. Unstained, untreated, and LPS-treated cells are represented in flow cytometry histograms. Percentages of marker positive cells and MFI are indicated within the histograms. (D) Same as in C, but represented as bar graphs from pooled data of two independent experiments.
4.2.5. IMMATURE MYELOID CELLS DIFFERENTIATED WITH CM\textsuperscript{293T} and CM\textsuperscript{B16} EXHIBIT CD11b\textsuperscript{+} GR-1\textsuperscript{+} PROFILE AND OTHER MDSC-ASSOCIATED MARKERS.

To validate the phenotype of \textit{ex vivo} differentiated MDSCs, the expression of several additional markers associated to MDSCs in other published studies was analysed. In addition, I sought to identify markers that could differentiate the \textit{ex vivo} MDSCs from immature BM-DC cultures.

CD62L, also known as L-selectin, is a homing receptor to secondary lymphoid organs, selectively expressed on MDSCs within the myeloid lineage (Highfill, Rodriguez et al. 2010). We found CD62L to be highly expressed in \textit{ex vivo} MDSCs (Fig. 4.5.A,B). B16-MDSCs exhibited enhanced Ly6G-CD62L co-expression, as compared to 293T-MDSCs (Fig. 4.6.A). While CD62L surface expression decreased over time in BM-DC cultures, the opposite was true for MDSC cultures (Fig. 4.6.B). CD62L proved to be a consistently reliable marker for MDSCs in our experimental system. CD49d is a subunit of integrin α4β1, another lymphocyte homing receptor that has recently been described to be predominantly expressed by a highly immunosuppressive M-MDSC subpopulation (Haile, Gamrekelashvili et al. 2010; Tsiganov, Verbina et al. 2014). In agreement with this data CD49d was expressed mainly by the M-MDSC population of our \textit{ex vivo} differentiated MDSCs (Fig. 4.6.C). Similar results were obtained with the marker CD64a/b, also known as FcyRI receptor, mainly present in the M-MDSC population of B16-MDSCs (Fig. 4.6.C). Immature BM-DCs expressed higher levels of CD64a/b on day 7/8 of culture as compared to MDSCs (Fig. 4.5.B and Fig. 4.6.D). As expected, BM-derived macrophages with M-CSF were pre-dominantly positive for this surface marker (Fig. 4.6.D). While CD64a/b is constitutively expressed on monocytes, macrophages, and DCs, granulocytes can up-regulate its expression upon activation (van Vugt, Kleijmeer et al. 1999; van der Meer, Pickkers et al. 2007). Accordingly, B16-G-MDSCs, in contrast to B16-M-MDSCs, exhibited increased CD64a/b surface expression upon TLR-4 ligation. No increase in CD64a/b expression was observed when analysing bulk MDSCs (data not shown). In addition, CD115 (M-CSF receptor) has been also identified in MDSCs (Huang, Pan et al. 2006), which was also the case in B16-MDSCs (Fig. 4.6.C).
Fig. 4.6. *Ex vivo* MDSC phenotypes are consistent with further MDSC-associated markers. (A) Flow cytometry density plots showing CD62L-Ly6G expression profiles on day 8 of culture. Percentages of CD62L and Ly6G expressing cells are indicated within the plots. (B) Flow cytometry histograms representing CD62L expression in day 5, 8, and 10 of cultures of BM-derived immature DCs (iDCs) and MDSCs. (C) Bar graphs represent CD49d (left) and CD64a/b (right) expression within the M-MDSC and G-MDSC populations of 293T- and B16-MDSCs. Cells were stained and analysed on day 7/8 of culture. Pooled data from a minimum two independent experiments. (D) Bar graphs represent CD64a/b expression in M-MDSC (left) and G-MDSC (right) populations of 293T- and B16-MDSCs. Cells were treated with LPS for 72h before analysis on day 8 of culture. Pooled data from three independent experiments.
4.2.6. M-MDSCs WITHIN EX VIVO MDSC CULTURES CONSERVE THEIR ABILITY TO PROLIFERATE AND DIFFERENTIATE

The data suggested that M-MDSCs and G-MDSCs in the ex vivo cultures do not only express different surface markers but also react differently to stimuli. The exact relationship between the two subpopulations remains unclear but it has been suggested that intra-tumour G-MDSCs represent the terminal differentiation stage of the intra-tumour M-MDSC population (Youn, Kumar et al. 2013; Condamine, Kumar et al. 2014). Alternatively, intra-tumour G-MDSCs have been described as pathologically activated precursors of neutrophils that are recruited to the tumour by secreted factors (Youn, Collazo et al. 2012). Another hypothesis is that G-MDSCs are recruited to the tumour where they differentiate into TANs (Fridlender, Sun et al. 2012). While there remains controversy on the origin of each tumour-associated myeloid cell type, it is clear that myeloid cells retain a high degree of plasticity. As the proportion of G-MDSCs in 293T- and B16-MDSC cultures increased over time in our system (Fig. 4.7.A), I tested whether ex vivo M-MDSCs in ex vivo cultures could act as precursors of G-MDSCs. To this end, the M-MDSC population was purified on day 5 of culture using a magnetic bead-based system (Miltenyi MACS MDSC purification kit). Purified M-MDSCs were cultured in CM for additional 72h, when they were re-analysed by flow cytometry. Immediately after purification M-MDSCs did not express Ly6G. In contrast, these cells strongly expressed Ly6G after 72h of culture (Fig. 4.7.B). This suggested that ex vivo differentiated M-MDSCs differentiate into G-MDSCs and can hence act as G-MDSC precursors. Assessing overall proliferation of MDSCs versus immature BM-DCs, I found that while BM-DCs continued proliferating, MDSC numbers remained stable after day 5 (Fig. 4.7.C). Interestingly, this loss of proliferation coincided with the increased presence of G-MDSCs (Fig. 4.7.A,C). Further, the viability of the two MDSC subpopulations was assessed. The G-MDSC population was less viable (Fig. 4.7.C), in agreement with reports demonstrating impaired G-MDSC viability in vivo (Condamine, Kumar et al. 2014).

In contrast to M-MDSCs, G-MDSCs do not proliferate well (Youn, Kumar et al. 2013). I therefore purified M- and G-MDSCs and assessed their proliferation after an overnight culture. Proliferation was assessed by Ki67 intra-nuclear staining, which was significantly higher in M-MDSCs than G-MDSCs (Fig. 4.7.D). This data suggested not only
that M-MDSCs could act as precursors of G-MDSCs but also that this differentiation process was accompanied by a loss of proliferation and viability. There is evidence suggesting that MDSC function can be reversed, through maturation to APCs (Kusmartsev, Su et al. 2008; Kerkar, Goldszmid et al. 2011; Steding, Wu et al. 2011). To address the question, 293T-MDSCs were cultured in standard DC differentiation medium for 72h. G-MDSC differentiation could be reverted towards DC differentiation, as shown by a loss of Ly6G and increase of CD11c surface expression (Fig. 4.7.E). Alternatively, it could be possible that these Ly6G-expressing cells arise from uncommitted myeloid precursors still present in MDSC cultures.
**A**

293T-MDSC

- **p = 0.04**
- **p = 0.004**

B16-MDSC

- **p = 0.01**
- **p = 0.007**

Days of culture of indicated MDSCs

**B**

293T M-MDSC

- **CM<sup>293T</sup>**
  - Day 5: G-MDSC, 2%
  - Day 10: G-MDSC, 49%

B16 M-MDSC

- **CM<sup>B16</sup>**
  - Day 5: G-MDSC, 5%
  - Day 8: G-MDSC, 25%
C

![Graph showing viability day 10 and dead cell staining with M-MDSC 70% viability and G-MDSC 22% viability.]

D

**Ki67 expression in B16-MDSCs**

![Graph showing % Ki67 expressing cells and MFI Ki67 comparing G-MDSC and M-MDSC subsets.]

**p = 0.0061**
**Fig. 4.7. Ex vivo MDSCs retain plasticity of differentiation.** (A) Bar graphs represent the relative proportion of M- and G-MDSCs in 293T-MDSC and B16-MDSC cultures of indicated days of culture. Relevant statistical comparisons are shown. (B) Ly6C-Ly6G expression profiles of purified M-MDSCs from 293T-MDSC and B16-MDSC cultures on day 5 (density flow cytometry plots on the left). On the right the same cells but incubated in CM\(^{\text{293T}}\) and CM\(^{\text{B16}}\) for three additional days. The percentages of G-MDSCs are shown within the graph. (C) Bar graph on the left represents the ratio of the number of cells on day 8 versus 5 in 293T-MDSC and B16-MDSC cultures. This represents the cell growth rate. On the right, dead cell staining with fixable viability stain (FVS) of M-MDSC and G-MDSC cultures. The proportion of viable cells is shown in the legend. (D) Bar graphs represent Ki67 expression within G-MDSC and M-MDSC subsets from B16-MDSC cultures, as indicated. Left graph represents the proportion of Ki67-expressing cells while the graph on the right represents Ki67 mean fluorescent intensities. (E) Day 5 293T-MDSC cultures were incubated for three days with either DC medium or CM\(^{\text{293T}}\), as indicated. Above, Ly6G-CD11c profiles of 293T-MDSCs incubated in CM\(^{\text{293T}}\) (left) or DC medium.
Below, histograms represent Ly6G (left) and CD11c (right) expression of 293T-MDSCs incubated in CM\textsuperscript{293T} or DC medium, as indicated. Percentages and mean fluorescent intensities are indicated in the legend. Relevant statistical comparisons are indicated. *, **, *** represent significant ($p<0.05$), very significant ($p<0.01$), and highly significant differences ($p<0.001$). Experiments were repeated at least twice.
4.2.7. **EX VIVO-DIFFERENTIATED B16-MDSCs PHENOTYPICALLY RESEMBLE IN VIVO MELANOMA-INFILTRATING MDSCs**

It has become increasingly clear that tumour-infiltrating and splenic MDSCs are not equivalent, neither in phenotype nor in function (Maenhout, Van Lint et al. 2013). As the strategy described here was to mimic the tumour microenvironment through the use of CM from modified tumour cell lines, it was hypothesized that *ex vivo* B16-MDSCs would resemble B16 tumour-infiltrating MDSCs *in vivo*. Hence, tumours and spleens from B16 melanoma-bearing mice were isolated and the MDSC phenotype was compared to day 5 *ex vivo* B16-MDSCs (Fig. 4.8.A-C). No significant differences in percentage of M- and G-MDSCs were found in spleen, tumour, or *ex vivo* MDSCs (Fig. 4.8.B). Several representative markers were used to assess MDSC phenotype, which included CD86, MHC II, CD62L, Arg-1, and PD-L1. While *ex vivo* B16-MDSCs phenotypically resembled tumour-infiltrating MDSCs, spleen MDSCs exhibited a distinct phenotype (Fig. 4.8.C).
A) Tumour cells

B) M-MDSC vs G-MDSC

- **CD45** vs **CD11b**
  - **Ly6C** vs **Ly6G**
  - **G-MDSCs**

- **M-MDSCs**

- **Bar graph**
  - **Ly6C+ Ly6G-**
  - **Ly6C+ Ly6G+**

- **Tumour**
  - **Spleen**
  - **In vitro**
**Fig. 4.8.** *Ex vivo* B16-MDSCs resemble tumour-infiltrating MDSCs in vivo. (A-C) Mice were s.c. inoculated with $5 \times 10^5$ B16 cells. When tumours reached sizes of circa 100 mm$^2$, single cell suspensions were generated from tumours and spleens. *In vivo* cells and *ex vivo* day 5 B16-MDSCs were simultaneously stained with fluorochrome-conjugated antibodies specific for the indicated markers and analyzed via flow cytometry. (A) Gating strategy for tumour and spleen cells. Shown are representative flow cytometry density plots from a tumour sample. CD45$^+$ CD11b$^+$ cells were gated as myeloid cells. (B) Bar graph representing percentages of M- and G-MDSCs in indicated samples. (C) Bar graphs representing percentages of indicated markers within the population of CD45$^+$ CD11b$^+$ M- or G-MDSCs of each tissue or *in vitro* cell sample.
4.2.8. *EX VIVO* B16-MDSCs EXPRESS MDSC HALLMARK PROTEINS iNOS, ARG-1, AND TGFβ

*Ex vivo* B16-MDSCs were phenotypically similar to tumour-infiltrating MDSCs in the tested markers. Further, the suppressive capabilities of the *ex vivo* MDSCs were assessed. MDSCs are known to partly mediate their suppressive functions by depleting amino acids such as L-arginine through the expression of iNOS and Arg-1 (Talmadge 2007; Rodriguez, Ernstoff et al. 2009; Raber, Thevenot et al. 2014). In addition, MDSCs recruit and expand Treg cells through secretion of TGFβ and IL10 (Huang, Pan et al. 2006; Filipazzi, Valenti et al. 2007). The expression of these proteins was assessed in *ex vivo* B16-MDSCs, control 293T-MDSCs, and immature conventional BM-DCs. Interestingly, only B16-MDSCs expressed significant levels of iNOS, as assessed by western blot as well as intracellular flow cytometry (Fig. 4.9.A). On the other hand, Arg-1 was expressed by all three myeloid cell types. Interestingly, analysis by western blot suggests an increased molecular weight of Arg-1 in immature BM-DCs as compared to MDSCs (Fig. 4.9.B).

Production of bioactive TGFβ was assessed via a bioassay previously developed in our laboratory (Arce, Breckpot et al. 2011). This assay is based on GFP expression by incubation of SMAD-GFP cells (293T cells containing a SMAD-dependent promoter driving GFP expression) with TGFβ. TGFβ production was found to be significantly higher in B16-MDSCs than in 293T-MDSCs and BM-DCs. Importantly, the conditioning medium CM<sub>B16</sub> did not contain bioactive TGFβ, suggesting that TGFβ was not required for MDSC differentiation in our system (Fig. 4.9.C). IL10 production could not be found in any of the three myeloid cell types, as assessed by ELISA (data not shown). This was consistent with published data, where MDSCs only produced IL10 upon activation by IFNγ, while TGFβ and iNOS were constitutively expressed but could be enhanced by IFNγ stimulation (Huang, Pan et al. 2006). Together this data suggested that *ex vivo* B16-MDSCs produced iNOS, Arg-1, and TGFβ, which are all molecules associated with MDSC suppressive function.
**A**

![iNOS Ab and GADPH Ab blots for DC, 293T-MDSC, and B16-MDSC. The iNOS Ab shows a band at 130 kDa.](image)

Dotted: unstained, 4%, 512
Filled: DC, 5%, 923
Thick: B16-MDSC, 30%, 2225
Thin: 293T-MDSC, 7%, 1080

**B**

![Arg-1 Ab and GADPH Ab blots for DC, 293T-MDSC, and B16-MDSC. The Arg-1 Ab shows a band at 40 kDa.](image)

Thin: unstained, 5%
Filled: DC, 72%, 5169
Thick: B16-MDSC, 63%, 4069
Dashed: 293T-MDSC, 56%, 3950
Fig. 4.9. Ex vivo B16-MDSCs express hallmark MDSC proteins iNOS, Arg-1, and TGFβ. (A-B) iNOS and Arg-1 protein expression were assessed by western blot (above) and intracellular flow cytometry (below). (C) Bioactive TGFβ was assessed by culturing SMAD-GFP cells with supernatants from myeloid cell cultures for 24 hours. Pooled data from three independent experiments.
4.2.9. *EX VIVO* MDSCs SUPPRESS ACTIVATED CD8 T CELL RESPONSES *IN VITRO*

While the extensive studies of the phenotype, proliferation, and differentiation of *ex vivo* MDSCs were important for the assessment of the MDSC differentiation system described in this thesis, the crucial characteristic of this cell type remains its suppressive capability. Firstly, I compared the capacities of MDSCs and BM-DCs to trigger a mixed lymphocyte reaction (MLR). In this way it could be tested whether the MDSCs could potentially act as stimulatory APCs. While immature BM-DCs from C57/BL6 mice were capable of inducing BALB/c CD8 and CD4 T cell proliferation, this was not the case for C57/BL6 MDSCs. This data suggested that *ex vivo* MDSCs were potentially poor APCs that were not competent in priming or activating T cells (Fig. 4.10.A). This result correlated with the fact that *ex vivo* MDSCs expressed low MHC I/II and co-stimulatory molecules (Fig. 4.5). However, this assay could not answer the question whether these *ex vivo* differentiated MDSCs could suppress activated T cells. Thus, standard T cell suppression assays were performed, to confirm their inhibitory activities over anti-CD3/CD28-activated T cells. This T cell suppression assay is widely used in the MDSC field, as it mimics the effect that MDSCs have on already activated lymphocytes. Hence, B16-MDSCs were co-cultured with anti-CD3/CD28-activated CD8 T cells, and their proliferation and IFNγ production was quantified. Suppression activities of bulk, monocytic, and granulocytic populations were assessed. All B16-MDSC subsets inhibited proliferation and IFNγ production in activated CD8 T cells within a wide range of MDSC:T cell ratios (Fig. 4.10.B). To further assess the MDSC suppressive activity compared to 293T-MDSCs and immature conventional BM-DCs Ki67 expression was assessed in previously anti-CD3/CD28-activated CD8 T cells. In contrast to conventional BM-DCs, which could increase proliferation of activated CD8 T cells, both MDSC types significantly inhibited Ki67 expression in target T cells (Fig. 4.10.C). MDSC numbers correlated to efficacy of T cell suppression (Fig. 4.10.B,C).
Fig. 4.10. Ex vivo MDSCs suppress proliferation of activated CD8 T cells. (A) Bar graphs represent percentage of proliferating CD4 and CD8 T cells in response to ex vivo MDSCs in a MLR. Briefly, BM cells from C57/BL6 mice were cultured with CM\textsuperscript{293T} and CM\textsuperscript{B16} for 5 days. Splenocytes from BALB/c mice were isolated and co-cultured with day 5 ex vivo MDSCs for 72h. Surface and intra-nuclear staining with CD4, CD8, and Ki67 antibodies was performed and cultures were analysed by flow cytometry. (B-C) CD8 T cells were isolated from spleens of healthy C57/BL6 mice and activated with anti-CD3/CD28 dynabeads. Suppression was assessed as function of proliferation loss. (B) CD8 T cells were incubated with CFSE prior to activation and co-culture with bulk, M- or G-MDSCs at decreasing ratios. 72h later CFSE dilution and IFNγ production were assessed. This experiment was performed by Julia Schwartz and Karine Breckpot. Pooled data from 3 experiments. (C) Similar to A, but loss of T cell proliferation was assessed by intra-nuclear Ki67 expression. Bar graphs represent one experiment of two similar repetitions. All samples in triplicate.
4.2.10. ADOPTIVE TRANSFER OF EX VIVO B16-MDSCs ENHANCES MELANOMA GROWTH IN VIVO

Ex vivo B16-MDSCs were capable of suppressing activated CD8 T cells in vitro. Further, it was tested whether ex vivo B16-MDSCs would retain their suppressive function in vivo, by enhancing B16 tumour growth. This would confirm the in vitro results in a more realistic experimental system. Thus, mice were subcutaneously injected with B16 melanoma cells, with one group of mice receiving three doses of ex vivo B16-MDSCs every 5–7 days. The first dose of B16-MDSCs was included in the initial B16 melanoma cell transfer. Mice that were injected with B16-MDSCs died significantly faster than control mice (Fig. 4.11.A). In addition, increased tumour growth rates were observed in the MDSC group (Fig. 4.11.B,C). Interestingly, in the first experiment, 2 out of 4 mice in the MDSC group died prior to establishing large tumours (Fig. 4.11.B). In the second experiment B16 cells were used that contained the firefly luciferase gene (B16-Fluc), so that tumour growth could be measured in an additional and unbiased way. In this experiment all mice were sacrificed due to large tumour burden. Average tumour growth was significantly faster in the B16-MDSC mouse group than in control mice (Fig. 4.11.C). 12 days after B16-Fluc transfer, mice were injected with luciferin to assess luciferase activity, which would correlate to the number of B16-Fluc cells present in the tumour (Fig. 4.11.D). While all mice had palpable tumours at this stage, the tumour in one control mouse was not large enough to emit a significant signal (Fig. 4.11.D). There was a trend on day 12 of increased photon emission by luciferase activity in B16-MDSC mice, albeit without significance (Fig. 4.11.E). Repetition of the assessment of luciferase activity at a later time-point may give statistically significant results. Nevertheless, this data suggested that ex vivo B16-MDSCs were capable of enhancing B16 melanoma growth in vivo as well as decreasing survival of B16 melanoma-bearing mice. Thus, the newly developed ex vivo B16-MDSC differentiation system was capable of producing large numbers of suppressive MDSCs that phenotypically and functionally resemble tumour-infiltrating MDSCs.
A

**Percent survival of 9-10 mice**

- B16
- B16 + B16-MDSCs

Days after subcutaneous B16 transfer:

- 0
- 16
- 20
- 22
- 24
- 26
- 32
- 34
- 36

*\( p=0.0336 \)

B

**Tumour growth B16 melanoma**

- B16
- B16 + B16-MDSCs

Days after subcutaneous B16 challenge:

- 8
- 11
- 14
- 16
- 18
- 21
- 22
- 28
- 32
- 35

C

**Tumour surface (mm<sup>2</sup>)**

- B16
- B16 + B16-MDSCs

Days after subcutaneous B16 transfer:

- 5
- 6
- 7
- 8
- 11
- 12
- 13
- 14
- 15
- 16
- 17
- 18
- 19

**\( p=0.0035 \)**
Fig. 4.11. Ex vivo B16-MDSC suppressive activity in vivo. (A-D) C57/Bl6 mice were subcutaneously inoculated with B16 melanoma cells in combination with (B16 + B16-MDSC) or without (B16 only control) B16-MDSCs. The B16-MDSC group additionally received B16-MDSCs every 5-7 days. Tumour volume and time of death were recorded.
(A) Kaplan-Meier survival plot representing pooled data from two independent experiments. (B) Tumour growth from experiment 1. 2 red symbols represent two mice that died prematurely without large tumour burden. (C-D) In this second experiment B16 cells expressing the firefly luciferase gene (B16-FLuc) were used. (C) The graph represents average tumour sizes from experiment 2. All B16-MDSC mice had established tumours larger than 150mm² at day 19. Error bars correspond to SEM. p-value represents column factor from 2-way anova analysis. (D) Light emission within mice, dorsal view (above, FW) and ventral view (below, RS). (E) Graphs represent photons/s/cm²/sr of luciferin emission. FW represents data from mice, dorsal view (left), RS represents mice, ventral view (right). Error bars correspond to SEM.
4.2.11. CM GENERATED FROM huGM-CSF AND huIL-4-EXPRESSING H1299 CELLS DIFFERENTIATES IMMATURE HUMAN MYELOID CELLS WITH CD8 T CELL SUPPRESSIVE CAPABILITIES.

As the development of the ex vivo mouse MDSC differentiation system showed promising results, I wondered whether the murine system could be translated to a human setting. Human MDSCs do not exhibit such a distinct phenotype as mouse MDSCs, as there is no homologue for GR-1 in humans. Human MDSCs are generally accepted to be CD11b+/CD33+HLA-DRlow with their main characteristic remaining their T cell suppressive activities. For this purpose, the human non-small cell lung carcinoma H1299 cell line was transduced with lentivectors co-expressing the huGM-CSF gene and the puromycin resistance gene. In addition, the selected huGM-CSF-expressing cells were transduced with a lentivector containing the huIL-4 gene as well as the blasticidin resistance gene. It is well-known that MDSCs express the IL4 receptor (IL4Rα) on their surface and aptamer-mediated blockade of IL4Rα induces mouse MDSC apoptosis in vivo and limits tumour progression (Roth, De La Fuente et al. 2012). Conditioning medium was collected and termed CMH1299. CM293T conditioning control medium was simultaneously produced by expressing the huGM-CSF and huIL-4 genes in 293T cells. The monocytic adherent fraction of PBMCs was incubated with CMH1299 for 3-5 days, after which cell viability began to decrease. A preliminary assessment of myeloid cell phenotype with increasing concentrations of CM was undertaken (Fig. 4.12.A). 10% CMH1299 was selected as a suitable culture concentration as cells in this condition had proliferated more than in other conditions (data not shown) and exhibited a phenotype consistent with MDSCs; CD33high CD14− CD83low DC-SIGNlow. In addition, on day 5 cells differentiated in 10% CM exhibited higher expression of CD33. Accordingly, CD33 expression was highest in myeloid cells differentiated in the presence of CMH1299, as compared to CM293T and RPMI only (Fig. 4.12.B-C). While DC-SIGN expression was low in all myeloid cell types, markers CD14 and CD83 were lower in CMH1299 and CM293T conditions. Importantly, on day 3 myeloid cells in CMH1299 were HLA-DR+. Differentiated cells in CM293T medium were HLA-DRlow, and in RPMI only the cells were HLA-DRhigh (Fig. 4.12.D). This data suggested that myeloid cell culture in CMH1299 yielded immature myeloid cells, which could be H1299-MDSCs. Further, it was tested whether these
immature myeloid cells possessed T cell suppressing capacities. To this purpose the lymphocyte fraction of PBMCs was activated with anti-CD3/CD28 activation beads before co-culture with H1299-MDSCs. H1299-MDSCs significantly inhibited human CD8 T cell proliferation, as measured by decreased nuclear Ki67 expression (Fig. 4.12.E). While H1299-MDSCs seemed to inhibit CD4 T cell responses, this was not found to be significant. Somewhat surprisingly, H1299-MDSCs were more suppressive in conditions with a higher lymphocyte:MDSC ratio. Taken together this preliminary data suggested that the ex vivo MDSC differentiation system could be translated to the human setting. However, this system will require further work by adding other cytokines to develop a highly efficient human MDSC differentiation system.
B

CD14 expression

unstained RPMI only 10% CM^{293T} 10% CM^{H1299}

CD33 expression

81% 11% 34% 61%

C

DC-SIGN expression

unstained RPMI only 10% CM^{293T} 10% CM^{H1299}

CD83 expression

2% 2% 87% 60% 59%

D

Filled: unstained
Dotted: RPMI only
Thin: 293T CM
Thick: H1299 CM

US: 5%, 420
RPMI: 67%, 1409
293T: 14%, 639
H1299: 4%, 455

Events

HLA-DR expression
Fig. 4.12. PBMCs cultured with CM$^{H1299}$ differentiate into immature myeloid cells with immunosuppressive capabilities. (A-D) The monocytic fraction of human PBMCs were cultured in CM$^{H1299}$ for 3-5 days before flow cytometric analysis. (A) Graphs represent percentages of indicated surface markers in myeloid cells for different concentrations of CM$^{H1299}$ on day 3 (left) and day 5 (right) of culture. No, indicates myeloid cells cultured in RPMI only. (B-C) Flow cytometry density plots representing the expression of the indicated markers in myeloid cells conditioned with RPMI only, 10% CM$^{293T}$ and CM$^{H1299}$ on day 3 of culture. (D) Histogram representing HLA-DR expression in myeloid cells conditioned with RPMI only, 10% CM$^{293T}$ and CM$^{H1299}$ on day 3 of culture. (E) Lymphocytes from human PBMCs were activated with anti-CD3/CD28 activation beads prior to co-culture with H1299-MDSCs. T cell proliferation was assessed by Ki67 intranuclear staining. Experiment only performed once, all values in triplicate.
4.3. DISCUSSION

The tumour micro- and macroenvironment affects the accumulation, differentiation, and function of many cell types, including myeloid cells. MDSCs have recently become prominent as one of the main tumour-induced suppressive cell types, hampering cancer immunotherapy approaches. Hence, it would be beneficial to assess immunostimulatory treatments on such suppressive cell types as MDSCs. The isolation, culture, and ex vivo differentiation of mouse tumour-infiltrating MDSCs remains challenging, expensive, time-consuming, and usually renders only low efficiencies (30-40%) (Escors, Liechtenstein et al. 2013). In this PhD study, I set out to establish a novel ex vivo differentiation system, using enhanced but endogenous GM-CSF expression from melanoma cells. GM-CSF is key to most ex vivo MDSC differentiation protocols (Valenti, Huber et al. 2006; Youn, Nagaraj et al. 2008; Highfill, Rodriguez et al. 2010; Marigo, Bosio et al. 2010; Morales, Kmiecik et al. 2010; Michels, Shurin et al. 2012; Kurkó, Vida et al. 2014), correlating its importance for in vivo MDSC mobilization in tumour-bearing hosts (Bayne, Beatty et al. 2012). Recombinant cytokines such as GM-CSF are usually produced by bacteria and thus exhibit different post-translational modifications as proteins produced in eukaryotic cells. I hypothesized that endogenous mouse GM-CSF expression by lentivector transduction could increase the efficiency of MDSC differentiation. High levels of endogenous GM-CSF were produced in this way (2.9±0.2 µg/ml, Fig. 4.2.B), which was delivered to mouse BM cells in combination with other B16 melanoma-derived factors, simulating altered myelopoiesis in a tumour-bearing host. Using this method large numbers of immature myeloid cells were obtained, which were equivalent to MDSCs in phenotype and suppressive function. Addition of high concentrations of recombinant GM-CSF alone was not as effective at inducing pure MDSC cultures (Fig. 4.4.C) and neither was addition of 293T supernatant in combination with recombinant GM-CSF (Fig. 4.4.B). This data has been further validated in collaboration with Prof. Karine Breckpot’s group at the Free University of Brussels. Using a colorectal cancer cell (CT26) system, recombinant GM-CSF in cancer cell conditioning medium was not as efficient as GM-CSF production from cancer cells themselves (Dufait, Schwarze et al. 2015).
Ex vivo differentiated MDSCs consisted of the two accepted subpopulations, M- and G-MDSCs, as assessed by Ly6C-Ly6G profiles and further differential markers such as CD49d (Fig. 4.4 and Fig. 4.6). While I did not show morphology images of sorted M- and G-MDSC populations of B16-MDSCs in this thesis, we showed that the morphology of sorted M- and G- CT26-MDSCs was consistent with monocytic and polymorphonuclear/granulocytic morphologies, respectively (Dufait, Schwarze et al. 2015). In most tumour models, the G-MDSC population preferentially expands, indicating that G-MDSCs represent the predominant MDSC population in tumour-bearing mice (Youn, Nagaraj et al. 2008; Talmadge and Gabrilovich 2013; Youn, Kumar et al. 2013). Accordingly, it was found in this PhD study that in ex vivo cultures the G-MDSC component increased over time until it was the predominant population (Fig. 4.7). When analysing MDSCs isolated from B16 tumour-bearing mice no significant differences between the percentage of the two MDSC subpopulations were found at different locations. Nonetheless, the predominant type within the tumour seemed to be M-MDSCs while in the spleen it was G-MDSCs (Fig. 4.8). The ex vivo MDSCs used for this experiment were from day 5 of culture, thus representing equal numbers of M- and G-MDSCs. MDSCs were isolated from tumours when they reached an average size of 90mm$^2$. Considering the accumulation of G-MDSCs over time it would be interesting to assess whether the G-MDSC population increases in tumours from mice with larger tumour burden. Using a 4T1 breast tumour model, it was shown that MDSCs within tumours are derived from splenic extra-medullary haematopoiesis (EMH) before infiltrating tumours. The spleen acted as a reservoir for G-MDSCs that could leave the spleen and circulate rapidly (Younos, Dafferner et al. 2012). Hence, it is tempting to hypothesize that the G-MDSCs within the spleen will be recruited to the tumour over time, leading to a reduction in the G-MDSC population in the spleen and an increase of the G-MDSC population within the tumour. Nevertheless, the frequency of M- versus G-MDSCs in various organs differs in various tumour models and also depends on progression of disease. It has to be stressed that in this PhD study I demonstrated that G-MDSCs are differentiated from M-MDSCs, and thus, both populations should not be considered as independent. This consideration is not taken in many of the published works. Data from a recent study working with collaborators in the colorectal cancer model did not find that implanting GM-CSF-expressing CT26 tumour cells led to different
percentages of G-MDSC populations in the spleen or tumours of these mice (unpublished data from (Dufait, Schwarze et al. 2015)). Interestingly, splenomegaly and an increase in the percentage of CD11b+ cells within tumours, but not CD11c+ or F4/80+ cells, were observed in mice implanted with CT26-GMCSF (Dufait, Schwarze et al. 2015). The increase of CD11b+ myeloid cells within CT26-GMCSF tumours further confirms the role of GM-CSF for mobilizing large amounts of myeloid cells within tumour-bearing hosts.

Tumour-infiltrating MDSCs are more prone to apoptosis, proliferate less, and lose plasticity of differentiation, as compared to splenic MDSCs (Younos, Dafferner et al. 2012). Similarly, M-MDSCs are more viable and retain higher proliferative capacities and differentiation plasticity than G-MDSCs (Escors, Liechtenstein et al. 2013; Youn, Kumar et al. 2013). There remains significant controversy about whether M- and G-MDSCs arise from independent precursors in vivo (Albeituni, Ding et al. 2013) or whether M-MDSCs could act as precursors to G-MDSCs (Ugel, Peranzoni et al. 2012; Youn, Kumar et al. 2013). I showed that in our system, purified M-MDSCs could serve as precursors for G-MDSCs. Moreover, this differentiation process was accompanied by a loss of both viability and proliferation (Fig. 4.7). Thus, G-MDSCs in tumours do not have a long life-span and need to be replenished by circulating or splenic MDSCs (Younos, Dafferner et al. 2012). This PhD thesis shows evidence that MDSC differentiation could be reverted to differentiation of conventional DCs by changing the medium (Fig. 4.7). It could be argued that DC differentiation was due to the proliferation of a small fraction of non-GR-1+ cells present within MDSC cultures. Thus, isolating M-MDSCs and G-MDSCs before culture in DC differentiation medium will be of value. Further, assessment of redirected myeloid cells in suppression or antigen-presentation assays would be of interest.

On the other hand, MDSCs are comprised of various myeloid progenitor cells at different stages of differentiation and conducting experiments with non-purified ex vivo differentiated MDSCs may therefore be of value. Using a system with immature MDSCs at various differentiation stages is advantageous to study MDSC targeting for therapy and also MDSC biology. How do MDSCs differentiate? Which tumour-derived factors induce changes in myeloid differentiation? Are there different precursors in vivo or a single common precursor? What causes the switch from splenic MDSCs to tumour-infiltrating MDSCs? As the study of MDSC biology is rather young and many questions
remain controversial, the system described in this PhD thesis will likely prove valuable to elucidate and clarify their biological mechanisms.

It was consistently found that *ex vivo* MDSC cultures contained a small fraction of CD11c⁺ cells. However, it was not assessed here whether they are DCs or can act as APCs, or even if they are CD11c⁺ MDSCs. It would certainly be of interest to purify these CD11c⁺ cells and test them in antigen presentation and suppression assays. So far, these experiments were not performed as bulk MDSCs were immunosuppressive *in vitro* and *in vivo*. Nevertheless, isolation of CD11c⁺ cells in MDSC cultures will be of value in the future. In addition, MDSCs are highly heterogenic and there are several researchers that claim that MDSCs can also be CD11c⁺ or CD11c<sub>low/interm</sub> (Umemura, Saio et al. 2008; Norian, Rodriguez et al. 2009; Zoso, Mazza et al. 2014). On the other hand, transferring these CD11c⁺ cells to a non-tumour environment could induce DC differentiation, which has been prevented so far in the tumour environment. This seems likely as culture of tumour-infiltrating MDSCs without tumour-derived factors drives their differentiation to DCs and macrophages. Similarly, transfer of tumour-infiltrating MDSCs to tumour-free mice results in DC and macrophage differentiation (Li, Pan et al. 2004; Gabrilovich and Nagaraj 2009; Narita, Wakita et al. 2009; Gabrilovich, Ostrand-Rosenberg et al. 2012). Our group and other researchers (Huang, Pan et al. 2006) found that MDSCs express the M-CSF receptor (CD115, Fig. 4.6), suggesting that they have the potential of differentiating into macrophages upon M-CSF ligation. Thus, MDSCs are not committed to development into immunosuppressive myeloid cells but can differentiate into immunostimulatory myeloid cells or immunosuppressive myeloid cells according to the specific signals they receive (Narita, Wakita et al. 2009).

Importantly, *ex vivo* B16-MDSCs phenotypically resembled *in vivo* tumour-infiltrating MDSCs induced by the same tumour type (B16 melanoma) as used for mimicking the tumour environment *in vitro* (Fig. 4.8). In contrast, splenic MDSCs isolated from B16 tumour-bearing mice exhibited a different phenotype from tumour-infiltrating and *ex vivo* B16-MDSCs. This is particularly important considering that peripheral and tumour-infiltrating MDSCs are not equivalent (Abe, Dafferner et al. 2010; Corzo, Condamine et al. 2010; Maenhout, Van Lint et al. 2013; Noman, Desantis et al. 2014). PD-L1 and Arg-1 expression were lower in splenic MDSCs than in tumour and *ex vivo* B16-MDSCs. Adapting our *ex vivo* MDSC differentiation system to colorectal cancer...
during a collaboration with Prof. Karine Breckpot’s group, it was confirmed that *ex vivo* CT26-MDSCs resembled tumour-infiltrating MDSCs rather than splenic subsets. Analogous to B16-MDSCs, tumour-infiltrating and *ex vivo* CT26-MDSCs expressed higher levels of PD-L1, Arg-1, and MHC II than splenic MDSCs (Dufait, Schwarze et al. 2015). This data agreed with a study using several murine tumour models, where tumour MDSCs exhibited enhanced PD-L1 expression. Interestingly, hypoxia could induce PD-L1 expression in splenic MDSCs. In addition, the authors did not observe differential expression of CD86, which is in contrast to the data presented here (Noman, Desantis et al. 2014). Of note, MDSC phenotypic differences may be caused by differences in tumour model or stage of tumour progression. In addition to hypoxia there are several other tumour-derived factors that can induce PD-L1 expression on myeloid cells in cancer patients. IL10 and VEGF, for example, have been shown to induce expression of PD-L1 in monocyte-derived DCs and PD-L1 blockade enhanced their T cell activation capabilities (Curiel, Wei et al. 2003). Similarly, the tumour environment can drive the up-regulation of Arg-1 expression in splenic MDSCs (Corzo, Condamine et al. 2010). Thus, splenic MDSCs seem to be activated by the tumour environment, through hypoxia-mediated and tumour-secreted factors, thus acquiring further suppressive functions.

Splenic MDSCs from tumour-bearing mice were shown to exhibit an intermediate phenotype in transcription levels between tumour MDSCs and immature myeloid cells from tumour-free mice. The authors found increased transcription of Arg-1, iNOS, Flt3L, and VEGF-A in tumour-infiltrating MDSCs (Abe, Dafferner et al. 2010). This suggests that splenic MDSCs are an intermediate stage between immature myeloid cells and pathologically activated MDSCs within the tumour. Nevertheless, as MDSCs in the spleen suppress T cells in an antigen-specific way they possess certain suppressive functions (Ugel, Peranzoni et al. 2012).

Further differences between 293T-MDSCs and B16-MDSCs highlighted the effect of tumour-derived factors. Using the system described in this PhD thesis, *ex vivo* MDSCs can be generated for a wide range of tumour types, which is important as MDSCs exhibit various phenotypes and functions according to tumour type (Poschke and Kiessling 2012; Solito, Marigo et al. 2014). In fact, Dufait et al. have already successfully applied this system to the study of colorectal cancer, in collaboration with our group. In agreement with data presented in this thesis, *ex vivo* differentiated CT26-MDSCs were
equivalent to tumour-infiltrating rather than splenic MDSCs. In this study, inhibition of iNOS or Arg-1 could overcome MDSC suppressive activity (Dufait, Schwarze et al. 2015).

*Ex vivo* B16-MDSCs expressed hallmark MDSC suppressive proteins Arg-1, iNOS, and TGFβ (Rodriguez, Hernandez et al. 2005; Huang, Pan et al. 2006; Umemura, Saio et al. 2008; Gabrilovich, Ostrand-Rosenberg et al. 2012; Raber, Thevenot et al. 2014) (Fig.4.9). Interestingly, *ex vivo* MDSCs did not express higher levels of Arg-1 than immature BM-DCs. Several studies showed that Arg-1 is not always essential for MDSC suppressive activity. In melanoma patients suppressive function may not depend on Arg-1 activity at all (Filipazzi, Valenti et al. 2007). In a study of human breast cancer MDSCs, suppressive function pre-dominantly depended on other enzymes such as IDO (Yu, Du et al. 2013). It has also been suggested that IL4 and IL13 are necessary for MDSC activation, up-regulation of Arg-1, and suppressive activity (Gabrilovich and Nagaraj 2009). Tumour-associated DCs have also been shown to use Arg-1 (Norian, Rodriguez et al. 2009). Thus, Arg-1 expression is dependent on the tumour environment and may therefore be expressed in immature DCs to similar levels as in certain MDSC types. Moreover, it has been suggested that the co-expression of Arg-1 and iNOS is a hallmark of MDSCs, rather than the expression of only one of the enzymes. This is due to the complex relationship of the two enzymes, which share the same substrate. iNOS activity in cells where L-arginine has been depleted by Arg-1 shifts production from predominantly NO to predominantly $O_2^-$ (Talmadge 2007). The *ex vivo* differentiated B16-MDSCs co-expressed Arg-1 and iNOS while immature BM-DCs and 293T-MDSCs only expressed Arg-1.

Further, *ex vivo* B16-MDSCs exhibited enhanced bioactive TGFβ secretion while IL10 secretion could not be detected (Fig. 4.9). Both cytokines are implicated in MDSC suppressive activities. However, while TGFβ is constitutively secreted by MDSCs, IL10 secretion is induced upon IFNγ stimulation (Huang, Pan et al. 2006). TGFβ secretion and iNOS activity can be further enhanced by IFNγ stimulation from activated T cells (Huang, Pan et al. 2006). This data highlights the effect of the inflammatory environment on MDSC activity, activating MDSCs to acquire further suppressive functions. Similarly, TLR4 signalling enhances MDSC activity and induces IL10 secretion, skewing macrophages to an M2 type with decreased IL12 secretion (Bunt, Clements et al. 2009). TLR4 signalling in immature DCs on the other hand leads to DC maturation, IL12 production, immune stimulation, and anti-tumour activities (Cisco, Abdel-Wahab et al. 2004; Bekeredjian-
Ding, Roth et al. 2006; Apetoh, Ghiringhelli et al. 2007; Breckpot and Escors 2009; Liechtenstein, Dufait et al. 2012). Treatment with LPS, a ligand of TLR4, led to a phenotypic maturation of immature BM-DCs but not of ex vivo differentiated MDSCs (Fig. 4.4). The only marker that was up-regulated in ex vivo MDSCs but not in BM-DCs was Ly6G. Interestingly, this result may suggest that TLR4 signalling in ex vivo MDSCs may enhance G-MDSC differentiation.

Importantly, ex vivo MDSCs did not activate T cells in a MLR in vitro (Fig. 4.10). Low MHCI/II and co-stimulatory molecule expression in MDSCs thus correlated with poor APC capabilities. As poor antigen presentation is not an indication of immune suppression, MDSCs were further assessed in suppression assays, where they inhibited activated CD8 T cell proliferation. Transferring ex vivo B16-MDSCs together with B16 melanoma cells enhanced tumour growth and decreased survival in mice (Fig. 4.11). Thus, it is clear that ex vivo differentiated B16-MDSCs exhibit T cell suppressive and tumour-promoting activities, mirroring their function in vivo.

In this PhD study, some preliminary results on the establishment of an ex vivo human MDSC differentiation system were presented. PBMCs differentiated with CM from transduced H1299 lung cancer cells exhibited an immature myeloid cell type (Fig. 4.12.A-D). Importantly, human ex vivo H1299-MDSCs could suppress activated CD8 lymphocyte proliferation in vitro. In this experiment H1299-MDSCs were more suppressive when present at lower ratios (Fig. 4.12.E). Several explanations for this data come to mind. One reason could be that MDSCs proliferate and activate in the presence of activated T cells (Nagaraj, Youn et al. 2013). Supporting this hypothesis, I have observed enhanced mouse ex vivo MDSC proliferation (Ki67 expression) in the presence of activated T cells (data not shown). Alternatively, MDSCs at large quantities could suppress each other, thus leading to reduced suppressive function. The simplest explanation would be due to human error, for example in labelling of FACS tubes or culture plates. Although H1299-MDSCs were suppressive, further work to improve the human system is needed and inclusion of further cytokines will be of value. So far, CM containing human GM-CSF and IL4 was used. Several groups found that IL6 and IL13 favour MDSC development (Highfill, Rodriguez et al. 2010; Marigo, Bosio et al. 2010). Next steps to improve the system will be the cloning of IL6 and other cytokines and growth factors to produce CM with several tumour-derived factors.
Taken together, a novel *ex vivo* system to efficiently differentiate large numbers of tumour-infiltrating MDSCs was established. The MDSCs were extensively studied according to what is generally accepted in the literature, and compared to their *in vivo* counterparts. B16-MDSCs effectively suppressed T cell proliferation *in vitro* and enhanced tumour growth *in vivo*. Importantly, this *ex vivo* MDSC differentiation system has already been validated by other researchers in collaboration with our group. Treatment of *ex vivo* differentiated B16-MDSCs predicted therapeutic efficacy of an anti-tumour mRNA strategy (Van der Jeught, Joe et al. 2014). Further, our system was translated for the study of colorectal cancer, by transducing a colorectal cancer cell line CT26 with our lentivector expressing mouse GM-CSF and the puromycin resistance cassette (Dufait, Schwarze et al. 2015).
CHAPTER 5: USE OF EX VIVO MDSCs IN IN VITRO ASSAYS AND HIGH-THROUGHPUT STUDIES FOR THE IDENTIFICATION OF THERAPEUTIC TARGETS

5.1. INTRODUCTION

In the last chapter an efficient ex vivo MDSC differentiation system was developed that could generate large numbers of MDSCs without the need of inducing tumours in mice. Isolation of tumour-infiltrating MDSCs only renders small numbers of MDSCs and thus several tumour-bearing mice need to be sacrificed to achieve sufficient numbers for in vitro assays, let alone high-throughput studies. As the ex vivo B16-MDSCs were found to be equivalent to B16 tumour-infiltrating MDSCs, they were used to further test whether the lentivector vaccines generated in Chapter 3 could counteract MDSC suppressive capacities. Furthermore, high-throughput quantitative proteomics studies were carried out to assess the differences between MDSCs and immature DCs, and identify novel molecular targets in MDSCs that could be therapeutically exploited.

5.1.1. REGULATORY AND SIGNALLING PATHWAYS IN MDSCs

The identification and exploitation of molecular targets in MDSCs for therapeutic intervention requires a deep understanding of the pathways that regulate MDSC differentiation, expansion and suppressive activities. In this introduction I will briefly summarise what is known about MDSC intracellular pathways and their exploitation as therapeutic targets.

As tumours grow they establish a microenvironment characterized by hypoxia, inflammation, low extracellular pH, and presence of high amounts of ROS, RNS, growth factors, and other tumour metabolites. I already discussed tumour-secreted factors such as VEGF, IL6, IL13, IL4, IL10, IFNγ, GM-CSF, and G-CSF, which influence myeloid differentiation and MDSC accumulation. These tumour-mediated effects activate several intracellular signalling pathways within MDSCs, thus mediating MDSC accumulation, differentiation, and function. Several intracellular pathways are of importance in this
regard and include STAT1, STAT3, STAT6, NFκB, PI3K/Akt, and ERK. Some of these pathways could be inhibited using selected drugs for therapeutic purposes. Signal transducer and activator of transcription 3 (STAT3) is one of the best-characterized MDSC signalling molecules. STAT3 is activated in a variety of tumour cells and tumour-associated immune cells (Yu, Kortylewski et al. 2007; Xin, Zhang et al. 2009). Many tumour-secreted factors, including GM-CSF, G-CSF, IL6, IL10, and IL1β induce signalling routes that use the Jak/STAT3 pathway, in this way enhancing MDSC development, accumulation, and function (Nefedova and Gabrilovich 2007; Corzo, Cotter et al. 2009; Condamine and Gabrilovich 2011) (Gabrilovich and Nagaraj 2009) (Fig. 5.1). Tumour-infiltrating MDSCs exhibit increased levels of STAT3 phosphorylation and its activation was associated with MDSC expansion in vitro and in vivo (Nefedova, Huang et al. 2004; Nefedova, Nagaraj et al. 2005). Further, sunitinib-mediated inhibition of STAT3 leads to a decrease in MDSC infiltration in a renal cell carcinoma mouse model (Xin, Zhang et al. 2009). Thus STAT3 signalling mediates MDSC expansion and tumour infiltration. STAT3 signalling is also involved in MDSC differentiation by inducing abnormal myeloid differentiation in cancer-bearing hosts (Gabrilovich 2004; Nefedova, Huang et al. 2004). G-CSF activates STAT3, which induces the expression of C/EBPβ, leading to myeloid progenitor proliferation and differentiation to functional MDSCs (Marigo, Bosio et al. 2010; Zhang, Nguyen-Jackson et al. 2010). Further STAT3-target genes include Bcl-xL, Cyclin D1, Survivin, c-myc. These proteins have anti-apoptotic and proliferative functions while blocking myeloid differentiation (Nefedova and Gabrilovich 2007). STAT3-target genes implicated in MDSC expansion include the S100A8 and S100A9 proteins, which inhibit DC differentiation and favour MDSC accumulation. S100A8/S100A9 was suggested to help in the formation of NADPH oxidase, which produces ROS in myeloid cells and in this way prevents myeloid differentiation. S100A8/A9 heterodimers facilitate the accumulation of MDSCs through blocking differentiation of myeloid precursors (STAT3-dependent) and MDSC chemoattraction to tumour (NF-κB-dependent) (Cheng, Corzo et al. 2008; Sinha, Okoro et al. 2008; Ostrand-Rosenberg and Sinha 2009). Interestingly, S100A9 over-expression can induce MDSC accumulation in mice without cancer (Cheng, Corzo et al. 2008). It has recently been demonstrated that the S100A protein family can be targeted to deplete MDSCs in vivo and activate the host immune response against cancer cells (Qin, Lerman et al. 2014). Activated STAT3 has also been
shown to down-regulate Protein kinase C βII (PKCβII) in myeloid cells. PKCβII is involved in DC differentiation, thus its inhibition favours MDSC development by preventing progenitor differentiation (Condamine and Gabrilovich 2011). Taken together, constitutive STAT3 activation prevents differentiation of myeloid cells. In fact, STAT3 signalling enhances proliferation and survival of myeloid progenitors, leading to MDSC accumulation (Corzo, Cotter et al. 2009). In addition, STAT3 signalling plays a role in inducing suppressive functions in MDSCs. STAT3 signalling in human MDSCs regulates Arg-1 activity (Vasquez-Dunddel, Pan et al. 2013) and induces IDO-mediated immunosuppression through the activation of the non-canonical NF-κB pathway (Yu, Wang et al. 2014). Further, STAT3 signalling up-regulates the expression of NADPH subunits (Nox2) in MDSCs. This mechanism facilitates increased release of ROS upon contact with other cells (Corzo, Cotter et al. 2009). It could therefore be argued that STAT3 is one of the main transcription factors leading to MDSC differentiation, accumulation, tumour infiltration, and suppressive function in tumour-bearing hosts (Corzo, Cotter et al. 2009) (Fig. 5.1).

Cytokines IFNγ and IL1β primarily signal through the STAT1 transcription factor, while IL13 and IL4 primarily signal through STAT6 (Condamine and Gabrilovich 2011) (Fig. 5.1). STAT1-mediated IFNγ signalling induces iNOS expression (Huang, Pan et al. 2006; Movahedi, Guilliams et al. 2008) and is implicated in Arg-1 activity (Condamine and Gabrilovich 2011), both used by MDSCs for T cell suppression. Blocking IFNγ overcomes MDSC-mediated immune suppression by inhibiting iNOS upregulation (Kusmartsev and Gabrilovich 2005). In addition, STAT1 activation by IFNγ induces the expression of Bcl2a1, leading to G-MDSC survival (Medina-Echeverz, Haile et al. 2014). Tumour-secreted IFNγ further causes induction or increased secretion of IL10, iNOS, and TGFβ (Huang, Pan et al. 2006). STAT6 activation by IL13 or IL4 causes increased Arg-1 activity and TGFβ production by MDSCs (Condamine and Gabrilovich 2011). The IL4Rα/STAT6 signalling pathway is considered to be important for MDSC survival as aptamer-mediated blockade of IL4Rα triggers apoptosis of MDSCs and limits tumour progression in a 4T1 mammary carcinoma mouse model (Roth, De La Fuente et al. 2012). On the other hand, a study using IFNγ-, IFNγR-, and IL4Rα-deficient mice suggested that MDSC accumulation and function do not depend on IFNγ- or IL4Rα-mediated signaling (Sinha, Parker et al. 2012).
In MDSCs, TLR signalling and hypoxia can activate the NFκB pathway, which in turn activates pro-inflammatory proteins Cox2 and PGE$_2$ (Eruslanov, Daurkin et al. 2010; Gabrilovich, Ostrand-Rosenberg et al. 2012) (Fig. 5.1). Inhibiting Cox2 prevents the differentiation from BM precursors to MDSCs (Eruslanov, Daurkin et al. 2010). PGE$_2$ is widely described in relation to MDSCs and is believed to regulate Arg-1 and iNOS activity (Rodriguez, Hernandez et al. 2005; Donkor, Lahue et al. 2009), thereby inducing immunosuppressive MDSC functions. S100A9 has been found to be important for MDSC recruitment to tumour tissues, and S100 protein signalling can be mediated by the NF-κB pathway. MDSCs also synthesize S100 proteins and secrete them. As MDSCs express surface glycoprotein receptors that can bind to S100 proteins, there exists an autocrine feedback loop for MDSC accumulation (Sinha, Okoro et al. 2008). Thus, the S100A family of proteins could be key molecular targets for anti-MDSC therapies.

Phosphoinositide 3-kinase (PI3K) signalling is known to regulate chemotaxis, phagocytosis, ROS production, and apoptosis in macrophages and neutrophils (Trikha and Carson 2014). Defective PI3K/Protein kinase B (Akt) signalling has recently been linked to accumulation of immunosuppressive immature myeloid cells in aging mice (Enioutina, Bareyan et al. 2011). MDSCs from tumour-bearing mice exhibit Akt hyperphosphorylation (Pilon-Thomas, Nelson et al. 2011) and Akt activation has recently been shown to play a role in MDSC activity and proliferation (Liu, Lai et al. 2012). The PI3K pathway thus regulates transcription factors that control proliferation and survival of MDSCs. It therefore has been suggested that PI3K may play a central role in regulating MDSC biology and that it may be an effective MDSC-depleting target (Trikha and Carson 2014). Extracellular signal-regulated kinase (Erk) signalling is also implicated in MDSC biology. Previous work from our laboratory has shown that activation of the Erk pathway in mouse and human DCs down-modulates positive co-stimulatory molecules (especially CD40 and MHC molecules). This led to differentiation of tolerogenic DCs that could skew T cell differentiation toward Treg cells. In addition, Erk activation in mouse DCs leads to production of bioactive TGFβ (Escors, Lopes et al. 2008; Arce, Breckpot et al. 2011). Others have also shown the regulatory role of Erk, leading to inhibition of DC maturation in mouse and human DCs (Puig-Kroger, Relloso et al. 2001). Erk also mediates immunosuppressive activities in macrophages (Xiao, Malcolm et al. 2002). An interesting study showed that tumour cell line-derived GM-CSF activated Erk within ex vivo MDSCs,
which led to MDSC expansion (Chalmin, Ladoire et al. 2010). Further confirming the role of these pathways was the observation that STAT3, p38/MAPK (mitogen-activated protein kinase), Erk1/2, and NF-κB exhibited increased phosphorylation and thus activation in MDSCs in vivo (Wu, Yan et al. 2012). However, the importance of these pathways may not be universal in MDSCs as tumour-associated myeloid cells differ between tumour types (Elpkek, Cremasco et al. 2014).

Hypoxia-mediated MDSC accumulation, differentiation, and function was recently linked with MAPK, PI3K/Akt, and NF-κB signalling (Zhu, Tang et al. 2014), linking these signalling pathways to MDSC activity and hypoxia. Thus, Gabrilovich and his colleagues have proposed a two-stage model of MDSC involvement in cancer progression (Condamine and Gabrilovich 2011; Gabrilovich, Ostrand-Rosenberg et al. 2012). The first stage is represented by altered myelopoiesis, which prevents myeloid differentiation and mobilizes large amounts of immature myeloid cells. Tumours secrete factors (such as GM-CSF, G-CSF, IL6, and VEGF) that signal through STAT3 and other transcription factors, mobilizing and recruiting immature myeloid cells to the tumour tissue (Fig. 5.1). The second stage is initiated within the tumour microenvironment. There, pro-inflammatory molecules such as IFNγ, IL1β, IL13, and toll-like receptor ligands (TLRLs) bind their receptors on MDSCs, initiating regulatory networks that lead to the acquisition of immunosuppressive activities. Alternatively, MDSCs can differentiate into TAMs, TANs, and tolerogenic DCs. In addition, hypoxia and other tumour metabolites influence the second stage of MDSC development (Gottfried, Kreutz et al. 2008; Gabrilovich, Ostrand-Rosenberg et al. 2012; Husain, Huang et al. 2013). NFκB, STAT1, and STAT6 activation, for example, lead to up-regulation of iNOS, Arg-1, NO (nitric oxide), and other immunosuppressive cytokines (Condamine and Gabrilovich 2011; Gabrilovich, Ostrand-Rosenberg et al. 2012). Importantly, immunosuppressive activities vary according to tumour type as this will influence which regulatory pathways are activated and to what extent.
**Fig. 5.1. Intracellular regulatory pathways within MDSCs.** Scheme represents the regulatory pathways and its effects on MDSC differentiation, accumulation, and function. From top to bottom, the highest level represents tumour-secreted factors. The second level represents the transcription factors that are activated by various tumour-secreted factors. The third level represents the genes and down-stream effector proteins that are up-regulated/activated by the transcription factors. The last level represents the effect of the up-regulated proteins on MDSCs. Figure adapted and modified from Gabrilovich et al, Nat Rev Immunol, Vol. 12(4), 253-68, 2012 (Gabrilovich, Ostrand-Rosenberg et al. 2012).

### 5.1.2. THE ROLE OF REACTIVE OXYGEN AND NITROGEN SPECIES IN MDSC BIOLOGY

Reactive oxygen species (ROS) are present at high levels in MDSCs from tumour-bearing mice in various tumour models and cancer patients (Nagaraj, Gupta et al. 2007; Corzo, Cotter et al. 2009). ROS include the superoxide anion ($\text{O}_2^-$), hydrogen peroxide ($\text{H}_2\text{O}_2$), and the hydroxyl radical (OH). Superoxide anion is either generated by mitochondrial electron leakage or NADPH oxidases (Kang, Lee et al. 2015). In leukocytes, the primary source of ROS in response to stimuli is NADPH oxidase (Nox2) assembly, which consists of two membrane proteins and three cytosolic subunits, which are associated with Rac (Rho family GTPase) (Groemping and Rittinger 2005). NADPH catalyzes the one electron reduction of oxygen to superoxide anion. The electrons are supplied by NADPH (Corzo, Cotter et al. 2009). In murine tumour-infiltrating MDSCs two subunits of Nox2 were found to be up-regulated (gp91$^{\text{phox}}$ and p47$^{\text{phox}}$) and this was
responsible for the induction of ROS and T cell suppression. Moreover, this process was STAT3-dependent (Corzo, Cotter et al. 2009).

Superoxide anions can react directly with proteins or can react with nitric oxide (NO) to produce peroxynitrite (ONOO\(^{-}\)), a RNS (Alvarez and Radi 2003). Superoxide anions can also be converted to H\(_2\)O\(_2\) by a free electron or superoxide dismutase (SOD) activity. H\(_2\)O\(_2\) can be converted to OH, which is detrimental to cells as it is the most reactive ROS. The OH radical attacks proteins, DNA, and membrane lipids, inducing irreversible changes. Superoxide anion and H\(_2\)O\(_2\) are milder as they cause reversible DNA and protein oxidation. To protect themselves from ROS damage, cells contain peroxidase enzymes (such as peroxiredoxin, glutathione peroxidase, and catalase) to reduce H\(_2\)O\(_2\) to water before it can be converted to OH radicals. Peroxidase enzymes obtain the reducing power from NADPH. Thus, NADPH is needed for ROS homeostasis and is supplied by energy metabolic pathways such as the pentose phosphate pathway (PPP) (Kang, Lee et al. 2015).

It is well established that ROS (such as H\(_2\)O\(_2\)) and RNS (such as NO) exert detrimental effects on NK and T cells (Mougiakakos, Johansson et al. 2009), and that MDSCs from cancer patients and tumour-bearing mice suppress T cells through these reactive molecules (Talmadge 2007; Kusmartsev, Su et al. 2008; Youn, Nagaraj et al. 2008; Gabrilovich and Nagaraj 2009). NADPH oxidase, Arg-1, and iNOS work in concert to produce peroxynitrite and H\(_2\)O\(_2\) to induce T cell blockage (Gabrilovich, Ostrand-Rosenberg et al. 2012). Peroxynitrite indirectly nitrates tyrosine residues but also directly reacts with tryptophan, methionine, and cysteine residues (Alvarez and Radi 2003). H\(_2\)O\(_2\), superoxide anion, NO, and peroxynitrite can oxidize cysteine thiols on proteins, which causes structural changes and thus controls protein activity (Kang, Lee et al. 2015). As ROS and peroxynitrite are highly reactive they are short-lived, and their action is hence limited to short distances. It has therefore been suggested that the immunological synapse during antigen presentation may be a good environment for the action of such molecules (Nagaraj, Gupta et al. 2007). There are three main ROS- and RNS-mediated molecular blocks acting on T cell activity. First, nitration (by peroxynitrite) of the CD8 TCR induces a conformational change, thus preventing its recognition of MHC peptides. This process negatively regulates antigen-specific CD8 T cell activity. Interestingly it has been shown that these cells are still responsive to unspecific
activation (Nagaraj, Gupta et al. 2007; Condamine and Gabrilovich 2011). Second, H$_2$O$_2$ leads to a loss of the CD3  chain and thus to truncated TCR signalling and T cell cytokine production (Schmielau and Finn 2001). Third, NO activity can block IL2R-mediated signalling, thus limiting T cell proliferation (Mazzoni, Bronte et al. 2002).

5.1.3. THE ROLE OF HYPOXIA IN MDSC BIOLOGY

Hypoxia alters the energy metabolism in cells/MDSCs (Corzo, Condamine et al. 2010; Liu, Bi et al. 2014), leading to abnormal respiratory metabolic pathways. This in turn influences the energy balance and hence ROS balance in cells (Kang, Lee et al. 2015). But hypoxia affects MDSCs in additional ways. The hypoxic tumour microenvironment recruits and activates MDSCs (Corzo, Condamine et al. 2010; Zhu, Tang et al. 2014). The interaction between HIF-α/MIF (Hypoxia inducible factor/Macrophage migration inhibitory factor) and NF-κB/IL6 axes plays an important role in the hypoxia-induced accumulation of MDSCs and tumour growth in HNSCC (head and neck squamous cell carcinoma) (Zhu, Tang et al. 2014). Further, hypoxia up-regulates COX-2, which leads to production of PGE$_2$. PGE$_2$ inhibits DC maturation, expression of co-stimulatory molecules, IL12 secretion or it can skew their differentiation to tolerogenic DCs. In addition, PGE$_2$ can stimulate MDSC immunosuppressive functions (such as Arg-1 expression) by binding to the EP-4 (Prostaglandin E receptor 4) receptor. PGE$_2$ secretion by MDSCs affects the capacity of CD8 T cells to recognize antigen on tumour cells and prevents tumour cell apoptosis (Rodriguez, Hernandez et al. 2005; Sinha, Clements et al. 2007; Serafini 2010; Chouaib, Messai et al. 2012).

Hypoxia within the tumour can further affect the activity of MDSCs, enhancing their suppressive capacities. Tumour-infiltrating MDSCs express higher levels of PD-L1 than splenic MDSCs and this is mediated by hypoxia. Blocking PD-L1 impaired antigen-specific and unspecific MDSC suppressive function (Noman, Desantis et al. 2014). Splenic MDSCs were shown to suppress T cell function in an antigen-specific way while tumour MDSCs could also suppress using antigen-unspecific mechanisms. Hypoxia conferred this capability unto splenic MDSCs. In addition, the hypoxic tumour microenvironment caused the up-regulation of iNOS and Arg-1 in splenic MDSCs (Corzo, Condamine et al. 2010; Liu, Bi et al. 2014).
MDSCs were also shown to differentiate into TAMs under hypoxic conditions (Corzo, Condamine et al. 2010; Noman, Desantis et al. 2014). It could therefore be argued that hypoxia plays an important role in the second stage of MDSC development, where MDSCs acquire additional suppressive capacities.

5.1.4. THE EFFECT OF IL12 ON MDSCs

It is becoming more evident that the IL-12R is expressed on tumour cells, myeloid cells, and the tumour stroma (Trinchieri 2003; Airoldi, Di Carlo et al. 2007; Pistoia, Cocco et al. 2009; Steding, Wu et al. 2011). While the effect of IL12 on T cells is well-characterized it is less so on myeloid cells. An analysis of the B16 tumour environment showed that myeloid cells formed the majority of cells that could respond to IL12 by expressing the IL12R-2β (Kerkar, Goldszmid et al. 2011). Mice deficient for IL12 produce macrophages that have a bias toward the M2 phenotype, suggesting a role for IL12 in macrophage polarization (Bastos, Alvarez et al. 2002). IL12 provided in microspheres altered the function of tumour-infiltrating macrophages. This was exhibited by a rapid reduction of tumour-supporting molecules such as IL10, MCP-1 (monocyte chemoattractant protein 1), MIF (migration inhibitory factor), and TGFβ. At the same time enhanced production of TNFα, IL15, and IL18 was observed (Watkins, Egilmez et al. 2007). However, it has to be noted that the authors purified macrophages by CD11b+ expression, indicating that this population did not only consist of macrophages but possibly all myeloid cells within the tumour. Inducible IL12 in adoptive CAR-T cells also led to an increase in tumour-infiltrating macrophages, which together with TNFα induction, was crucial to the anti-tumour response. Importantly, this treatment was also effective against tumours that had lost TAA expression (Chmielewski, Kopecky et al. 2011). An increase in myeloid cell tumour infiltration is only positive if these cells possess anti-tumour cytotoxic activities. IL12 released by tumour-specific T cells was found to act directly on BM-derived cells within the tumour and convert tumour-infiltrating myeloid cells into functional APCs. The authors demonstrated enhanced expression of genes associated with antigen presentation in bulk tumour-infiltrating myeloid cells exposed to IL12-secreting T cells. Myeloid cells purified from tumours treated with IL12-secreting T cells could stimulate T cell proliferation ex vivo, while cells
purified from mock-treated tumours could not (Kerkar, Goldszmid et al. 2011). In a subsequent study using the same engineered T cells, IL12 induced the up-regulation of CD95 (Fas) on macrophages, MDSCs, and DCs within the tumour. Fas expression caused Fasl-expressing CD8 T cells to proliferate via a co-stimulatory reverse signal. This led to the collapse of the tumour stroma and infiltration of CTLs and memory CD8 T cells (Kerkar, Leonardi et al. 2013). Other researchers recently showed that adenoviral-mediated delivery of IL12 decreased MDSC numbers by 50% as well as altered their phenotype (higher CD80 and MHC II) in a glioma mouse model. In addition, there was an increase in DC numbers. Interestingly, MDSC depletion did not abrogate IL12-mediated prolongation of survival (Thaci, Ahmed et al. 2014). Nonetheless, the specific effects of IL12 on MDSCs are poorly known, and it therefore remains an interesting area to research. Importantly, IL12 exerts not only positive effects on T cells but also on myeloid cell populations within the tumour. This could be partly explained by the fact that tumours use several mechanisms that block IL12 production by DCs and macrophages.

5.1.5. THE ROLE OF PD-L1 IN MDSC BIOLOGY

Many tumours express PD-L1 and PD-L1 negative tumour cells up-regulate PD-L1 expression upon treatment with IFNγ (Blank, Gajewski et al. 2005; Yang, Chen et al. 2008; Yang, Li et al. 2009), again emphasizing the importance of inflammation in tumour escape. MDSCs in patients (Andorsky, Yamada et al. 2011; Christiansson, Söderlund et al. 2013; Zhang, Wang et al. 2013) and mice (Noman, Desantis et al. 2014) express elevated PD-L1, although this may vary depending on tumour type (Noman, Desantis et al. 2014). PD-L1 was also up-regulated in tumour-infiltrating macrophage samples from human B cell lymphoma patients. The authors performed immunohistochemistry studies and classified macrophages as CD68+ cells (Chen, Chapuy et al. 2013). Interestingly, in this thesis, both ex vivo murine macrophages and MDSCs expressed CD68. PD-L1 was up-regulated on tumour-associated blood monocyte-derived myeloid dendritic cells from ovarian carcinoma patients (tissue and draining lymph nodes). And PD-L1 mediated T cell suppression, as PD-L1 blockade partially reversed T cell suppression (Curiel, Wei et al. 2003). In contrast, other studies did not encounter differences in PD-L1 expression between MDSCs from tumour-bearing patients and mice, and immature myeloid cells
from healthy counterparts (Youn, Nagaraj et al. 2008; Zhang, Wang et al. 2013). Of note, these studies were performed on circulating or spleen MDSCs and thus had not been further activated by the tumour environment. Data from other researchers and data presented in this thesis has shown that tumour-infiltrating MDSCs express higher levels of surface PD-L1 than splenic MDSCs (Noman, Desantis et al. 2014). The importance of PD-L1 for MDSC-mediated immune suppression varies. Some studies show that PD-L1 blockade in MDSCs can overcome their suppressive activities (Chikamatsu, Sakakura et al. 2012; Noman, Desantis et al. 2014) while others only observed up-regulated IL2 production but no T cell proliferation (Christiansson, Söderlund et al. 2013).

5.1.6. THE EFFECT OF CHEMOTHERAPY ON MDSCs

Several chemotherapeutic agents have immunostimulatory properties, playing a role in their anti-tumour efficacy. Many of these chemotherapeutic drugs can up-regulate maturation and function of mouse and human DCs at non-cytotoxic doses, including vincristine, vinblastine, paclitaxel, doxorubicin, methotrexate, mitomycin C, and 5-aza-2-deoxycytidine (Kaneno, Shurin et al. 2009). Interestingly, this maturation was dependent on IL12 in the case of paclitaxel, methotrexate, doxorubicin, and vinblastine. In addition, they regulate signal transduction pathways in DCs and increase tumour recognition by DCs and CTLs (Shurin, Tourkova et al. 2008; Shurin, Tourkova et al. 2009; Kaneno, Shurin et al. 2011; Michels, Shurin et al. 2012). Paclitaxel at non-cytotoxic concentrations suppresses the induction of tumour-mediated regulatory DC differentiation in vitro and in vivo. Interestingly, regulatory DC induction was assessed by a down-regulation of CD11c and up-regulation of CD11b, which does not necessarily correlate with immunostimulatory activity (Zhong, Gutkin et al. 2014). Nevertheless, paclitaxel has been shown to promote the differentiation of MDSCs into DCs in vitro (Michels, Shurin et al. 2012) and was shown to decrease accumulation of tumour-infiltrating MDSCs in melanoma-bearing mice, without alterations in BM haematopoiesis. Interestingly, this effect was associated with inhibition of p38 MAPK activity, S100A9 expression, and TNFα production, which restored CD8 T cell effector functions and led to increased survival and diminished tumour burden (Sevko, Michels
et al. 2013). Similarly, docetaxel induces the up-regulation of several maturation markers in MDSCs (MHCIi, CD11c, CD86) (Kodumudi, Woan et al. 2010).

Certain chemotherapeutic treatments at cytotoxic concentrations selectively inhibit or target MDSCs, which significantly enhances their efficacy (Suzuki, Kapoor et al. 2005; Vincent, Mignot et al. 2010; Sevko, Michels et al. 2013). 5-fluorouracil (5-FU) induces MDSC apoptosis in vitro and in vivo, enhancing tumour-infiltrating CTL and anti-tumour responses (Vincent, Mignot et al. 2010). Gemcitabine at clinical doses reduces splenic MDSC numbers in tumour-bearing mice and increases CD8 and NK mediated anti-tumour immune responses. Interestingly, other immune cells such as CD4/8, NK, macrophages, and B cells were unaffected by this treatment (Suzuki, Kapoor et al. 2005). Certain chemotherapeutic agents deplete MDSCs and inhibit MDSC differentiation by triggering cell death in MDSCs or their precursors, including gemcitabine, 5-fluorouracil, docetaxel, and doxorubicin (Suzuki, Kapoor et al. 2005; Kodumudi, Woan et al. 2010; Vincent, Mignot et al. 2010; Alizadeh and Larmonier 2014; Alizadeh, Trad et al. 2014). Doxorubicin selectively eliminated MDSCs in blood, spleen, and tumour of mice (mammary cancer model) and the remaining MDSCs were impaired in suppressive activities. MDSCs from patients (bearing a variety of tumour types) were killed by doxorubicin in vitro (Alizadeh, Trad et al. 2014). Importantly, certain chemotherapeutic treatments selectively target MDSCs but do not affect conventional DCs. To-date, the mechanisms that cause this selective cytotoxicity are not clear.
5.1.7. AIMS OF CHAPTER

In the previous chapter, *ex vivo* MDSCs were phenotypically and functionally studied and found to be equivalent to tumour-infiltrating MDSCs. The aim of this chapter is the establishment of protocols using the newly developed *ex vivo* MDSCs for discovery of novel anti-cancer therapeutic targets that inhibit MDSC suppressive function. First, the lentivector vaccines from chapter 3, particularly the IL12-based lentivectors, will be evaluated to characterise their effects on MDSCs. Further, high-throughput mass spectrometry based proteomic analysis of *ex vivo* MDSCs is presented, followed by validation of P450R expression as a novel MDSC-specific therapeutic target for chemotherapy drugs. In this chapter, the applicability of *ex vivo* differentiated MDSCs for research of MDSC biology and evaluation of MDSC therapeutic targets is demonstrated.
5.2. RESULTS

5.2.1. LENTIVECTOR VACCINES EXPRESSING IL12 AND SILENCING PD-L1 CONVERT EX VIVO MDSCs TO APCs, CAPABLE OF INDUCING CTL AND TH1 RESPONSES IN VITRO.

In the previous chapter it was demonstrated that the ex vivo MDSCs developed in this PhD thesis were capable of suppressing T cell responses. MDSCs are highly plastic cells and pro-inflammatory cytokines can convert MDSCs into efficient APCs (Bronte, Apolloni et al. 2000; Bronte, Serafini et al. 2001; Kerkar, Goldszmid et al. 2011). Consequently, it was assessed whether transduction of MDSCs with the lentivectors developed in chapter 3 could inhibit MDSC-mediated T cell suppression and enhance their antigen presenting capacities. For this purpose MDSCs were transduced in vitro before their use in MLRs or in OVA-specific co-culture assays. For MLRs C57/BL6 MDSCs were transduced with lentivectors expressing the GFP reporter gene and co-cultured with BALB/c splenocytes. Interestingly, the MDSCs were only capable of inducing significant Th1 and CTL responses when transduced with IL12-p1-GFP (Fig. 5.2.A). Further, a co-culture assay using OVA-specific CD4 and CD8 T cells from OT-II and OT-I mice was performed, respectively, as responder cells. MDSCs were transduced with lentivectors containing the model antigen OVA before co-culture with OVA-specific T cells at a ratio of 1:10. Similar to the results in MLRs, only IL12-p1-based lentivectors were capable of significantly enhancing CTL activation above control. While IL12-p1-based lentivectors were also effective at inducing Th1 responses, no significance was achieved when pooling two independent experiments (Fig. 5.2.B-C). It could be argued that IL12 secretion by transduced MDSCs could have autocrine effects and cause proliferation and maturation of DC precursors, instead of causing a change within MDSCs. To test this, day 5 B16-MDSCs were treated with recombinant IL12 and tested for CD11c expression 72 hours afterwards by surface staining and flow cytometry. No change in surface CD11c expression was observed (Fig. 5.2.D). This result suggested that T cell responses were not induced by proliferation or maturation of DC precursors that may exist in the MDSC preparations.

Some reports have shown that IL12 provided in trans to MDSCs induce the up-regulation of conventional maturation and antigen presentation markers such as CD80, CD86, and MHC II (Kerkar, Goldszmid et al. 2011; Steding, Wu et al. 2011; Thaci, Ahmed
et al. 2014). Hence, it was assessed whether IL12 addition in trans to the ex vivo MDSC cultures would up-regulate the maturation markers CD86 and MHC II. Interestingly, no change in these markers was found (Fig. 5.2.D). Further, it was assessed whether IL12 provided in cis by lentivector-transduction would have an effect on the MDSC phenotype. Ex vivo B16-MDSCs were transduced with lentivectors expressing selected cytokines (IL12, IFNγ, and IL10) and p1. Constructs co-expressing GFP were used for these experiments to study the MDSC phenotype of GFP+ transduced cells. A lentivector encoding only GFP was used as control. Transduction efficiencies ranged from 10-45% (Fig. 5.2.E). The expression of maturation markers in transduced (GFP+) MDSCs versus non-transduced (GFP-) MDSCs within the same cultures was compared. Interestingly, IL12-, IL10- and especially IFNγ-containing lentivectors up-regulated CD11c, CD86, and MHC II within transduced MDSC populations (Fig. 5.2.F). In these experiments, none of the surface marker changes in response to IL12-based lentivectors reached statistical significance. Ly6G expression on the other hand was not affected by lentivector transduction.

Taken together, these data suggested that ex vivo MDSCs can be transduced by lentivectors and that IL12-p1-based lentivectors induce antigen-presenting capacities. Importantly, enhanced T cell activation does not necessarily correlate with increased surface expression of markers CD11c, MHC II, and CD86 on MDSCs. Rather, the T cell stimulatory effect of IL12 and the microRNA p1 in MDSCs may directly act on the T cells themselves. This issue could not be solved within the time frame of this PhD thesis.
**A**

Bar graphs showing IFNγ+ CD4 T cells and IFNγ+ CD8 T cells for different conditions.

**B**

Bar graphs showing the percentage of IFNγ+ CD4 T cells and IFNγ+ CD8 T cells for different conditions.

**C**

OT I responses by selected transduced MDSCs

- **GFP-liOVA**: 81% 17%
- **IFNγ-p1-liOVA**: 54% 14%
- **IL12-p1-liOVA**: 29% 70%

Grz B expression vs. IFNγ expression
Fig. 5.2. Lentivectors expressing IL12 and microRNA p1 convert ex vivo MDSCs into APCs. (A) Bar graphs represent percentages of Th1 and CTL responses in response to transduced ex vivo MDSCs in a MLR. Briefly, BM cells from C57/BL6 mice were cultured with CM for 5 days, and MDSCs were transduced with the indicated lentivectors. Splenocytes from BALB/c mice were isolated and co-cultured with transduced MDSCs for 72h. Surface and intracellular staining was performed with CD4, CD8, and IFN-γ antibodies and cultures were analysed by flow cytometry. Pooled data from two independent experiments. (B) Bar graphs represent percentages of OVA-specific Th1 and CTL responses in response to transduced ex vivo MDSCs. Briefly, MDSCs were transduced on day 5 of culture with IiOVA-containing indicated lentivectors. Splenocytes were isolated from OT-II and OT-I mice and co-cultured with transduced MDSCs for 72h. Surface and intracellular staining was performed with CD4, CD8, and IFNγ antibodies and cultures were analysed by flow cytometry. Pooled data from two independent experiments. (C) Flow cytometry density plots show CD8 T cell responses in response to MDSCs transduced with indicated lentivectors. Percentages of GrzB and IFNγ-positive
cells are indicated within the dotplots. Grz B, Granzyme B. (D) Flow cytometry histograms representing expression of indicated markers in B16-MDSCs on day 8 of culture, untreated or treated with recombinant IL12 for 72 hours. Percentages and MFI are indicated within the histograms. Rec IL12, recombinant IL12. (E-F) B16-MDSCs were transduced on day 5 of culture with indicated GFP-expressing lentivectors and flow cytometry was performed 72 hours later. UT, untransduced cells. (E) Bar graphs represent percentages of GFP+ cells within transduced MDSC cultures on day 8 of culture. Pooled data from two independent experiments. (F) Bar graphs represent percentages of indicated surface markers within transduced (GFP+) and untransduced (GFP-) MDSCs within the same population. Pooled data from two independent experiments (except MHCII staining which shows data from one experiment).
5.2.2. IL12 IN COMBINATION WITH PD-L1/PD-1 SIGNALLING BLOCKADE OVERCOMES MDSC-MEDIATED CD8 T CELL SUPPRESSION WHEN PROVIDED IN TRANS.

Lentivector transduction of MDSCs could convert them into APCs, capable of priming naïve T cells. IL12 and the microRNA p1 were provided in cis in these experiments. While priming of naïve T cells will be important in prophylactic vaccination, this may not be the case during therapeutic intra-tumour administration. T cells found within the tumour are infiltrating effector cells, although they may be rendered dysfunctional by inhibitory factors or immunosuppressive cells (Zhou, Xiao et al. 2010). Additionally, the majority of transduced cells will be tumour cells following intra-tumour injections and not myeloid cells (Emeagi, Van Lint et al. 2012). Therefore, it was assessed whether direct transduction of MDSCs in the tumour would be required to counteract their activities over activated T cells. For this purpose, standard suppression assays with anti-CD3/anti-CD28-activated CD8 T cells were performed, in which cultures were treated with soluble IL12, anti-PD1 antibody, and anti-CTLA-4 antibody. Soluble IL12 was obtained from 293T cells that were previously modified with lentivectors to express the IL12 gene (2.5ng/mL IL12 in supernatant). Supernatant from 293T cells expressing GFP was used as a control, while isotype antibodies were used as a control for CTLA-4 and PD-1 blocking antibodies. Soluble IL12 could overcome MDSC inhibitory activities over activated CD8 T cells (Fig. 5.3.A,B). In contrast to soluble IL12, CTLA-4 or PD-1 antibodies alone did not significantly increase CD8 T cell/MDSC ratios. A consistent synergistic effect was observed when PD-1 antibody was added together with soluble IL12, albeit without significance in these particular experiments (Fig. 5.3.A). Addition of MDSCs to activated T cell cultures completely inhibited secretion of IFNγ, while addition of soluble IL12 reversed this process and strongly increased IFNγ secretion (Fig. 5.3.B). This data suggested that MDSCs did not have to be directly transduced with IL12-based lentivectors to overcome their immunosuppressive function. However, direct transduction with PD-L1 silencing microRNA may still be beneficial. MDSCs secrete TGFβ to dampen the immune response and ex vivo MDSCs secrete large amounts of this cytokine (Fig 4.9.C). As TGFβ impairs the ability of macrophages to produce and secrete IL12 (Takeuchi, Alard et al. 1998), and IL12 inhibits TGFβ production by TAMs (Watkins, Egilmez et al. 2007), I wondered whether addition of IL12 in trans could inhibit TGFβ
secretion by B16-MDSCs. Thus, B16-MDSCs were treated with recombinant IL12 for 24 hours before quantifying secreted bioactive TGFβ in supernatants. Although there was a trend in decreased bioactive TGFβ, pooling the data from independent experiments did not achieve statistical significance (Fig. 5.3.C).

Taken together, IL12 (a known effective anti-tumour agent) induction and inhibition of PD-L1/PD-1 interaction in vitro overcomes MDSC suppressive activities over naïve and effector T cells. Although these ex vivo assays are good indicators of the effect of IL12-based lentivectors on immune cells within the tumour, they cannot be directly linked to the in vivo outcome of the disease. Nevertheless, so far all assays using ex vivo MDSCs and in vivo anti-tumour effects consistently pointed to the favourable effect of IL12-p1-based lentivectors on T cells and disease progression.
**Fig. 5.3. Soluble IL12 can overcome MDSC inhibitory activities.** (A-B) Standard suppression assays were performed, in which ex vivo MDSCs were added to activated CD8 T cells in indicated conditions at a ratio of 1:1. –b, no anti-CD3/CD28 activatory beads were added; +b, anti-CD3/CD28 activatory beads were added; No, indicates T cell-MDSC co-cultures with no added condition; IC, Isotype control was added; CTLA4 Ab, CTLA-4 antibody was added; PD-1 Ab, PD-1 antibody was added; GFP, supernatant from 293T-GFP cells was added; IL12, supernatant from 293T-IL12 cells was added; IL12 PD-1 Ab, supernatants from 293T-IL12 cells together with PD-1 antibody was added. (A) Bar graphs represent the ratio of CD8 T cells over MDSCs within the co-cultures. After 72h of co-culture cells were stained with anti-CD8 and anti-CD11b antibodies and analysed by flow cytometry. (B) Bar graphs represent the amount of secreted IFN- antibody was added. (A) Bar graphs represent concentration in supernatants was measured by ELISA. (C) Bar graphs represent secreted bioactive TGFβ in B16-MDSCs. B16-MDSCs were treated with recombinant IL12 (10ng/mL) for 24 hours. Supernatants were then transferred to and quantified with a TGFβ-reporter cell line. Standard deviations are represented as error bars.
5.2.3. *Ex vivo* MDSCs Differ from Immature BM-DCs in Key Metabolic Pathways

The data presented in this thesis so far suggested that the newly developed *ex vivo* MDSCs are phenotypically and functionally equivalent to tumour-infiltrating MDSCs, and that they can be used to predict therapeutic outcomes in the melanoma mouse model. It was therefore reasoned that *ex vivo* MDSCs may be used to address mechanistic studies of MDSC behaviour. To validate our *ex vivo* MDSCs as well as identify MDSC differential intracellular pathways and specific therapeutic targets, high-throughput analytical techniques were applied. This could be possible as the *ex vivo* MDSC differentiation protocol yields high numbers of MDSCs (in contrast to purification of *in vivo* tumour-infiltrating MDSCs). B16-MDSCs were compared with immature BM-DCs by unbiased quantitative proteomic analyses and hence differentially activated pathways in the two myeloid cell types could be identified. Relative protein expression levels were quantified using iTRAQ isobaric tags coupled to 2D nano-liquid chromatography tandem mass spectrometry. The myeloid proteome of conventional immature BM-DCs was first compared with that of B16-MDSCs, using triplicates of each cell type. Further, the proteomic differences within MDSCs caused by the tumour-environment were evaluated. For this purpose non-neoplastic 293T-MDSCs were compared with melanoma B16-MDSCs. The dataset files were processed using ProteinPilot™ 4.5 software from AB Sciex. ProteinPilot™ 4.5 uses the algorithm Paragon™ (v.4.0.0.0) (Shilov, Seymour et al. 2007) for database search and Progroup™ for data grouping. As described in Chapter 2, each MS/MS spectrum was searched against a database of murine protein sequences (Uniprot complete mouse proteome). In this way, 3002 proteins were detected in the two myeloid cell types (B16-MDSC and BM-DC) with a false recovery rate (FDR) of 1%. 64 (2% of overall identified proteins) differentially expressed proteins were identified, using stringent cut-off conditions. While 28 proteins were up-regulated in B16-MDSCs, 36 were down-regulated, as compared to immature BM-DCs (Table 5.1).
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### Down-regulated proteins in B16-MDScs

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Table 5.1. Differentially expressed proteins from immature BM-DCs and ex vivo B16-MDSCs. iTRAQ isobaric tags coupled to 2D nano-liquid chromatography tandem mass spectrometry was used to identify the proteomes of the two myeloid cell types. Tables indicate the abundance of the indicated proteins from B16-MDSCs, relative to immature BM-DCs. All proteins were identified with >99% confidence (corresponding to a protein score cutoff >2.0). DC1, DC2, and DC3 together with B16-1, B16-2, and B16-3 correspond to the three independent biological replicates from BM-DCs and B16-MDSCs, respectively. The ratio of protein expression levels between DCs and B16-MDSCs are indicated and correspond to the protein reporter ion intensity originating from DC2 (Tag114), DC3 (Tag115), B16-1(Tag116), B16-2(Tag117), B16-3(Tag118) relative to DC1(Tag113). Proteins were considered to show a significant upward or downward trend if their expression ratios were >1.3 or <0.77, respectively. Ratios indicating differential expression (p-value <0.05) are in bold.
Relative abundance determined differential protein identification (Fig. 5.4.A). The proportion of nuclear proteins decreased two-fold in B16-MDSCs, while membrane-associated proteins increased two-fold in B16-MDSCs relative to immature BM-DCs (Fig. 5.4.B). Further, programmes STRING 9.1, DAVID Bioinformatics resources 6.7, and PANTHER were used to perform molecular network and pathways analyses of the differentially expressed proteins. In this way, insight was gained into the differentially activated and deactivated intracellular pathways of MDSCs, compared to DCs. This systems biology approach allowed the grouping of up-regulated proteins within B16-MDSCs into several localized groups within the cell (Fig. 5.4.C). A range of membrane receptors were shown to be up-regulated within B16-MDSCs, including c-type lectin receptors, adhesion molecules, and TLR-associated molecules (Chi3l3, Clec10a, Mgl2, Thbs1, and CD180). Interestingly, Chi3l3, Clec10a, and Mgl2 up-regulation could be linked to NOS and ROS production by Arg-1, iNOS, and S100 family proteins (Fig. 5.4.D), in agreement with data published in the literature as shown in the introduction. While Arg-1 and iNOS are well-established proteins within MDSC biology, S100 family proteins have been recently found to be specific targets for MDSC depletion in vivo in tumour-bearing mice (Cheng, Corzo et al. 2008; Qin, Lerman et al. 2014). In addition, these molecules were linked to phagocyte migration to sites of inflammation, chemotaxis, immune regulation, clathrin-dependent endocytosis, and Src signalling. The Src signalling pathway is linked to cell motility and division. CD180 was linked to TLR signalling, while thrombospondin 1 (Thbs1) was associated to integrin family proteins, negative regulation of DCs, and responses to hypoxia. Response to hypoxia is important in MDSC biology as MDSCs are known to function and even differentiate in hypoxic tumour environments (Corzo, Condamine et al. 2010). Enhanced protein expression of clathrin light chain A (Clta), alpha-actinin-4 (Actn4), vesicle-fusing ATPase (Nsf), synaptosomal-associated protein (Snap 23), and vesicle-associated membrane protein-associated protein A (VAPA) in B16-MDSCs indicated further involvement of endocytosis, vesicle trafficking, and fusion in MDSC function. STRING analyses of these proteins (Fig. 5.4.D,E) showed association with intracellular signalling pathways SRC, Grb2 (Growth factor receptor-bound protein 2), Ras, Stat3, NF-κB and MAPKs, all of which are known to be active in MDSCs (Gabrilovich, Ostrand-Rosenberg et al. 2012). Some SRC family members such as Fyn kinase have been recently predicted to play a role in MDSCs.
(Aliper, Frieden-Korovkina et al. 2014). STAT3, for example, is a known melanoma MDSC-specific target (Emeagi, Maenhout et al. 2013). RNA helicase Ddx3x, splicing factor 3A (Sf3a1), Lrcc59 (mRNA splicing), lamin B2 (Lmnb2), fibrillarin (Fbl), and glucosidase II subunit β (Prkcsh) are associated with transcription, splicing, and translation. Enhanced expression of these proteins suggested that ex vivo B16-MDSCs were not quiescent and could function under hypoxic conditions. In addition, enzymes associated with aerobic cellular respiration and glycogen/glucose metabolism were down-regulated in B16-MDSCs, compared to BM-DCs. This was represented by a decrease in expression of coenzyme Q10 (ubiquinone), NADH dehydrogenase flavoprotein 2 (Ndufv2), creatine kinase (Ckb), glycogen phosphorylase (Pygl) and phosphoglucomutase-1 (Pgm1) (Table 5.1). Not only did B16-MDSCs down-modulate proteins linked to glycogen/glucose metabolism but also showed enhanced expression of proteins associated with lipid metabolism, indicating a shift in energy production within this cell type. Enhanced expression of ApoB receptor (lipoprotein endocytosis) and peripilin-3 (Plin3, lipid storage, hydrolysis and metabolism) were linked to lipid metabolism. In addition, mitochondrial proteins associated to lipid metabolism and amino acid synthesis, such as aldehyde dehydrogenase (Aldh3), glycine amidinotransferase (Gatm), ornithine aminotransferase (Oat), cis-aconitic acid decarboxylase (IRG1) and methylenetetrahydrofolate dehydrogenase (Mtfhd2), were found to be increased in B16-MDSCs. Lysosomal enzymes were also down-modulated in B16-MDSCs, as well as enzymes involved in β-oxidation (Table 5.1.B). These included peroxysomal acyl-CoA oxidase (Acox3), trifunctional enzyme subunit β (Hadhb), and carnitine O-palmitoyltransferase (Cpt1a).

Very interestingly, it was found that MDSCs up-regulated a group of ROS scavenger proteins and detoxifying enzymes, such as cytochrome p450 reductase (Por or P450R), epoxide hydrolase 1 (Ephx-1), heme oxygenase 2 (Hmox2) and superoxide dismutase 2 (Sod2). These enzymes can be linked to ROS production, protection from oxidative damage, and in NADP/NADPH-dependent metabolism. This is important for MDSCs, to counteract the generation of highly toxic metabolites generated by NOS and ROS production as well as lipid metabolism (Fig. 5.4.E).

Further, the proteome of B16-MDSCs was compared to the proteome of 293T-MDSCs (Table 5.2). The percentage of identified differentially expressed proteins
between B16-MDSCs and DCs was 2%. This percentage was further reduced to 0.4% (11 of 3100 identified proteins) when quantitatively comparing the two different ex vivo MDSC cell types. While 3 proteins were up-regulated, 9 were down-regulated in B16-MDSCs (Table 5.2 and Fig. 5.4.G). Up-regulated proteins in B16-MDSCs possessed antioxidant properties, such as peroxiredoxin 6 (Prdx6) and superoxide dismutase 1 (Sod1). These proteins play a role in protection of cells from oxidative stress. In addition, D-3-phosphoglycerate dehydrogenase exhibited enhanced protein expression in B16-MDSCs, which is a known regulator of amino acid synthesis. Interestingly, similar to the results with BM-DCs, B16-MDSCs showed down-modulation of proteins involved in aerobic energy metabolism (slc2a6, a hexose transporter; CD38, an ADP-riboxyl cyclase).

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Uniq Cov 95% Uniq pept
Table 5.2. Differentially expressed proteins from ex vivo B16-MDSCs and 293T-MDSCs.
iTRAQ isobaric tags coupled to 2D nano-liquid chromatography tandem mass spectrometry was used to identify the proteomes of the two myeloid cell types. Table indicates the abundance of the indicated proteins from 293T-MDSCs, relative to B16-MDSCs. All proteins were identified with >99% confidence (corresponding to a protein score cutoff >2.0). 293T.1, 293T.2, and 293T.3 together with B16.1, B16.2, and B16.3 correspond to the three independent biological replicates from 293T-MDSCs and B16-MDSCs, respectively. The ratio of protein expression levels between the two cell types are indicated and correspond to the protein reporter ion intensity originating from B16.2 (Tag114), B16.3 (Tag115), 293T.1(Tag116), 293T.2(Tag117), 293T.3(Tag118) relative to B16.1(Tag113). Proteins were considered to show a significant upward or downward trend if their expression ratios were >1.3 or <0.77, respectively. Ratios indicating differential expression (p-value <0.05) are in bold.
PHAGOCYTE MIGRATION TO INFLAMMATORY AND WOUND SITES S100 PROTEIN FAMILY

C-TYPE LECTIN RECEPTORS CHEMOTAXIS AND IMMUNE REGULATION

NOS AND ROS PRODUCTION

RECEPTOR-MEDIATED ENDOCYTOSIS AND SRC SIGNALLING

PROTECTION AGAINST OXIDATIVE STRESS

Mitochondrial superoxide dismutase

SRC, Stat3, JAK, LcK SIGNALLING
Fig. 5.4. **Unbiased quantitative proteomics comparing proteomes from ex vivo MDSCs and conventional immature BM-DCs.** (A) Pie charts represent percentages of identified proteins according to cellular localization. Above: all proteins identified in overall myeloid proteome. Below: Differentially expressed proteins, between immature BM-DCs and B16-MDSCs. (B) Bar graphs represent the ratio between the percentage of differentially-expressed proteins between B16-MDSCs and BM-DCs over the total detected myeloid proteins, grouped according to cell location. Highest relative changes in protein expression are indicated by arrows. (C) Schematic representation of B16-MDSC up-regulated proteins, according to cellular location, biological relationships, and pathways. String 9.1, DAVID, and Panther programs were used to assess differential protein relationships. Direct pathways between indicated protein groups are indicated by arrows. Detoxifying enzymes are indicated in red. Green boxes indicate pathways that are predicted to be activated, according to biological reactions between up-regulated proteins. (D-E) String 9.1 networks are represented, using the confidence view. Thicker lines connecting proteins indicate a higher confidence of protein interaction. Up-regulated B16-MDSC proteins are indicated with arrows and represent nodes of interactions. Proteins that are predicted to interact with up-regulated proteins are grouped according to interaction and function. (F) Same as in C, but representing B16-MDSC up-regulated proteins, as compared to non-neoplastic control 293T-MDSC proteome.
5.2.4. P450 REDUCTASE, UNCOVERED BY QUANTITATIVE PROTEOMICS, ACTS AS A MDSC-SPECIFIC CHEMOTHERAPY TARGET

Quantitative proteomics comparing B16-MDSCs with BM-DCs and 293T-MDSCs confirmed and highlighted several MDSC-associated pathways. Further, I strove to validate newly identified proteins through this method. P450R (or Por) expression was highly up-regulated in MDSCs, relative to conventional immature BM-DCs, and was selected for further analysis as a potential MDSC-specific target (Table 5.1 and Fig. 5.5.A.). First, enhanced P450R expression in MDSCs was confirmed by western blot analysis (Fig. 5.6.B). P450R was selected as a candidate of interest as it plays a role in the activation/metabolism of chemotherapeutic pro-drugs, leading to cytotoxicity and cell growth arrest of tumour cells (Kivisto, Kroemer et al. 1995; Martinez, Williams et al. 2008). In addition, some chemotherapeutic agents have been found to selectively diminish MDSC populations in experimental animals (Suzuki, Kapoor et al. 2005; Vincent, Mignot et al. 2010; Sevko, Michels et al. 2013). As the mechanism for MDSC selective cytotoxicity by certain chemotherapeutic drugs is unknown, the hypothesis that differential P450R expression was crucial in this process, was tested. Paclitaxel, Docetaxel, and Irinotecan are chemotherapeutic agents with differing therapeutic efficacies in melanoma patients. Importantly, P450R acts differentially on these three chemotherapeutic agents. P450/P450R convert the pro-drug Paclitaxel into its toxic form and it is an effective anti-melanoma agent that also depletes MDSCs (Rodriguez-Antona and Ingelman-Sundberg 2006; Sevko, Michels et al. 2013). On the other hand, Docetaxel is not effective in melanoma patients and not processed in the same way as Paclitaxel by P450 cytochromes (Tas, Camlica et al. 2003; Vaclavikova, Soucek et al. 2004; Rodriguez-Antona and Ingelman-Sundberg 2006). Irinotecan is neutralized by P450R and activated by carboxylesterases and could thus serve as a control (Wu, Yan et al. 2002). MDSCs and conventional immature BM-DCs were treated with the three chemotherapeutic agents and the effect on viability was tested and compared. Published cytotoxic concentrations over cancer cell lines of each chemotherapeutic agent were used and viability was assessed after 24 hours of treatment by trypan blue staining. Interestingly, Paclitaxel (200nM) was selectively cytotoxic to MDSCs while BM-DCs remained viable. In contrast, Docetaxel (50nM) and Irinotecan (32μM) did not
exhibit a significantly different effect on the two myeloid cell populations (Fig. 5.5.C). Hence, Docetaxel and Irinotecan affected BM-DCs and MDSCs similarly, while Paclitaxel selectively killed MDSCs.

As Paclitaxel is activated by P450R into its toxic form, it was tempting to hypothesize that P450R expression is crucial to its MDSC-selective cytotoxicity. To confirm that indeed P450R expression within target cells conferred selective Paclitaxel-mediated cytotoxicity towards MDSCs, P450R expression was silenced within myeloid cell populations. For this purpose a lentivector encoding a shRNA specific for P450R was constructed (Fig. 5.5.D). BM cells were transduced, followed by differentiation towards conventional DCs or towards MDSCs. While BM-DC sensitivity to Paclitaxel was not affected by silencing of P450R, B16-MDSCs became resistant to Paclitaxel treatment (Fig. 5.5.E). Importantly, using a PD-1-specific shRNA as control did not confer any protection on B16-MDSCs (data not shown). As expected, silencing P450R in myeloid cells enhanced the cytotoxic effect of Irinotecan. Taken together, this data suggested that P450R expression was enhanced in MDSCs and that it causes the selective cytotoxicity of certain chemotherapeutic treatments such as Paclitaxel. Thus the in vivo efficacy of Paclitaxel in melanoma patients may be enhanced by selective MDSC depletion, through P450R expression.
A

THERAPEUTIC TARGET BY ANTINEOPLASTIC DRUG ACTIVATION

Cytochrome P450 reductase
STEROID, VITAMINS, CARCINOGEN AND DRUG METABOLISM

DNA REPAIR
CHROMOSOMAL STABILITY

B

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<td>GADPH</td>
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Myeloid cells in culture

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<tr>
<th>Treatment</th>
<th>BM-DC</th>
<th>293T-MDSC</th>
<th>B16-MDSC</th>
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<tr>
<td>Paclitaxel 200nM</td>
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<tr>
<td>Docetaxel 50nM</td>
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<td>p=0.0755</td>
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<td>Irinotecan 32μM</td>
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<td>p=0.1571</td>
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Fig. 5.5. High expression of P450R renders B16-MDSCs susceptible to Paclitaxel. (A) String 9.1 network depicting P450R and its interactions. (B) Western Blot performed with anti-P450R antibody in BM-DCs, 293T-MDSCs, and B16-MDSCs. GADPH expression was used as a control as it was expressed at same levels according to quantitative mass spectrometry. P450R was therefore very highly expressed in MDSCs, compared to BM-DCs. (C) Myeloid cells were treated overnight with indicated chemotherapeutic drugs, at known cancer cell cytotoxic concentrations. Bar graphs represent the ratio of drug-treated over untreated myeloid cells as a percentage. Error bars correspond to standard deviations. (D) Above, schematic representation of the lentivector construct used to deliver P450R-silencing shRNAs. Below, detection of P450R or GADPH by Western Blot after transduction of B16 cells with P450R-silencing lentivectors. Three shRNAs were tested and P450R-shRNA 2 (indicated with an asterisk) was selected for myeloid cell transduction. (E) BM cells were transduced with P450R-shRNA 2, differentiated, and
treated overnight with indicated chemotherapeutic drugs. Bar graphs represent the ratio of drug-treated over untreated myeloid cells as a percentage. Error bars correspond to standard deviations. These experiments were performed by Mr Fabio Caliendo. LTR, long-terminal repeat; U6, U6 promoter; shRNA, short hairpin RNA coding sequence; PGK, phosphoglycerolate promoter; SIN, self-inactivating LTR. *, **, *** indicate significant, very significant, and highly significant differences, respectively.
5.3. DISCUSSION

In Chapter 3 IL12-based lentivectors slowed down tumour growth and prolonged survival in a rapidly growing B16 tumour model. IL12 exerts multiple effects over a variety of cell types within the tumour. It is becoming increasingly clear that IL12 also affects immature and suppressive myeloid cells within the tumour environment, thus contributing to tumour regression (Kerkar, Goldszmid et al. 2011). As MDSCs comprise about 5% of cells within B16 tumours (data not shown), purification of sufficient tumour-infiltrating MDSCs for functional experiments with a high number of different treatment conditions is highly impractical. Therefore, a novel system to assess anti-cancer treatments ex vivo on MDSCs was developed during the course of this PhD thesis (Chapter 4). Using ex vivo differentiated B16-MDSCs, it was found that the IL12-p1-based lentivector could confer T cell stimulatory activities upon this immunosuppressive cell type (Fig. 5.2). Lentivectors containing cytokines IFNγ, IL10, or TGFβ did not induce significant changes in T cell activation. While transduction of B16-MDSCs with IL12-p1-IiOVA lentivectors triggered T cell responses to OVA-presenting MDSCs, this was not accompanied by significant phenotypic myeloid maturation. Nevertheless, it was shown that transduction with the IL12-p1-based lentivector could confer antigen presenting capabilities upon B16-MDSCs, correlating with anti-tumour activities of this lentivector.

Further, IL12 did not have to be produced by MDSCs themselves to overcome MDSC suppressive activities (Fig. 5.3). This is important for application in the therapeutic experimental setting, as the majority of cells transduced during intra-tumour lentivector injection are tumour cells (about 80%) (Emeagi, Thielemans et al. 2012). In standard suppression assays (using CD3/CD28 activated CD8 T cells), addition of IL12 to culture medium led to an increase in the percentage of activated CD8 T cells over MDSCs and an increase in IFNγ secretion (Fig. 5.3). It is well established that IL12 stimulates proliferation of activated T cells while resting T cells do not proliferate in the presence of IL12. In addition, IL12 induces IFNγ production by NK cells, CD4 and CD8 T cells and polarizes naïve T cells into Th1. (Bertagnolli, Lin et al. 1992; Lee, Suen et al. 1998). Thus, addition of IL12 to the co-culture of MDSCs and activated T cells did not allow the assessment of the effect of IL12 on one cell population alone. On the contrary, it could imply a role for IL12 to act on either T cell, MDSC, or both cell types. As the effect of IL12
on T cells is well established, IL12 likely has a direct effect on survival and proliferation of activated CD8 T cells in this experiment. What the direct effect of IL12 on MDSCs is remains to be elucidated, but it is clear that IL12 retains its stimulatory effects even in the presence of highly suppressive MDSCs.

Nevertheless, other researchers showed that pre-treatment of tumour-infiltrating MDSCs with recombinant IL12 can overcome their suppressive activity over CD3/CD28 activated CD4 T cells. MDSCs were washed prior to co-culture with activated T cells, suggesting that IL12 had a direct effect on the suppressive activities of tumour-infiltrating MDSCs (Steding, Wu et al. 2011). In contrast to data presented in this thesis, Steding et al. found an increase in surface markers MHC II and F4/80 but not CD80 or CD86 on tumour-infiltrating MDSCs following IL12 treatment in vitro. Interestingly splenic MDSCs from tumour-bearing mice up-regulated CD80, CD86, MHCII, and F4/80 expression in response to IL12, indicating significant differences between spleen and tumour MDSCs. On the other hand, adenovirus-mediated IL12 led to up-regulation of all 4 markers in vivo in tumour-infiltrating MDSCs (Steding, Wu et al. 2011). Of note, adenoviral vectors are highly immunogenic and thus may have a significant effect on the maturation of myeloid cells (Barouch and Nabel 2005). Interestingly tumour-infiltrating MDSCs showed a decrease in iNOS, Arg-1, and IFNγ following intramuscular injection of adenoviral vectors encoding IL12, representing a possible mechanism for overcoming MDSC suppressive activity (Steding, Wu et al. 2011). Studies transferring engineered T cells secreting IL12 have similarly shown effects on tumour-infiltrating myeloid cells. Myeloid cells from treated mice could stimulate CD8 proliferation in vitro while cells from mock-treated mice did not (Kerkar, Goldszmid et al. 2011). Taken together, the data from this thesis and other studies suggests that IL12 has direct immunostimulatory effects on tumour-infiltrating MDSCs, overcoming their suppressive functions and converting them into efficient APCs with potential anti-tumour activities. Aiding this effect may be the positive feedback loop of IL12, as IL12-mediated IFNγ induction induces myeloid cells to produce more IL12 (Yoshida, Koide et al. 1994).

Addition of PD-1 blocking antibodies to suppression assays did not significantly enhance the stimulatory activity of IL12 (Fig. 5.3). In vivo there were significant synergistic effects of IL12 expression and PD-L1 silencing, especially in prophylactic vaccinations (Fig. 3.7). Blocking of PD-L1 has been shown to impair MDSC suppressive
activity (Chikamatsu, Sakakura et al. 2012; Noman, Desantis et al. 2014). It could be hypothesized that IL12 may mask the adjuvant effect of PD-1 blocking in the experiments performed in this thesis. Importantly, functional assays involving two cell types do not reflect the complexity of the in vivo situation since they do not take into account various other cell types and molecules present within the tumour microenvironment. Accordingly, it was shown that PD-L1 silencing in cells of non-haematopoietic origin at the injection site confers adjuvant effects in vivo, leading to enhanced T cell responses (Fig. 3.10).

Taking advantage of the newly developed MDSC differentiation system two quantitative proteomic experiments were performed, comparing BM-DCs and B16-MDSCs as well as MDSCs from different “environments”. In this way the changes elicited in the two-step development of MDSCs were dissected, the first step being the mobilization of immature myeloid cells and the second step being further acquisition of MDSC-associated characteristics depending on the tumour environment (Talmadge 2007; Gabrilovich, Ostrand-Rosenberg et al. 2012). The proteomic data and analyses highlighted several important changes between MDSCs and DCs. MDSCs up-regulated a range of membrane receptors, which could be linked to TLR signalling, phagocyte migration to inflammatory sites, immune regulation, and blocking of myeloid differentiation and maturation. This data revealed that MDSCs are not quiescent cells but are involved in cell cycle progression, growth, proliferation, active movement, transcription, splicing, translation, and protein transport. These processes require high energy levels but MDSCs down-regulated aerobic cell respiratory processes and glycogen/glucose metabolism. To provide energy MDSCs up-regulated proteins involved in lipid metabolism. Lipid metabolism provides energy and aids in amino acid synthesis but also produces toxic metabolites. Thus, detoxifying and ROS scavenger proteins such as P450R, Hmox2, and Sod2 were elevated in MDSCs (Table 5.1 and Fig. 5.4). Sod2 is elevated in TAMs and tumour cells, thus representing a potential TAM/MDSC-specific therapeutic target (Hartmann, Tousseyn et al. 2013). S100 family proteins represent known and important MDSC targets (Cheng, Corzo et al. 2008; Sinha, Okoro et al. 2008; Qin, Lerman et al. 2014) and they could be linked to up-regulated proteins in B16-MDSCs (Fig. 5.4.D). This data correlates with the S100 autocrine feedback loop in tumour-bearing hosts, causing enhanced MDSC accumulation (Sinha, Okoro et al. 2008).
Similarly, a connection to hallmark MDSC enzymes iNOS and Arg-1 as well as transcription factor STAT3 could be established (Fig. 5.4.D,E). STAT3 is one of the main transcription factors leading to MDSC differentiation, accumulation, tumour infiltration, and suppressive function in tumour-bearing hosts (Corzo, Cotter et al. 2009) (Fig. 5.1). After finishing this PhD thesis, further in-depth proteomic studies were performed, which highlighted mitochondrial dysfunction and MHC II down-modulation, together with other proteins involved in chemotaxis, immune cell trafficking, intracellular signalling, and reduced antigen presenting capacities (data not shown).

As expected, comparing the proteomes of 293T- and B16-MDSCs resulted in the identification of fewer differentially expressed proteins. Particularly important were changes in energy metabolism and proteins involved in the generation and neutralization of ROS and RNS. The B16-MDSC proteome “emphasized” on detoxifying processes, while 293T-MDSCs up-regulated proteins involved in the mitochondrial electron transport chain and NADPH oxidase activities. Again, after finishing this PhD thesis, further in-depth analyses were carried out which linked ROS production and the regulation of energy metabolism by driving the carbohydrate flux to the pentose phosphate pathway (PPP)(data not shown). Interestingly, Arg-1 expression was enhanced in 293T-MDSCs as detected by proteomics (Table 5.2). Differential Arg-1 expression supports the theory that MDSCs acquire further suppressive activities upon tumour infiltration.

Important intracellular signalling molecules that were linked to MDSC development shown during the analyses of all the proteomic data included STAT3, MAPK/ERK, PKC, SRC, NF-κB, Ras, Grb2, mTOR, and Akt (Fig. 5.4, Fig. 5.5, and data not shown). Further, proteomic analyses uncovered major changes in the mitochondria. On one hand these changes produce more ROS species and on the other they neutralize ROS species. Interestingly, similar processes have been observed in cancer cells. Cancer cells exhibit mitochondrial metabolic reprogramming and higher ROS levels than other cells. And much of cellular glucose is metabolized through the PPP in cancer cells (Kang, Lee et al. 2015). The detrimental effects of ROS and RNS on NK and T cells are well established. Tumours and MDSCs secrete these molecules to induce immune suppression. As MDSCs up-regulate proteins with anti-oxidative properties they protect themselves from this highly toxic environment.
Differentially expressed proteins and conclusions from the proteomic analyses were confirmed by published results. Nevertheless, P450R was selected to further validate the proteomic data, due to its role in the activation/metabolism of chemotherapeutic pro-drugs. B16-MDSCs showed enhanced P405R expression, which conferred selective cytotoxicity of the chemotherapeutic agent paclitaxel. In contrast, docetaxel and irinotecan did not selectively deplete MDSCs (Fig. 5.5.B,C). Interestingly, docetaxel and irinotecan alone or in combination do not appear to be effective in melanoma patients (Tas, Camlica et al. 2003). Importantly, silencing of P450R in MDSCs abolished the selective cytotoxicity of paclitaxel (Fig. 5.5.D,E). This mechanistically explains for the first time why the pro-drug paclitaxel selectively depletes MDSCs in vivo, which is thought to contribute to improved anti-melanoma efficacy in patients compared to other chemotherapeutic treatments.

The effect of paclitaxel on MDSCs may be tumour-type dependent. In an EL4 thymoma mouse model several chemotherapeutic agents (including paclitaxel) were compared for their effect on MDSC levels within spleen and tumour beds. Interestingly, in this study, the only agents significantly depleting MDSCs in vivo were gemcitabine and 5-fluorouracil. Paclitaxel on the other hand did not lead to a significant reduction in MDSC levels (Vincent, Mignot et al. 2010). It would be of interest to assess whether EL4-MDSCs express high levels of P450R. Other researchers showed that paclitaxel prolongs survival and reduces tumour burden in a ret transgenic melanoma mouse model. Paclitaxel administration in this spontaneous melanoma model reduced the number of tumour-infiltrating MDSCs as well as their T cell suppressive activities. Interestingly, MDSC NO production and STAT3 activation but not MDSC numbers were reduced in metastatic lymph nodes. Importantly, paclitaxel did not affect DC or macrophage populations within tumours. Thus, paclitaxel mediated its effect in vivo in a tissue and cell type specific manner. Tumour-infiltrating MDSCs exhibited reduced p38 MAPK activity, TNFα production, and S100A9 expression, following paclitaxel treatment (Sevko, Michels et al. 2013). As previously discussed, STAT3 and S100A9 are important molecules in MDSC biology and p38 MAPK activation in myeloid cells reduces DC immunostimulatory capacities (Fig. 5.4, (Cheng, Corzo et al. 2008; Corzo, Cotter et al. 2009; Zhao, Falk et al. 2009). This data was confirmed by data presented in this chapter showing selective cytotoxicity of paclitaxel to MDSCs, compared to BM-DCs (Fig. 5.5.C).
As the authors previously published *in vitro* data showing paclitaxel-mediated MDSC differentiation to functional conventional DCs, it was suggested that the *in vivo* decrease of MDSCs could be due to differentiation (Michels, Shurin et al. 2012; Sevko, Michels et al. 2013). Although MDSC and DC markers were not assessed following paclitaxel treatments in this thesis it was clear that paclitaxel mediated selective cytotoxicity. Of note, Sevko et al. used non-cytotoxic concentrations while cytotoxic concentrations were used in this thesis. It would be of interest to assess the B16-MDSC phenotype following paclitaxel treatment at cytotoxic concentrations.
CHAPTER 6: CONCLUSIONS, LIMITATIONS, AND FUTURE PERSPECTIVES

6.1. INITIAL OBJECTIVES AND FURTHER DEVELOPMENT OF THESIS

Initially the objective of this thesis was to develop and test lentivector vaccines modulating co-stimulation during antigen presentation. This project developed from previous results of our laboratory. Karwacz et al. found that PD-L1 silencing inhibited TCR down-modulation during antigen presentation in vitro and in vivo. T cells activated by PD-L1-silenced DCs exhibited enhanced proliferation and produced increased IFNγ and IL17. These T cells thus represented a hyper-activated T cell population with a pro-inflammatory phenotype. Therapeutic transfer of PD-L1 silenced DCs in an EG7 mouse lymphoma model (with OVA surrogate tumour antigen expression) led to reduced tumour growth and an increase in life span. However, overall survival after one month remained the same and PD-L1 silencing had to be combined with DC molecular activators to increase therapeutic efficacy. Thus, it was suggested that while PD-L1 silencing in DCs may speed up T cell expansion and cytokine secretion, additional factors were needed to convey effector functionality to activated T cells (Karwacz, Bricogne et al. 2011). I therefore set out to construct and test lentivectors that would not only deliver an antigen in combination with PD-L1 silencing but would also ensure cytokine signalling to confer acquisition of effector T cell capacities. Lentivector constructs containing a wide range of T cell polarizing cytokines were constructed and tested in vitro in antigen presenting assays and in vivo in healthy and tumour-bearing mice.

While early efforts in cancer immunotherapy approaches were mainly focused on enhancing the number of tumour-specific immune effector cells, little attention was paid to the effect these treatments could have on the immunosuppressive tumour micro- or macro-environments. The crucial question to ask is whether stimulated immune effector cells would be able to eliminate cancer cells within the immunosuppressive tumour environment. The immunosuppressive mechanisms are manifold and include amongst others, the recruitment and differentiation of immunosuppressive cell types and the up-regulation of negative co-stimulatory
molecules by cancer cells and immune cells. Lentivector vaccination can lead to increased PD-L1 expression on tumour cells and tumour-infiltrating leukocytes, which can be counteracted by blocking of PD-L1 signalling, thus leading to a partial recovery of T cell effector functions and enhanced lentivector efficacy (Zhou, Xiao et al. 2010). Thus, the lentivectors constructed in this thesis, by silencing PD-L1 in transduced cells, counteracted this route of immune suppression. While immunosuppressive cells use PD-L1 for suppression this is not the only suppressive mechanism. As the effects of the immunosuppressive tumour environment have become more prominent, research has shifted to pay more attention to it.

Lentivector delivery of TAAs enhances the infiltration of CD4 and CD8 T cells into tumours but at the same time attracts immunosuppressive cell types, such as Tregs and MDSCs. Further, tumour-infiltrating CD8 T cells exhibit high PD-1 expression, secrete low levels of IFNγ and TNFα, and are deficient in degranulation (Zhou, Xiao et al. 2010). The lack of clinical efficacy of immunotherapeutic approaches is now generally believed to be due to immunosuppression, for which MDSCs are largely responsible. Thus, MDSC biology has become of increasing interest in the fields of experimental and clinical cancer immunology (Escors, Liechtenstein et al. 2013; Iclozan, Antonia et al. 2013). Considering these issues I realized the importance of cancer immunotherapy regimens to contain elements initiating TAA-specific responses while at the same time ensuring that these approaches can overcome the immunosuppressive elements of the tumour environment, especially those of MDSCs. Thus, an ex vivo MDSC differentiation protocol was developed to test anti-cancer immunotherapeutic treatments in vitro, circumventing the need to purify tumour-infiltrating MDSCs from a significant number of tumour-bearing mice. The ex vivo MDSC differentiation system produces large numbers of MDSCs ex vivo, which are equivalent to tumour-infiltrating MDSCs. The establishment, assessment, and application of these ex vivo differentiated MDSCs in in vitro assays and high-throughput proteomic studies thus constituted the second part of this thesis.
6.2. CONCLUSIONS

Silencing of PD-L1 in combination with presentation of TAAs leads to the hyper-activation of T cells but not effective anti-tumour activities. It was hypothesized that additional signals needed to be conveyed in cis to T cells and thus lentivectors containing cytokine transgenes were constructed. In this context, the most effective cytokine was IL12, which could be predicted by the DC-T cell as well as MDSC-T cell assays. This suggested that there is an advantage of using both these assays for the preclinical assessment of cancer treatments. Further, vaccination of healthy mice with lentivectors encoding OVA transgenes as well as using tumour models with surrogate antigen OVA expression had limited predictability. A B16 melanoma model without surrogate antigens was therefore used, which revealed the beneficial effects of the IL12-p1-based lentivectors. In this experimental model, induction of anti-cancer immune responses is more challenging, making it a more realistic and more relevant tumour model. Cancer cells express either mutated or altered levels of self-antigens and thus cancer immunotherapy treatments need to overcome the natural tolerance towards these antigens. In addition, mice were vaccinated only once, both in prophylactic and therapeutic settings, as it was argued that this regimen would allow for the identification of treatments that could have an effect in difficult conditions.

IL12 is a known and potent anti-tumour molecule but its clinical use by systemic administration has been limited by high toxicity. The approach presented in this thesis allows for local administration, circumventing toxicity and harnessing its activity at the site of injection or within the tumour. As MDSCs decrease IL12 production by tumour-resident myeloid cells, IL12 delivery counteracts the skewing of the tumour microenvironment. IL12 has anti-tumour as well as immunostimulatory properties, allowing for a multitude of anti-tumour mechanisms within the tumour. Initially I was somewhat surprised that the IFNy-p1-based lentivectors did not exhibit anti-melanoma protective effects as IFNy is central to the endogenous anti-tumour immune response. Due to the intensified study of the immunosuppressive mechanisms it is now becoming clear that IFNy also initiates immunosuppressive mechanisms, to limit inflammatory damage to tissue. Inflammation and IFNy play a role in the induction of MDSC responses
and IFNγ induces MDSCs to up-regulate immunosuppressive molecules TGFβ, iNOS, and IL10 (Huang, Pan et al. 2006; Ostrand-Rosenberg and Sinha 2009).

For any successful cancer immunotherapy strategy, to achieve an effective and potent TAA presentation to effector cytotoxic T cells is crucial, especially CD8 T cells (Bodey, Bodey et al. 2000; Escors, Liechtenstein et al. 2013). Additionally, it is fundamental to ensure that these effector cells will not be inactivated within the tumour. PD-L1 is up-regulated on tumour cells of various cancer types and on tumour-infiltrating myeloid cells. PD-L1 up-regulation is caused by tumour-derived factors as well as the hypoxic tumour environment (Curiel, Wei et al. 2003; Noman, Desantis et al. 2014). This suggests that therapies targeting PD-L1 should be beneficial, which in fact they are (Zhou, Xiao et al. 2010; Brahmer, Tykodi et al. 2012). PD-L1 acts at two levels. PD-L1 plays a role first during antigen presentation by APCs to T cells. Secondly, during antigen recognition on target cells by effector cells. Thus the prophylactic and therapeutic vaccination regimens have to be viewed from different angles and need different requirements. In the prophylactic setting, transduced cells with haematopoietic and non-haematopoietic origins present TRP1 in the context of reduced PD-L1 ligation but increased IL12 secretion. This may lead to hyper-activated TRP1-specific T cells with effector capacities conveyed by IL12. Thus tolerance to TRP1 can be broken, which induces CD4 and CD8 T cells that secrete IFNγ in response to TRP1-presenting DCs. It was shown that transduction of cells of non-haematopoietic origin at the injection site plays a crucial role for enhancing antigen-specific T cell responses. Once B16 tumours are transferred to vaccinated mice, TRP1-specific effector T cells recognize and eliminate B16 tumour cells. Nevertheless, in the end most tumours escaped, apart from one exception in which a mouse exhibited efficacious recall responses 5 months later. In the therapeutic experimental protocol mainly tumour cells are transduced during intra-tumour lentivector injection. The recognition and elimination of tumour cells by TAA-specific T cells is facilitated through inhibition of PD-L1 in transduced tumour cells. In addition, a number of tumour-resident myeloid cells and stroma cells are transduced, assuring that CTLs will not be deactivated once they reach the tumour. In vitro assays using ex vivo B16-MDSCs suggested that once transduced, tumour-infiltrating MDSCs would be capable of activating TAA-specific effector T cell responses. Taken together the data presented in this thesis showed that
the engineered lentivectors could break tolerance to the endogenous TAA TRP1 and prolong survival in prophylactic and therapeutic experimental protocols.

To further elucidate the mechanisms and effects of the IL12-p1-based lentivectors not only their immunostimulatory effects were assessed but also their efficacy to overcome the effects of immunosuppressive MDSCs. During the course of this thesis, an easy and reproducible system was developed for the ex vivo differentiation of MDSCs resembling in vivo cancer-specific immunosuppressive tumour-infiltrating subsets. Importantly, this ex vivo differentiation system included immature stages of myeloid cells, which conserved high proliferative capacities and plasticity of differentiation. Ex vivo B16-MDSCs were highly immunosuppressive in vitro and in vivo and their responses to treatments could predict the anti-tumour efficacy of the IL12-p1-based lentivectors. The use of ex vivo B16-MDSCs in in vitro assays showed that IL12 does not need to be secreted by MDSCs themselves to overcome their T cell suppressive functions. This further suggested that direct intra-tumour vaccination is a valid strategy. The ex vivo differentiated MDSCs were further validated through use of high-throughput analytical techniques, leading to the identification of proteins that have been described in MDSC biology. This strategy uncovered that P450R is responsible for the selective MDSC cytotoxicity of Paclitaxel, representing a previously unknown MDSC-specific target. I therefore propose that systematic assessment of anti-cancer treatments on the viability and differentiation of MDSCs, as well as their effects on immune effector cells such as T or NK cells, will be an effective method to assess anticancer regimens within the preclinical setting.
6.3. THE SIGNIFICANCE OF THE RESULTS WITHIN THE FIELD

IL12 has direct anti-tumour cytotoxic effects in addition to its immunostimulatory effects on a wide range of immune cells. Due to high toxicity following systemic IL12 administration several groups have established protocols to locally administer IL12 to the tumour. Strategies involve the transfer of transduced DCs (Zhao, Bose et al. 2011) and T cells (Chmielewski, Kopecky et al. 2011; Kerkar, Goldszmid et al. 2011; Kerkar, Leonardi et al. 2013) or direct vector injection (Steding, Wu et al. 2011; Thaci, Ahmed et al. 2014) and microsphere administration (Watkins, Egilmez et al. 2007). An approach similar to the one described in this thesis was the adoptive transfer of DCs engineered to secrete IL12. This strategy slowed down B16 tumour growth in vivo in mice through priming of CD8 T cell responses (Zhao, Bose et al. 2011). However, I propose that direct intra-tumour vaccination may be more beneficial than infusion of transduced DCs as it ensures that cancer, tumour stroma, and infiltrating myeloid cells present TAAAs in a positive co-stimulatory context. This proposition is further supported by a recently submitted study by Goyvaerts et al., where vaccines specifically targeting APCs were inferior to broad tropism lentivirus vaccines (Goyvaerts, Lienenklaus et al. 2015). In addition, intra-tumour vaccination is cheaper than autologous DC therapy and thus more amenable to translation to the clinic. While there are several strategies combining TAA-specificity and IL12 production by engineered T cells, so far no approach has combined PD-L1 silencing, IL12 induction, and TAA presentation in a single delivery vehicle. As the efficacy of combination therapies is becoming more prominent, strategies like the one presented in this thesis are becoming more important.

DC ex vivo differentiation protocols were established in the 1990s, enormously boosting the development of cancer immunotherapy treatments, especially of DC-based approaches (Escors, Liechtenstein et al. 2013). These protocols mainly rely on recombinant GM-CSF addition to murine BM or purified monocytes (Inaba, Inaba et al. 1992; Zhou and Tedder 1996). These protocols were simple and reproducible, leading to a rapid increase in the knowledge of DC biology and the number of publications on DCs in the last decade (Escors, Liechtenstein et al. 2013) (Fig. 6.1). From this moment on the effect of immunomodulating (stimulatory and suppressive) strategies were systemically studied. Intracellular signalling, antigen presentation, and DC maturation in response to
stimuli were assessed. The increasing insight into cellular and molecular mechanisms of antigen presentation and T cell polarization led to the development of cancer vaccines through a more rational approach (Liechtenstein, Dufait et al. 2012; Escors, Liechtenstein et al. 2013; Liechtenstein, Perez-Janices et al. 2013). As DCs can be genetically modified with various gene carrier systems, including viral vectors, their maturation status can be modified, thus affecting their polarization of adaptive immune responses (Dullaers, Breckpot et al. 2004; Breckpot and Escors 2009). Nowadays, immunotherapeutic strategies are frequently assessed in \textit{in vitro}/\textit{ex vivo} DC-T cell assays before their use in \textit{in vivo} experimental models (Gruber, Kan-Mitchell et al. 2000; Dyall, Latouche et al. 2001; Arrighi, Pion et al. 2004; Yu, Kovacs et al. 2004; Escors, Liechtenstein et al. 2013). Increasing knowledge of DC-based mechanisms led to their use in anti-cancer immunotherapeutic strategies (Klein, Bueler et al. 2000; Akazawa, Shingai et al. 2007; Escors, Lopes et al. 2008; Breckpot, Aerts-Toegaert et al. 2009; Hu, Dai et al. 2010; Chiang, Hagemann et al. 2011; Karwacz, Bricogne et al. 2011) with promising results in pre-clinical models (Schnurr, Galambos et al. 2001; Hegmans, Hemmes et al. 2005; Liu, Shivakumar et al. 2008). However, promising therapeutic activities could not always be translated into clinical success (Gitlitz, Beldegrun et al. 2003; Zarour and Kirkwood 2003; Escors, Liechtenstein et al. 2013).

What are the reasons for the apparent discrepancy between pre-clinical and clinical efficacy? One reason is that \textit{in vitro} models do not reflect the \textit{in vivo} context and many factors influencing immune responses are disregarded. The second reason likely reflects the major cause for this discrepancy. DCs loaded with TAAs and/or modified to mature must counteract strong and systemic immunosuppressive activities once transferred to cancer patients. Nevertheless, the possibility of differentiating DCs \textit{ex vivo} had a huge impact on cancer immunology (Steinman and Banchereau 2007; Escors, Liechtenstein et al. 2013). Here I propose that we need to go a step further and establish reproducible \textit{ex vivo} MDSC differentiation protocols to model a strong immunosuppressive factor in vaccine evaluation. The use of \textit{ex vivo} differentiated MDSCs will likely have a substantial influence on cancer immunology, hopefully analogous to the establishment and use of \textit{ex vivo} differentiated DCs. Whether the use of MDSCs will be of equal importance to cancer immunology as the use of DCs will have to be assessed in the future. Nevertheless, the importance of MDSCs has steadily been
increasing, as reflected by the increase in the number of publications on these cells in recent years (Escors, Liechtenstein et al. 2013) (Fig. 6.1). Thus, in this PhD thesis I proposed that the establishment and use of MDSC-T cell assays could compliment the use of DC-T cell assays for the assessment of anti-cancer treatments. This would ensure that anti-cancer regimens could overcome immunosuppressive characteristics of MDSCs, making it a more faithful model in cancer immunotherapy research. This approach could save resources, time, and effort, as it would prevent the further development of treatments that are inhibited by MDSC activities. In fact, it is becoming clear that certain immunotherapeutic and chemotherapeutic treatments partially owe their efficacy to their inhibition of MDSC expansion (Serafini, Meckel et al. 2006; Xin, Zhang et al. 2009; Iclozan, Antonia et al. 2013). Of significance, here it is shown that ex vivo MDSCs were amenable to lentivector modification, acquiring APC characteristics, leading to stimulation of antigen-specific immune responses. Our group and others have shown that splenic and tumour-infiltrating MDSCs are not equivalent (Maenhout, Van Lint et al. 2013; Aliper, Frieden-Korovkina et al. 2014; Dufait, Schwarze et al. 2015), highlighting the advantages of this novel ex vivo MDSC differentiation system. Importantly, the ex vivo MDSC differentiation system has already been used by our close collaborators. The use of ex vivo B16-MDSCs predicted the therapeutic anti-melanoma efficacy of mRNA encoding soluble proteins (Van der Jeught, Joe et al. 2014). Further, the system was adapted to the study of colorectal cancer (CT26 tumour model). In this study we showed that MDSC suppressive activity was dependent on Arg-1 and iNOS, by selectively inhibiting these molecules during suppression assays and in vivo in mice. This study confirmed that the ex vivo MDSC differentiation system developed in this PhD thesis renders a high MDSC differentiation efficiency and produces large numbers of immunosuppressive MDSCs with high proliferative potential. The study by Dufait et al. further showed that the novel ex vivo MDSC differentiation system can be applied to different tumour models, facilitating MDSC research in a tumour-relevant manner (Dufait, Schwarze et al. 2015).

Several studies have analysed MDSCs using high-throughput methods such as proteome characterization by mass spectrometry. These studies used MDSCs purified from spleens or blood of tumour-bearing mice, thus not representing tumour-infiltrating MDSC populations (Boutté, Friedman et al. 2011; Boutté, McDonald et al. 2011;
Chornoguz, Grmai et al. 2011; Burke, Choksawangkarn et al. 2014). Nevertheless, these studies were useful in identifying characteristics of circulating and peripheral MDSCs. As the differences between circulating and tumour-infiltrating MDSCs have become prominent, researchers have started using purified tumour-infiltrating MDSCs in proteomic studies. A recent study compared splenic and tumour-infiltrating MDSCs by gene arrays, further emphasizing the difference between these MDSC populations. This study predicted key transcription factors, kinases, and proteases within the MDSC populations following the inferred interactomes from gene expression arrays (Aliper, Frieden-Korovkina et al. 2014). This study complemented and reinforced the validity of our system. While this is a very reliable way of assessing in vivo MDSCs, using the ex vivo MDSCs developed in this thesis substantially facilitates the application of MDSCs in high-throughput studies. Due to the laborious nature of using tumour-infiltrating MDSCs in studies requiring large numbers of sample, their use in this setting has been limited. Use of MDSCs in high-throughput studies will aid in clarifying several issues in MDSC biology as well as facilitate the discovery of MDSC-specific targets. A main challenge in MDSC biology remains their heterogeneity, making it difficult to distinguish them from other myeloid cell types. Thus the use of ex vivo MDSCs along-side other myeloid cell types will be able to clarify fundamental differences. This approach was used in this thesis to identify differential protein expression between immature BM-DCs and ex vivo B16-MDSCs and the same strategy can be applied to other myeloid cell types. Other important issues to clarify are the MDSC activation mechanisms upon tumour infiltration as well as the tumour-specific differences, which our group and other researchers have attempted to clarify (Aliper, Frieden-Korovkina et al. 2014).
Figure 6.1. Evolution of number of publications for conventional DCs and MDSCs. Bar graphs represent the approximate number of publications dealing with DCs or MDSCs as a function of the date of publication. PubMed (http://www.ncbi.nlm.nih.gov/pubmed) was searched for entries whose titles contained terms “dendritic cell” or “myeloid-derived suppressor cells”. Reproduced from Escors et al., Oncoimmunology, Vol. 12(10), e26148, 2013 (Escors, Liechtenstein et al. 2013).
6.4. LIMITATIONS AND FUTURE PERSPECTIVES

While limited efficacy in preventing tumour establishment in the prophylactic setting (a single survivor mouse) and no cure rates were achieved here, it was proposed that these stringent conditions allow for a more realistic and practical vaccination protocol. I strictly implemented a single vaccination regimen in a rapidly growing tumour model lacking surrogate tumour antigens. The use of OVA antigen is a convenient way of assessing antigen presentation but its use in vivo obscures the efficacy of anti-cancer immunotherapeutic treatments. Conducting pre-clinical research in this more realistic setting would be beneficial for alleviating the discrepancies between preclinical research and clinical efficacy.

Another reason for the discrepancies between preclinical and clinical efficacy is the fact that immunosuppressive mechanisms are not included in pre-clinical evaluation. A significant amount of time and resources could be saved if anti-cancer treatments would be assessed in more realistic pre-clinical assays. Thus, MDSC-T cells assays using ex vivo MDSCs were performed in this thesis, showing that IL12-p1-based lentivectors could overcome the immunosuppressive functions of these cells. While these assays take into account the immunosuppressive effect of MDSCs, they remain another approximation to reality. Additional cell types and immunosuppressive mechanisms exist in the tumour that may prevent MDSCs acquiring APC activities. All in vitro assays are undertaken in a highly controlled environment. While this is important to dissect effects from an experimental point of view, it does not reflect the in vivo situation within tumour-bearing hosts.

It was not possible to further improve the human MDSC ex vivo differentiation protocol within the time frame of this PhD thesis. Human models are generally more complicated to establish and this holds true also for DC ex vivo differentiation protocols. Human DCs are differentiated from PBMCs, in this way limiting the initial supply of immature myeloid precursors and making the system less efficient. Nevertheless, it remains a good DC differentiation protocol of key clinical relevance (Escors, Liechtenstein et al. 2013). Addition of several endogenously produced growth factors and cytokines will surely improve the human ex vivo MDSC differentiation system presented in this thesis. An improved ex vivo human MDSC differentiation system will
represent a significant step forward. Once this system is fully developed, human MDSCs can be generated by patient-specific tumour cells. The patient-specific ex vivo differentiated human MDSCs could then be tested in mouse models with patient-derived xenografts.

The first successful cancer immunotherapy clinical trials using lentivectors have recently been published (Kalos, Levine et al. 2011; Porter, Levine et al. 2011; Maude, Frey et al. 2014). It is evident that the use of lentivector-based treatments in cancer patients is feasible, effective, and possibly safe, although a number of issues need to be taken into account. Translation of lentivector constructs for use in clinical practice entails elaborate production and purification procedures, safety measures, and high costs. Thus, ensuring GMP (good manufacturing practice)-grade lentivector production remains a barrier to lentivector-based treatments. Lentivector production systems should be improved, as the establishment of stable producer cell lines remains a challenge. Stable producer cells lines would decrease production costs and lead to higher quality and safer vector particles. Significant steps forward have been undertaken very recently to implement such an efficient GMP lentivirus production system (Sanber, Knight et al. 2015). In this way, lentivectors can be continuously produced without the need of transiently transfecting the transfer, packaging, and envelope plasmids. Safety measures need to ensure that no replication competent retroviruses can arise in treated patients and that no insertional mutagenesis or other genotoxic events are initiated by these treatments (Liechtenstein, Perez-Janices et al. 2013). Nevertheless, improvement of lentivector production is a worthy pursuit to facilitate the application of lentivector-based treatments in human gene therapy.

Gene therapy techniques have become more efficient and safer over the last decade and a milestone in cancer immunotherapy using lentiviral vectors has recently been achieved. Clinical trials with CAR T cells have shown highly promising clinical responses and have emphasized the viability of cancer immunotherapeutic strategies using lentivectors (Kalos, Levine et al. 2011; Porter, Levine et al. 2011; Maude, Frey et al. 2014). As cancer immunotherapy has been largely ineffective in the last decades, the emphasis has shifted to encompass research on the immunosuppressive mechanisms within tumour-bearing hosts. Pre-clinical MDSC research has been hampered by the fact that splenic MDSCs are not equivalent to tumour-infiltrating MDSCs and that purification
of tumour-infiltrating MDSCs is expensive, time-consuming and inefficient. The development of this novel \textit{ex vivo} MDSC differentiation system facilitates research on MDSC biology and identification of targets for therapy. Using these cells an MDSC-specific target was identified by quantitative proteomics, P450R. Currently our laboratory is continuing the work initiated in this PhD thesis by silencing other proteins identified by this proteomic study, especially the ones involved in ROS generation and protection against oxidative damage. These studies of MDSC biology are possible as \textit{ex vivo} MDSCs and their precursors can be transduced by lentivectors. By silencing or activating genes of interest we will gain enhanced insight into MDSC biology and identify further MDSC-specific targets.

Characterization of MDSCs will in turn facilitate the discovery of MDSC therapeutic targets, which remains the most prominent application of MDSC \textit{ex vivo} differentiation systems. Several strategies are being developed to target MDSCs for cancer immunotherapy. These strategies include MDSC differentiation/maturation, MDSC depletion, MDSC inhibition, and inhibition of MDSC recruitment (Albeituni, Ding et al. 2013; Jiang, Guo et al. 2014). Further, MDSCs could be used as vehicles to deliver cancer-targeting molecules to the tumour site (Chandra and Gravekamp 2013; Chandra, Jahangir et al. 2013). The wide variety of MDSC-targeting therapies and the issue of cancer-specific MDSC phenotypes require large numbers of tumour-specific MDSCs for high-throughput drug testing. Moreover, MDSC biology can equally be applied to the fight against pathogenic infections or in the field of autoimmune diseases. An efficient way to generate \textit{ex vivo} MDSCs in the context of chronic inflammation could surely enhance MDSC research in the context of pathogenic infections. A differentiation system could be developed to mimic chronic inflammation mediated by the pathogenic agent in question. The application of MDSCs in autoimmune diseases, on the other hand, would be based on the adoptive transfer of \textit{ex vivo} differentiated immunosuppressive MDSCs.
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APPENDIX A: FIRST AUTHOR PUBLICATIONS