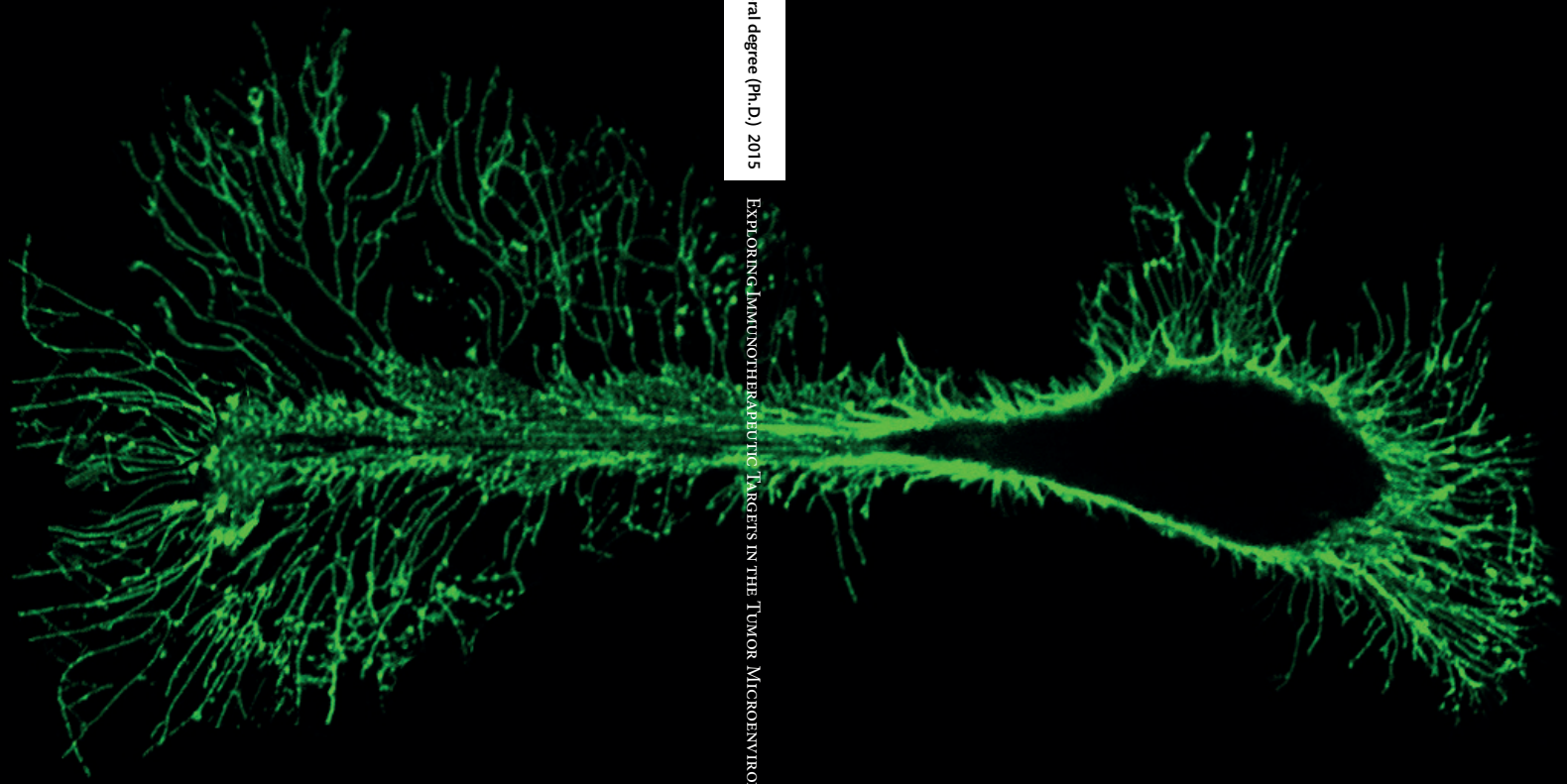

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From The Department of Microbiology, Tumor & Cell biology
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EXPLORING IMMUNOTHERAPEUTIC TARGETS IN THE TUMOR MICROENVIRONMENT

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Department of Microbiology, Tumor and Cell biology

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Stockholm 2015

To my parents,

for giving me my wings

Σα βγεις στον πηγαιμό για την Ιθάκη,
να εύχεσαι να ’ναι μακρύς ο δρόμος,
γεμάτος περιπέτειες, γεμάτος γνώσεις.
Κ. Καβάφης

As you set out for Ithaka,
hope the voyage is a long one,
full of adventure, full of discovery.
K. Kavafis

ABSTRACT

The immune system has developed along with the evolution of increasingly complex cellular organisms to sustain homeostasis and protect from threats. Cancer, a detrimental side effect of increasing organismic complexity, typically sequesters the immune system and hijacks its functions for its own prosperity. Cancer immunotherapy aims to harness the intrinsic potential of the immune system for the therapeutic benefit of cancer patients.

The focus of this thesis is to identify and evaluate new immunotherapeutic targets in the tumor microenvironment, which can be modulated to restrict tumor growth and metastasis.

Paper I describes a novel mechanism of interaction between marginal zone macrophages (MZMs) and marginal zone B cells (MZBs) in the spleen, which can be modulated by antibodies (Abs) to scavenger receptor MARCO on MZMs. This study demonstrates that MARCO targeting diminishes antigen (Ag) uptake by MZBs, which results in reduced Ag deposition in the splenic follicles. As anti-MARCO Abs can also be found in systemic lupus erythematosus (SLE), this interaction may affect subsequent adaptive immune responses to both self- and foreign antigen.

Paper II identifies MARCO as a specific marker for a tumor-promoting macrophage subtype in the tumor microenvironment of mammary carcinoma, melanoma and colon carcinoma tumor models. Targeting MARCO on tumor-associated macrophages (TAMs) by Abs, inhibits tumor growth and metastasis and reprograms TAMs to an anti-tumor phenotype. Treatment with anti-MARCO mAbs in combination with checkpoint inhibitor, anti-CTLA-4 ab, may provide a promising approach for cancer immunotherary with clinical relevance for human breast cancer and melanoma.

Paper III reveals the molecular mechanism through which tumor cells imitate immune cells, during epithelial-mesenchymal transition (EMT), prompting their targeted metastasis through the lymphatics. This study demonstrates that TGF-β, a known inducer of EMT, regulates the chemotactic axis CCR7/CCL21, directing preferential lymphatic dissemination of breast cancer cells.

Paper IV evaluates dendritic cell-derived exosomes in a vaccination approach to re-activate adaptive anti-tumor responses. This study shows that dendritic cell-derived CD1d expressing exosomes loaded with α-Galactosylceramide (α-GalCer) can sensitize NKT cells and lead to subsequent activation of B cell and effector T cell responses, restricting tumor growth.

In summary, the work presented in this thesis describes novel targets in the tumor microenvironment that can be used in immunotherapeutic approaches to re-activate endogenous mechanisms of innate and adaptive immunity against cancer. Additionally, it gives new insight into gene regulatory pathways controlling metastatic tumor spread, as well as utilizes custom designed biological molecules in anti-tumor vaccination strategies.

Increasing our understanding of the intricate mechanisms regulating the immunosuppressive tumor microenvironment will reveal new knowledge and novel targets that can contribute to the design of prospective cancer immunotherapies.

LIST OF SCIENTIFIC PAPERS

I. Prokopec K*, **Georgoudaki AM***, Sohn S, Wermeling F, Grönlund H, Lindh E, Carroll MC, Karlsson MCI
Marginal zone macrophages regulate antigen transport by B cells to the follicle in the spleen via CD21
Submitted

II. **Georgoudaki AM**, Prokopec K, Boura V, Hellqvist E, Sohn S, Östling J, Dahan R, Harris RA, Rantalainen M, Klevebring D, Sund M, Egyhazi Brage S, Fuxe J, Rolny C, Li F, Ravetch JV, Karlsson MCI
Reprogramming tumor associated macrophages by antibody targeting inhibits cancer progression and metastasis
Submitted

III. Pang MF, **Georgoudaki AM**, Lambut L, Johansson J, Tabor V, Hagikura K, Jin Y, Jansson M, Alexander JS, Nelson CM, Jakobsson L, Betsholtz C, Sund M, Karlsson MCI, Fuxe J
TGF-β1-induced EMT promotes targeted migration of breast cancer cells through the lymphatic system by the activation of CCR7/CCL21-mediated chemotaxis
Oncogene, 2015 May 11. doi: 10.1038/onc.2015.133.

IV. Gehrmann U, Hiltbrünner S, **Georgoudaki AM**, Karlsson MCI, Näslund TI*, Gabrielsson S*
Synergistic induction of adaptive antitumor immunity by codelivery of antigen with α-galactosylceramide on exosomes
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Georgoudaki AM, Khodabandeh S, Puiac S, Persson CM, Larsson MK, Lind M, Hammarfjord O, Nabatti TH, Wallin RP, Yrlid U, Rhen M, Kumar V, Chambers BJ
CD244 is expressed on dendritic cells and regulates their functions
Immunology & Cell Biology, 2015 Jul;93(6):581-90

Lindmark E, Chen Y, **Georgoudaki AM**, Dudziak D, Lindh E, Adams WC, Loré K, Winqvist O, Chambers BJ, Karlsson MCI
AIRE expressing marginal zone dendritic cells balances adaptive immunity and T-follicular helper cell recruitment
Journal of Autoimmunity, 2013 May;42:62-70

CONTENTS

LIST OF ABBREVIATIONS

α-GalCer	alpha-galactosylceramide
Ab	Antibody
ADCC	Antibody-dependent cellular cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
ADP	Adenosine diphosphate
Ag	Antigen
AP-1	Activator protein-1
APC	Antigen presenting cells
ATP	Adenosine triphosphate
BCR	B-cell receptor
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
CD40L	CD40 ligand
CDR	Complementarity determining regions
CRI	Cancer-related inflammation
CSF-1(R)	Colony stimulating factor-1 (receptor)
CTL	Cytotoxic T lymphocytes
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CXCR	CXC chemokine receptor
DAMP	Danger associated molecular pattern
DC	Dendritic cell
EGF	Epidermal growth factor
EMT	Epithelia-to-Mesenchymal Transition
Fab	Fragment, antigen binding
Fc	Fragment, crystallizable
FcγR	Fc gamma receptor
FDC	Follicular dendritic cell
FOB	Follicular B cell
GC	Germinal center
GITR	glucocorticoid-induced TNFR-related protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IC	Immune complexes
ICD	Immunogenic cell death
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cells
(i)NKT	Invariant natural killer T cells
iNOS	Inducible nitric oxide synthase
IRF	Interferon regulatory factor 1
IVIG	Intravenous immunoglobulin
KIR	Killer-cell immunoglobulin-like receptors
KO	Knock out
LPS	Lipopolysaccharide
M-CSF	Macrophage colony-stimulating factor
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MARCO	Macrophage receptor with collagenous structure
MDSC	Myeloid derived suppressor cell
MHC	Major histocompatibility complex
MHC-I/-II	MHC class I/II
MMP	Matrix metalloproteinases
Mo-MDSC	Monocytic-MDSC
MyD88	Myeloid differentiation primary response gene 88
MZ	Marginal zone
MZB	Marginal zone B cells
MZM	Marginal zone macrophages
MΦ	Macrophages
NALP3	NACHT, LRR and PYD domains-containing protein 3
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer
$NKT_{(fh)}$	Natural killer-like T (follicular helper) cell
NLR	Nod-like receptor
NOD	Nucleotide-binding oligomerization domain
PAMP	Pathogen associated molecular patterns
PD-(L)1	Programmed death-(ligand) 1
pMHC	Peptide-MHC complex
PMN-MDSC	Polymorphonuclear-MDSC
PRR	Pattern recognition receptor
RLR	RIG-I-like receptors
SIGN-R1	Specific intracellular adhesion molecule-3-grabbing non-
SLE	Systemic lupus erythematosus
SR	Scavenger receptor
SRCR	Scavenger receptor cysteine rich
STAT	Signal transducer and activator of transcription
TAM	Tumor associated macrophages
TAN	Tumor associated neutrophils
TCR	T cell receptor
TD	Thymus-dependent
T_{fh}	T follicular helper cell
TGF-β	Tumor growth factor beta
T_h	T helper cell
TI	Thymus-independent
TI-I/-II	Thymus –independent Type I/II
TIL	Tumor infiltrating lymphocytes
TLR	Toll-like receptor
TNF(R)	Tumor necrosis factor (receptor)
TRAIL	TNF-related apoptosis-inducing ligand
Treg	T regulatory cell
VEGF	Vascular endothelial growth factor

1 INTRODUCTION

1.1 THE IMMUNE SYSTEM

The immune system is an ensemble of sophisticated sensors and effectors that are in place to ensure tissue homeostasis and protect from invasion by infectious pathogens. Threats can be detected by a plethora of molecular sensors, which vary depending on the context of the threat. Accordingly, these sensors also target different responders with various functional activities. The common goal of these immune responses is to restore homeostasis or eliminate threats by inducing inflammation. Although infectious pathogens are the most commonly considered instigators, inflammation also occurs during tissue injury, cancer, autoimmune disease and metabolic deregulation [1].

The existence of the immune system was observed as early as 430 BC by the Athenian Thucydides who described that patients who recovered from the plague were not struck a second time or to the same extent by the disease. This observation describes the concept of immunity, which refers to the physiological state where an organism exhibits lack of susceptibility towards unwanted or noxious agents. The term originates from the Latin *immunitas,* which means "to be exempt from". References to active immunity date back to the early 1700s, when the practice of variolation in the Ottoman Empire (Turkey) was reported in the *Philosophical Transactions* of the Royal Society of London.

In 1796, Edward Jenner, set up an experiment that would for the first time demonstrate the workings of active immunotherapy and what we today refer to as immunological memory by showing that exposure to cowpox induced protection to smallpox. In 1884 Robert Koch identified pathogens as the underlying cause of infectious diseases postulating the "Germ theory". This would set the basis for what Louis Pasteur in 1891 termed vaccination (*fr. latin vacca;* cow), posthumously crediting Jenner for his contribution and expanding the term to include immunizations against various different pathogens. In parallel, great advances were made in the field of immunology, such as the discovery by Ilya Metchnikov in 1866 of leukocytes (white blood cells) with the capacity to ingest pathogens. He termed this process phagocytosis and the cells exerting this function phagocytes, an important component of the innate immune system [2]. This marked the birth of the branch of cellular immunity and earned Metchnikov the Nobel prize in 1908.

Further efforts to elucidate the mechanisms of induced immunity lead Emil von Behring and Shibasaburo Kitasato in 1890 to identify antitoxins, soluble factors in the serum, that were able to neutralize bacterial toxins. Antitoxins, later termed antibodies (Abs), could confer passive immunity to naïve individuals. Paul Ehrlich was the first to suggest the lock-and-key model describing the mechanism of molecular interactions through which Abs bound their ligands. Antibodies, together with the complement and antimicrobial peptides, constitute humoral immunity. The generation of such pathogen-specific immune responses would later be attributed to the adaptive immune system, which is acquired during the lifetime of an individual.

The above-mentioned discoveries come together to form our current view on the concept of immunology. As it is understood today, the immune system is a set of interactive networks which have evolved to eliminate potential threats (infection) and restore homeostasis (following trauma or cancer), while retaining sufficient tolerance to avoid reactivity to self (autoimmunity) or otherwise innocuous agents (allergy). A vital prerequisite for this to occur is the ability of the immune system to discriminate "self" from "non-self". Hence, recognition is an important capacity of both the early, innate immune system and the late, specific adaptive immune system and holds the key to preserving the balance between immune tolerance and immune-mediated elimination.

Below follows a description the two branches of the immune system, which collaborate to orchestrate immune cascades with the aim to restore immunity and homeostasis. Particular emphasis will be given to the cells and mechanisms relevant to this thesis.

1.1.1 Lymphoid organs

Immune cells arise from the $CD34^+$ hematopoietic stem cell lineage whose developmental niche is primarily the bone marrow. The bone marrow, along with the fetal liver and the thymus are termed primary lymphoid organs and are sites where immune cells are generated. Upon differentiation from common progenitors, the pluripotent hematopoietic stem cells, immune cells diverge to different lineages and egress from the bone marrow. In the periphery they populate different tissues, as well as secondary lymphoid organs, which function as major hubs where immune responses are generated. The secondary lymphoid organs include the spleen, lymph nodes, Peyer's patches, mucosa/gut-associated lymphoid tissue (MALT/GALT) and tonsils. Secondary lymphoid organs are dispersed throughout the body providing a network for efficient communication, facilitating immune interactions. These organs are interconnected by the lymphatic vessels. Some sites, however, are "protected" from possible adverse effects of inflammatory responses and therefore immune cell presence is restricted [3]. These sites are known as immune privileged and include the central nervous system, testis and eyes. These sites are instead guarded by other protective mechanisms, such as immunoglobulins (Igs) and complement.

The spleen, the largest peripheral lymphoid organ, is an important site where innate immune cells migrate to orchestrate adaptive immune responses. The splenic red pulp contains macrophages, which are responsible for scavenging aging erythrocytes and uptake of antigen (Ag) from the blood. The white pulp is the lymphoid compartment of the spleen, which consists of highly organized structures, known as the B cell follicles and the surrounding T cell areas, much resembling a lymph node. B cell follicles are the sites where Ag-specific adaptive immune responses are initiated. In mice, located between the white and red pulp is the marginal sinus, which directly connects the spleen to the circulation and is in strategic proximity of the marginal zone (MZ) of the spleen surrounding the follicles. Specialized cells populating the marginal zone, the marginal zone macrophages (MZMs), the marginal zone B cells (MZBs) and dendritic cells (DCs), surveil the blood entering the marginal sinus and capture antigens Ags through pattern recognition receptors (PRRs) [4,5] **(Figure 1)**. MZBs

shuttle Ags from the MZ into the follicles for Ag-presentation on follicular dendritic cells (FDCs) and activation of adaptive T and B cell responses through germinal center (GC) formation. The MZ is a dynamic structure where MZMs and MZBs are interdependent for their retention in the MZ and their trafficking to specialized splenic compartments upon Ag uptake [6]. Similar interactions are observed at other sites of immune surveillance, for example between subcapsular macrophages and follicular B cells (FOBs) in the lymph nodes, and peritoneal macrophages and B1 B cells in the peritoneal cavity. This common interaction between the two cell types suggests an important role for macrophages in regulating antigen availability and presentation by B cells, which will be addressed further in Paper I.

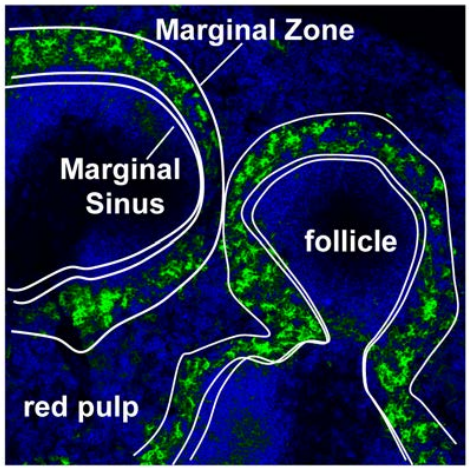

Figure 1. The marginal zone of the spleen
Immunofluorescence staining on histological section of a mouse spleen. Green indicates MZM expressing MARCO, lining the outer side of the marginal sinus of a B cell follicle, B cells are stained with B220 (blue).

1.1.2 The innate immune system

The innate immune system is the body's primary line of defense. It partly consists of physical barriers, such as the skin and mucosal surfaces, and anti-microbial peptides, which act by inhibiting pathogens at sites of entry. Additionally, the innate immune system consists of cells such as monocytes, macrophages, DCs, neutrophils, eosinophils, basophils and mast cells which are of myeloid origin, but also some DCs, natural killer (NK) cells and the recently described innate lymphoid cells (ILCs), which are lymphocytes. The innate immune system is characterized by rapid and broad responses against threats. The "self" vs. "non-self" discriminating ability of the innate immune system lies in the vast array of molecular PRRs. Some PRRs of ancestral defense systems (such as Ig-like structures, scavenger receptor cystein-rich domains and Toll-like receptors) are preserved through evolution. More specifically, the mechanistic basis for the innate immune system's efficiency depends on the recognition of common conserved molecular signatures/structures on pathogens, known as pathogen-associated molecular patterns (PAMPs), by PRRs. PRRs discriminate molecular patterns signaling altered self, non-self and damage/danger [7]. PRRs can be divided into membrane bound; such as scavenger receptors (SR), toll-like receptors (TLR) and C-type lectin receptors, cytoplasmic; such as NOD-like receptors (NLR) and retinoic acid-inducible gene (RIG)-I-like receptors (RLR), and secreted; complement receptors and others [8]. Charles

Janeway proposed the Infectious non-self theory, whereby the immune system recognizes noxious foreign agents through PRRs on antigen presenting cells (APCs). Later, Polly Matzinger suggested the revised 'Danger model', suggesting that the immune system can distinguish between dangerous and innocuous stimuli of either self- or foreign origin [9]. Similarly to PAMP recognition, PRRs are also capable of recognizing endogenous components signaling 'damage'. These signals are released into the surrounding extracellular space, actively secreted or exposed on the surface of dying, stressed or injured cells: these include calreticulin (CRT), adenosine triphosphate (ATP) and high mobility group protein B1 (HMGB1). They are known as danger-associated molecular patterns (DAMPs) and have recently been shown to function as adjuvants (generating "sterile" inflammation) for the induction of immune responses during cancer immunotherapy, a concept described as immunogenic cell death (ICD) [10]. Release of extracellular ATP by apoptotic cells functions as a "find me" signal, but ATP can also mediate an array of immunoregulatory responses and is thus of particular interest for this thesis. ATP release during ICD can activate the immune system, however its catabolite adenosine has the opposite effect, immunosuppression. The catabolic activity of ectonucleotidase CD39 is responsible for the reversible conversion of ATP to ADP (adenosine diphosphate) and ADP to AMP (adenosine monophosphate), while CD73 handles the irreversible conversion of AMP to adenosine [11].

1.1.3 The adaptive immune system

The adaptive immune system arose as a prerequisite for survival during the evolution of increasingly complex organisms and evolved to fulfill the need for a more specialized line of defense. In contrast to innate immune responses, adaptive immune responses are not self-sufficient. They depend on and are tightly interwoven with components of the innate immune system for the orchestration of functional cascades of events that aim to restore homeostasis. Another basic difference between the two branches of immunity, is the fact that adaptive immune responses are antigen-specific and encompass a memory component to previously encountered Ags. The main cellular components of the adaptive immune system are the T and B cells, which arise from common lymphoid progenitors in the bone marrow and diverge into separate lineages in the periphery. Depending on the type of antigen, different types of immune responses are orchestrated by the secretion of different cytokines, leading to the mobilization of various subsets of immune effector cells. These processes ultimately induce clonal expansion of activated T and B cells, as well as deployment to the site of inflammation where they exert various effector functions. Thus, the peripheral lymphoid organs, the spleen and the lymph nodes, are the sites where cellular components of the innate immune system activate cellular and humoral responses of adaptive immunity.

1.1.4 Lymphocytes

The cells of the lymphoid lineage originate from common lymphoid progenitors in the bone marrow, which give rise to T cells, γδ T cells, NKT cells, NK cells, innate lymphoid cells (ILC), subsets of DCs and B cells. The relevant subsets for this thesis are described below.

1.1.4.1 T cells

T cells are part of the lymphoid hematopoietic lineage. T lymphocyte precursors (thymocytes) originate from common lymphoid precursors in the bone marrow, which migrate to the thymus where T cell development occurs. Like the innate branch, the functionality of the adaptive immune system relies on efficient recognition. Therefore, T cells express the T cell receptor (TCR) on their surface, which is generated through complex gene rearrangements of the Tcr loci during T cell development in the thymus. The TCR consists of one α and one β-chain, it has a single unique antigen-recognition site and binds to antigenic peptides in complex with the major histocompatibility complex (MHC) on the surface of APCs. Upon engagement of the Ag-specific TCR with its cognate Ag, the cell expressing that TCR undergoes extensive proliferation, known as clonal expansion, and part of its progeny will go on to mount a directed immune response against the specific target, whether it be an infected cell or cancer cell. Other progeny will form a memory compartment that can instantly be deployed upon re-encounter of the Ag. Consequently each individual acquires a unique repertoire of Ag-specific T cell clones depending on their antigenic encounters.

More specifically, T cell precursors in the thymus express a functional TCR as well as co-receptors CD4 and CD8. During the first step, T cells whose TCR will engage self-peptide-MHC (pMHC) complexes on the cell surface of cortical thymic epithelial cells (cTECs) are selected, while absence of this "survival" signal results in death by neglect. This process is termed positive selection and ensures the functionality of the selected clones. Selected clones undergo a second step, negative selection, during which T cells with a TCR that engages self-pMHC on the surface of medullary TECs too strongly are eliminated through apoptosis. This is known as negative selection and aims to eliminate autoreactive clones. Selection is thus, regulated by a certain threshold of activation.

Thereafter, selected naïve T cells egress from the thymus and populate peripheral lymphoid tissues, such as the spleen and lymph nodes. There, induction of adaptive immune responses relies on the activation of the innate immune system and antigen presentation. During Ag-presentation by APCs to naïve T cells in peripheral lymph nodes, three signals are required for lymphocyte activation to occur. The first signal is provided by engagement of the TCR with the peptide-MHC complex and the second signal is provided by the activated DC in the form of co-stimulatory molecules CD80 and CD86. Depending on the context of the immune response the DC will provide a third activation signal in the form of soluble mediators, such as cytokines, which will polarize T cells to different effector phenotypes. pMHC-I complexes are recognized by $CD8^+$ T cells, while pMHC-II complexes are recognized by $CD4^+$ T cells. Thus, $CD8^+$ T cells become cytotoxic effectors, while $CD4^+$ T cells polarize into various helper populations driven by the up-regulation of different transcription factors depending on the inflammatory mediators in the microenvironment (more specifically: cytokines interferon γ (IFNγ) and IL-12 drive the expression of transcription factor *T-bet* which leads to T helper 1 (T_h1) polarization. Similarly, IL-4/*GATA-3* drive T_h2, IL-6/IL-21/IL-23/*RORγT* drive T_h17 and TGF-β/*Foxp3* drive T_{reg} polarization).

1.1.4.2 Natural Killer cells

NK cells were discovered in 1975 by Rolf Kiessling, Eva Klein and Hans Wigzell at Karolinska Institutet [12,13] and Ronald Heberman et al. simultaneously, at the University of Pittsburgh [14,15]. They are cells of lymphoid origin with the capacity to exert cytotoxic activity against virus infected or tumor cells without the need for prior activation. As such, they are attractive candidates for cancer immunotherapy [16]. They are potent producers of IFNγ and have cytotoxic granules containing perforin as well as granzyme B. NK cell recognition and binding to self-pMHC complexes on the surface of other cells leads to NK cell inhibition. This is mediated by the inhibitory killer cell Ig-like receptors (KIR) in human NK cells and by the c-type lectin-like Ly49 family of receptors in mice, and by NKG2A, which is conserved between the species. NK cells are educated to recognize a vast repertoire of MHC-I loaded with self-peptides. However, in the absence of self-pMHC-I recognition, lack of inhibitory receptor signaling leads NK cell activation, according to the "missing self hypothesis" [17]. This can occur e.g. in an MHC-I mismatch transplantation setting or in tumors that have down-regulated MHC-I as an immune escape mechanism. NK cells also express CD16 (FcγRIIIA/B in humans, FcγRIII in mice), a receptor involved in antibody-dependent cellular cytotoxicity (ADCC), and can be activated by binding of the Fc-part of Abs, as well as activating receptors such as DNAM1, NKp30, NKp44, NKp46 and NKG2D, whose ligands are present on tumor cells and infected cells. Thus, the final fate of NK cell responses results from the net balance of activating vs. inhibitory signal input.

1.1.4.3 Natural Killer T cells

NKT cells are innate lymphoid cells that share characteristics of both NK cells, such as expression of NK1.1 and NKG2D, and T cells as they express a TCR [18]. They are a small population making up 0.1% of the cells in the human circulation. The TCR of NKT cells is restricted to CD1d, an MHC-like presentation molecule which presents lipid and glycolipid Ag. Like NK cells, upon activation they secrete perforin and granzymes upon activation but they are also potent producers of cytokines. Unlike T cells, their TCR is less variable, only consisting restricted α and β chains. There are two types of NKT cells, Type I invariant NKT cells (iNKT) that bind the prototypic glycolipid Ag α-galactosylceramide (α-GalCer), and Type II diverse NKT cells which bind sulfatide. The study of NKT cells has been facilitated by the generation of CD1d-tetrameres loaded with α-GalCer. However, deciphering between the actions of the two subpopulations is still complex. There are two mouse models that allow the *in vivo* study of NKT cells. Jα18 knock-out (KO) mice are deficient in iNKT cells, while CD1d KO lack both subtypes. Thus the observed differential outcomes while comparing immune responses in these two strains has helped shed light onto the action of Type II NKT cells. In homeostasis, CD1d presents self-lipids to NKT cells. In mice, iNKT cells are classified into two major categories based on the expression of CD4 and CD8. The majority are $CD4^+$ and the remaining are $CD4^-CD8^-$, and differ in their ability to secrete cytokines [19]. They have been described to contribute to both pro- and anti-inflammatory innate immune responses depending on the cytokine context in their activation milieu and the lipid Ag they recognize. According to their function NKT cells

can be further classified into NKT_H1, NKT_H2, NKT_H17 and NKT_{FH} cells, and upon activation they become rapid producers of various cytokines [20]. In response to α-GalCer, NKT cells secrete IFNγ, inducing the maturation of DC into APCs by providing co-stimulation via the CD40-CD40L interaction. This interaction also leads to the release of interleukin 12 (IL-12) from activated DC which further propagates $CD8^+$ T cells through DC-cross-priming via CD70 [21]. Notably, many solid tumors express CD1d on their surface and the role of NKT cells in tumor immune surveillance is compelling, as suggested by their low number and loss of functionality in cancer patients [22]. Moreover, iNKT cells have been reported to kill tumor-associated macrophages, a major immunosuppressive cell type found in tumors (described in 1.2.1), thus contributing to anti-tumor immunity [23]. Due to this, most studies on iNKT cells in cancer immunotherapy utilize the so far best-characterized agonist, α-GalCer, which has been widely explored both experimentally and recently also in clinical trials as an adjuvant in DC-based vaccine approaches [24,25]. However, attempts to enhance NKT cell mediated anti-tumor responses by administration of soluble α-GalCer have been shown to cause anergy and therefore more controllable delivery systems and other agonistic analogues are being explored (see Paper IV) [26,27]. Examples of such are the co-administration of α-GalCer and tumor-Ag or the adoptive transfer of a-GalCer–loaded DCs or exosomes with tumor-Ag, both of which induced durable NKT cell cytokine responses [28,29].

1.1.4.4 B cells

B cells were discovered by Max Cooper in 1965 as a separate type of lymphocytes than T cells [30,3130,3130,31] that develop from common lymphocyte precursors in the bone marrow [29,30]. In the bone marrow, they transition from pro-B cells, late pro-B cells, large pre-B cells, small pre-B cells and immature B cells that egress the bone marrow into the blood stream and migrate to peripheral lymphoid organs where they later mature [32]. During their development in the bone marrow B cells interact with stromal cells and recognize Ags through the highly specific B cell receptor (BCR), which is a membrane-bound Ig that arises from the random rearrangement of the gene segments of the Ig locus. The BCR consists of two pairs of a heavy and a light chain (κ, kappa or λ, lambda) which come together to form two identical antigen-binding regions that directly bind to antigenic peptides, the complementarity determining regions (CDRs) [33]. Similar to T cells, the cost of the high specificity of a vast array of BCRs is the generation of autoreactive B cell clones. Upon engagement of the BCR with self-Ags, B cells are eliminated by apoptosis or undergo editing of the Ag-specificity of their BCR to avoid self-reactivity and are then released into the periphery. This gives rise to a wide repertoire of receptors specificities (up to 10^8 different ones), which accommodate a vast heterogeneity of Ags. Although central tolerance provides an efficient checkpoint it is not infallible, as 4% of immature B cells in the periphery are autoreactive. Several mechanisms, collectively known as peripheral tolerance, are put in place to compensate for the escape of those autoreactive clones from central selection. These include suppression by T_{regs}, induction of anergy in the absence of co-stimulation or presence of co-inhibition, and immune privileged sites.

The main innate-like B cell subset is the B1 B cells that populate the peritoneal cavity, the MZBs lining the marginal sinus of the spleen and the FOBs, which are found in the follicles of the spleen and lymph nodes. FOBs express CXCR5, which is important for their retention in the follicles in response to CXCL13. While FOBs recirculate, MZBs are mostly regarded as resident in the MZ. MZBs comprise a rather scarce, highly specialized B cell subpopulation in the spleen. They localize in close proximity to MZMs and DCs filtering the incoming blood for particulate Ags. MZBs are generally identified as $B220^{+}$ $CD21^{hi}$ (complement receptor 2) $CD23^{lo}$. Additionally they also express CD1d and can thus recognize lipid Ags. Like other innate-like cells, they are readily activated and respond with rapid induction of IgM production and plasma blast formation. MZBs and MZMs are interdependent and this cross talk is important for effective capture and coordinated early IgM responses to bacterial Ags [34,35]. MZBs are important for the continuous shuttling of Ags into the follicles via complement receptors and deposition on the FDCs so that GC reactions can give rise to Ag-specific adaptive effector and memory responses [36,37]. Ag-loaded CXCR5-expressing MZBs respond to FDC-produced CXCL13 by migrating into the follicle and depositing Ag in the form of immune complexes (IC) on FDCs for subsequent activation of FOBs. IC deposition is mediated through the interaction of complement receptors CD21 on MZBs and CD35 on FDCs. FOBs bind and internalize Ags through the BCR, process it and present it on MHC class II on their surface. The additional activation signal is delivered by an activated $CD4^{+}$ T helper cell which travels to the T/B border of the spleen, recognizes the peptide-MHCII complex and engages in a cognate T/B cell interaction. This leads to up-regulation of CD40L on T cells, which provides the co-stimulatory signal upon binding to CD40 on B cells, licensing B cells to enter the GC reaction and resulting in plasma cell formation and antibody production [38]. During the GC reaction B cells undergo class switch recombination and affinity maturation where B cells with high affinity to the Ag are selected. The selected B cells then undergo somatic hypermutation (SHM) where random mutation in the CDRs of the BCR further increase its specificity and affinity. A specific subtype of T cells, of particular importance for the GC reaction, is the T follicular helper cells (T_{fh}). T_{fh} initiate the GC response by providing survival, proliferation and differentiation signals to FOBs after cognate interaction. They also, secrete cytokines which determine the subclass (isotype) of the BCR and generated Abs. The resulting high affinity B cells will then differentiate into antibody-producing plasma cells or memory B cells.

Depending on the type of Ag encountered, different modes of B cell activation can occur. Antigen types include: Thymus-dependent (TD), such as TNP-KLH and Thymus-independent (TI). TD Ag activation involves the B cell receiving $CD4^{+}$ T cell help in the form of co-stimulatory CD40-CD40L interaction, as described above. TI Ag can be further subdivided into TI type I or TI type II. TI-I Ag (TLR-ligand polysaccharides and cytosine-phosphodiester-guanin (CpG)) lead to polyclonal B cell activation through engagement of TLR4 on the B cell surface. TI-II Ag are long polysaccharides (e.g. dextran, NP-Ficoll) that can simultaneously bind and activate multiple B cells. B cell receptors, B1 B cells and MZBs are mostly associated to TI Ag responses [39], while FOB are associated to TD Ag responses. MZB cells also highly express the MHC-like molecule CD1d, which is involved in lipid Ag

presentation to NKT cells in the splenic MZ and red pulp [40].

The role of B cells in cancer is disputable, with studies reporting both positive and deleterious influence of B cells on anti-tumor immunity. In a melanoma tumor model it was shown that B cells were essential for the mediation of $CD4^+$ and $CD8^+$ anti-tumor responses [41]. Another study describes a mechanism through which B cells drive carcinogenesis through the generation of immune complexes that bind FcγR, thus contributing to chronic inflammation [42]. Moreover, B cells secrete anti-inflammatory cytokines, such as IL-10 and TGF, which have profound immunosuppressive effect on other tumor-infiltrating immune cells [43]. The role of B cells in the regulation of macrophages in the tumor microenvironment (TME) is described in 1.2.1.

1.1.5 Myeloid cells

Myeloid cells originate from common myeloid precursors in the bone marrow, which give rise to granulocytes, monocytes, macrophages and DCs. The relevant subsets for this thesis, with particular focus on macrophages, are described below.

1.1.5.1 Granulocytes

Granulocytes are cells of the myeloid hematopoietic lineage and consist of subsets with distinct morphology and function, namely eosinophils, mast cells, basophils and neutrophils; which are the most abundant subset in humans, accounting for 70% of all circulating leukocytes. Neutrophils are short-lived, rapid responders to inflammatory cues, which migrate to sites of inflammation through a process known as chemotaxis. At site, they engulf microbial pathogens in phagosomes, which then fuse with intracellular granules containing reactive oxygen species (ROS), eliminating the pathogen. Alternatively, granules containing antimicrobial enzymes are released into the extracellular space. During their high turn over rate, dying neutrophils also have the ability to externalize their nuclear content forming neutrophil extracellular traps (NETs) that capture pathogens immobilizing them to be engulfed by other phagocytes. Eosinophils, basophils and mast cells perform immune responses against extracellular pathogens by engaging IgG and IgE antibodies via their Fc receptors.

1.1.5.2 Dendritic cells

In 1973, Ralph Steinman and Zanvil Cohn discovered a novel cell type, the DCs, which since then have been described to be the fundamental cellular sensors of the immune system and the bridge between innate and adaptive immune responses (Nobel prize 2011 for R.S.) [44-46]. DCs belong to the myeloid lineage, they originate from the bone marrow and develop into immature tissue-specific subsets that populate and surveil the periphery for potential invaders [47]. Cytokines fms-like tyrosine kinase three ligand (Flt3L) and granulocyte-monocyte colony stimulating factor (GM-CSF) are important for DC differentiation. There are two major subsets of DC in mouse, the myeloid DCs (mDCs) and the plasmacytoid DCs (pDCs). mDCs (also known as conventional DCs) are subdivided into $CD8\alpha^+$ or $CD8\alpha^-$ and originate from precursors in the blood, while inflammatory DCs arise from circulating inflammatory

monocyte precursors [48]. pDCs are potent responders to TLR7 and TLR9 stimuli, such as viral RNA and bacterial CpG-ODNs (oligodeoxynucleotides), to which they respond by producing type I interferons (IFNα, IFNβ).

Immature DC are phagocytic cells, which typically express high levels of Ag-presenting molecules MHC-I and -II and CD1d, integrin CD11c, intracellular adhesion molecule 1 (ICAM-1) and low levels of co-stimulatory molecules CD80, CD86 and CD40. Ag-recognition by PRRs, leads to phagocytic uptake and lysosomal degradation, upon which antigenic peptides are loaded on MHC class II molecules from the endoplasmic reticulum and transported to the cell surface of the DC for subsequent presentation to naïve T cells [49]. This process leads to DC maturation, at which point the expression of co-stimulatory molecules is up-regulated and IL-12 is secreted to assist T cell activation. Additionally, the expression of chemokine receptor CCR7 is up-regulated, which detects chemotactic gradients of CCL19 and CCL21, and induces DC migration to lymph nodes for Ag-presentation to occur [50]. $CD8\alpha^+$ DCs are capable of Ag cross-presentation through loading on MHC class I molecules. Ag-presentation is mediated through the formation of the pMHC/TCR complex. This induces changes in the cell membrane topology and causes the rearrangement of MHC-II, and co-stimulatory molecules CD80 and CD86 on the DC and of TCR, co-receptors CD4, CD8 and co-stimulatory molecule CD28 on the T cell, assembling a unique structure at the DC/T cell contact interface, the immunological synapse [51]. The immunological synapse potentiates the relay of three signals that determine the outcome of the interaction: 1) pMHC/TCR-mediated T cell activation (CD4/MHC-II or CD8/MHC-I), 2) CD80/CD28 or CD86/CD28 co-stimulation and 3) cytokine production.

Due to their inherent ability to coordinate innate and adaptive responses, DCs or DC-exosomes are potential tools for therapeutic cancer vaccine approaches aiming to induce tumor-specific cytotoxic responses and induction of immunological memory (Paper IV) [52].

1.1.5.3 Monocytes

Circulating monocytes in the blood can be classified into two types; the inflammatory and the resident monocytes. Inflammatory monocytes originate from the bone marrow and respond to inflammatory stimuli by differentiating into macrophages or monocyte-derived DCs (in human $CD14^+CD16^-$, in mouse $CCR2^+Ly6C^{hi}CX3CR1^{low}$) [53]. Resident monocytes, on the other hand, are thought to originate from circulating inflammatory monocytes and are important for tissue homeostasis and repair (in human $CD14^+/midCD16^+$, in mouse $CCR2^-Ly6C^-CX3CR1^{hi}$) [54]. They rely on the macrophage-colony stimulating factor (M-CSF) for their survival and differentiation. Besides the bone marrow, monocytes also populate the spleen and are responsible for local replenishment of tissue-resident macrophages. The spleen also constitutes a reservoir of inflammatory monocytes that can be easily mobilized in response to soluble mediators and recruited to distal inflammatory sites [55]. The release of monocytes into the circulation, as well as the recruitment to sites of inflammation, are dependent on the chemokine CCL2.

1.1.5.4 Macrophages

Macrophages are tissue-resident cells of the mononuclear phagocyte system with extreme functional diversity as is evident by the various highly specialized subpopulations across the different anatomical locations of the body. The name macrophages originates from the Greek: big eaters, from *makros* "large" + *phagein* "eat", and describes their function as professional phagocytes responsible for the uptake and clearance of pathogens and endogenous debris through a wide array of PRRs. Recent studies have unveiled a broad spectrum of functions of macrophages, which far exceed their phagocytic activity, as indicated by their important role in the regulation of tissue homeostasis, inflammation and adaptive immune responses [56].

Subsets of macrophages

Until recently, tissue-resident macrophages were regarded as descendants of circulating monocytes, which derive from hematopoietic stem cell precursors in the bone marrow. However, recent fate-mapping studies have shown that most tissue resident macrophages (in the lung, liver, peritoneum, spleen red pulp, bone marrow) originate from precursors in the yolk sac or the fetal liver during embryogenesis. These populations are self-maintained independently from circulating monocytes in steady state during adulthood [57].

The spleen is our largest peripheral lymphoid organ and is extensively infiltrated by many different highly specialized subsets of tissue-resident macrophages. Depending on their sub-anatomical localization in the spleen these subsets exert different functions related to Ag uptake, recycling and presentation. Red pulp macrophages are derived from the fetal liver and are responsible for clearance of aged erythrocytes and recycling of heme [58]. White pulp macrophages (tingible body macrophages) are responsible for the clearance of B cells licensed by FDCs in the GC. The MZ of the spleen is the interface between the blood circulation and resident lymphocytes of the spleen. More specifically, as the anatomical structure of the MZ provides a site for the blood flow to be filtered for Ags, the macrophages guarding the marginal sinus are important for the uptake and clearance of self- and foreign Ags[59]. Another specialized macrophage subset, the metallophilic macrophages (MMs) expressing CD169 (sialoadhesin, Siglec-1), lines the inner side of the MZ surrounding the follicle. Although the origin of MZMs is unclear, they are dependent on the nuclear liver-X receptor α (LXRα) and arise early during development [60]. Of particular interest for this thesis and more in detail described below, are the MZMs. MMs are important for the initiation of humoral responses to TD Ags [61].

MZMs are characterized by their expression of PPRs, amongst which class A scavenger receptors SR-A and the MARCO (macrophage receptor with collagenous structure), as well as the C-type lectin SIGN-R1 (specific intercellular adhesion molecule-3-grabbing non-integrin receptor 1). Due to their strategic localization in the MZ in close proximity to MZBs and DCs, they have an important role in bridging innate and adaptive immune responses. More specifically, SIGN-R1, an orthologue to human DC-SIGN (dendritic cell specific intracellular adhesion molecule-3-grabbing non-integrin), on MZMs binds different glycoproteins such as the capsular polysaccharide of *Streptococcus pneumonia (S.*

pneumonia) [62] and polysaccharide dextran [63,64]. SR-A and MARCO on MZMs are involved in binding and clearance of meningococci [65]. SIGN-R1 also enhances the clearance of apoptotic cells through complement opsonization and is important for maintaining self-tolerance [66]. Although expressed on the same cell, SIGN-R1 and MARCO, exhibit opposing regulation, with MARCO being up-regulated while SIGN-R1 is down-regulated upon activation [67]. After bacterial or viral Ag uptake, macrophages in the MZ respond by producing a variety of pro-inflammatory mediators (Type I interferons, IL-1β and TNFα) that ensure the propagation of the immune response. However, the same macrophages play an important role in mediating the anti-inflammatory effect of intravenous Ig (IVIG), a widely used treatment for many chronic autoimmune diseases. Binding of IVIG to SIGN-R1 leads to engagement of the Fcγ receptor IIb (FcγRIIb), a negative regulator of inflammation, which leads to an increased activation threshold and thereby suppression of autoimmunity [68]. MZMs are important for the suppression of innate and adaptive immune responses to apoptotic cells and maintenance of peripheral tolerance [69,70]. Scavenger receptor MARCO is essential for the uptake, clearance and maintenance of tolerance to apoptotic cells [69] and presence of autoantibodies against the receptor is indicative of systemic lupus erythematous (SLE) [71,72].

Similarly to MM, $CD169^+$ macrophages, the subcapsular sinus macrophages in the lymph nodes, are strategically located at site that allows immune surveillance and antigen capture from the lymph [73]. They play an important role in bridging innate and adaptive immunity as they have been shown to activate B cells by presenting opsonized Ags [74,75], to cross-present apoptotic tumor cells to $CD8^+$ T cells [76] and to present lipid Ags to NKT cells [77]. During an immune response $CD169^+$ macrophages in the lymph nodes are mobilized into B cell follicles and during the GC reaction localize at the T-B border, supporting a role in Ag transport [78]. Medullary macrophages in the lymph nodes, which are responsible for filtering the lymph for particulate Ags, are also known to express CD169, SIGN-R1, SR-A and MARCO.

Peritoneal macrophages (originating from the fetal liver) are restricted to the transcription factor Gata-6 and upon TLR-stimulation with microbial products up-regulate scavenger receptor MARCO amongst other PRRs. Due to the easy accessibility peritoneal macrophages have been extensively studied both in their naïve state as well as activated upon administration of inflammatory agents in the peritoneal cavity.

Macrophage activation and regulation

Since macrophage function is highly context dependent, they exhibit great plasticity and diversity in phenotype and expression pattern of different molecules [79,80] and are very responsive to soluble mediators in their microenvironment. Thus, depending on the micro environmental context, macrophages are differently polarized along a continuous spectrum of activation states. Reflecting the effect of cytokines IFNγ and IL-4 in inducing T_h1 vs. T_h2 immune responses [81], macrophages were classified into classically activated (M1) [82] or alternatively activated (M2) [83,84] subsets depending on phenotype and function [85]. Later studies on macrophage activation performed in mouse strains with known predisposition toward either T_h1 or T_h2 responses, led to the distinction between the M1 and M2 activation

states based on phenotypic characteristics [86,87]. This classification was further developed to include several phenotypic marker and functional characteristics that distinguish the two activation states [88-91]. However, an increasing amount of data demanded a more informative classification based on the basic functions macrophages employ to preserve homeostasis, host defense, wound healing and immune regulation. This was further extended to include distinct transcriptional pathways regulating the different activation states [92,93]. Thus, in recent years, the M1/M2 dichotomy, which represents two extremes of a linear activation spectrum was replaced by a more correct representation of the polarization status as a continuous spectrum where all intermediate activations states can be encountered [94]. This is illustrated as a color wheel where the basic functions are depicted as primary colors and shared functions are represented by many different shades in-between, making up a spectrum of activation states that are related to different functions [79] **(Figure 2)**.

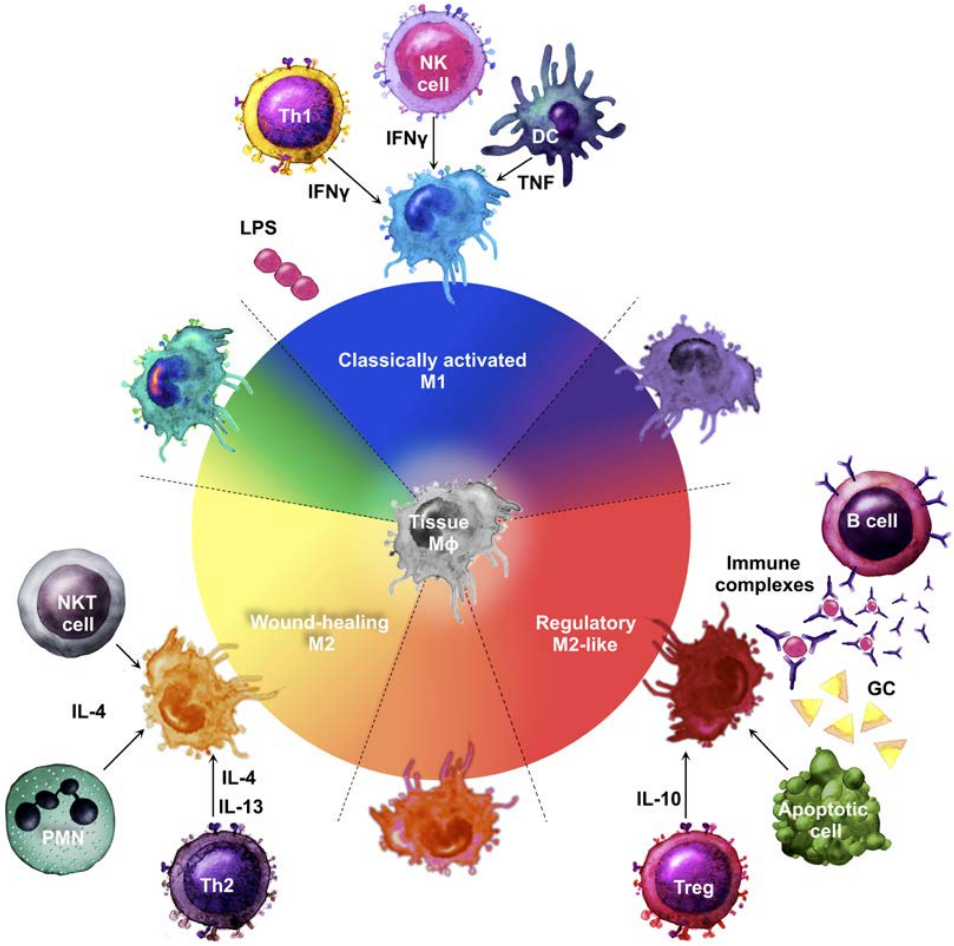

Figure 2. The colorful wheel of macrophage polarization
Macrophages can be polarized to a spectrum of different activation states. This is illustrated as a colorful wheel where the main macrophage activation modes are shown in primary colors: classical M1 activation in blue, wound-healing M2 in yellow and regulatory M2-like in red. Typically, M1 activation occurs in response to T_h1-type cytokines, like IFN and TNF during infection, while M2 is induced during T_h2-responses, by IL-4 and IL-13, e.g. in responses to parasites. M2 macrophages do not constitute a uniform population, thus intermediate colors represent activation states with shared characteristics. M2-like immunoregulatory macrophages are induced by immune complexes plus LPS or IL-1β, but also by IL-10, TGF-β and glucocorticoids. Finally, TAMs are a heterogeneous group and depending on the TME they can share characteristics of different activation states, illustrated here as secondary colors. Adapted from reference #78.

M1 (or classically activated) macrophages are considered pro-inflammatory and respond to IFNγ and TLR stimulation, such as lipolysaccharide (LPS), or tumor necrosis factor (TNF), produced by adaptive immune effector cells by exerting microbicidal and tumoricidal activities [95,96]. IFNγ, produced by NK, NKT cells or macrophages, activates STAT1 and interferon regulatory factors (IRF1 and 8), which up-regulate the transcription of cytokine receptor genes (IL-15RA, IL-2RA and IL-6R), cell activation molecules (CD38, CD69 and CD97) and cell adhesion molecules (integrins, mucin 1). LPS binds to TLR4 which signals through myeloid differentiation primary response gene 88 (MyD88), activating transcription factors nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB), activator protein 1 (AP-1) and signal transducer and activator of transcription 5 (STAT5) to induce the production of pro-inflammatory cytokines, chemokines and Ag presentation molecules. They also produce high levels of IL-12 and low levels of IL-10. They contain inducible nitric oxide synthase (iNOS), which leads to bacterial or cell lysis and they have antigen Ag-presenting capacity as indicated by the expression of high levels of MHC-II and co-stimulatory molecule CD86. M1 macrophages are potent producers of chemokines CCL2, CXCL9 and CXCL10 [97-99] and pro-inflammatory cytokines IL-1β, IL-6, IL-23 and TNFα. Lately, GM-CSF (granulocyte macrophage colony-stimulating factor) was added to the arsenal of stimuli leading to M1 polarization [100]. GM-CSF signaling lead to the activation and nuclear translocation of STAT5, IRF5 and NF-κB, which ultimately leads to the production of pro-inflammatory cytokines (IL-6, IL-8, G-CSF, M-CSF, TNF and IL-1β) and up-regulation of molecules associated with Ag presentation, complement- and antibody-mediated phagocytosis and migration, such as CD14, FcγRIA and CD163. Thus, M1 macrophages drive a so-called Type I immunity through the secretion of mediators that attract Th1 cells, which can be beneficial in the case of tumoricidal or microbicidal activity but can also cause tissue damage [101]. Therefore, they are considered important mediators of host defense but are also common culprits in several autoimmune pathologies **(Figure 3)**.

On the contrary, M2 (alternatively activated) macrophages can be elicited by a broader range of stimuli and have thus been subdivided further. More specifically, M2a macrophages typically express the IL-4Rα, which is important for their polarization to M2 through binding to cytokines IL-4 and IL-13, primarily produced by eosinophils, basophils, NKT cells and macrophages, leading to STAT6 activation. M2b macrophages bind immune complexes through Fc-receptors expressed on their surface in combination with TLR stimulation or IL-1R ligands. Engagement of Fc-receptors recruits tyrosine kinase Syk, which activates PI3K (phosphoinositide 3-kinase). M2c macrophages are induced by IL-10, TGF-β (both potent anti-inflammatory cytokines) or glucocorticoids. IL-10 is produced by macrophages in response to TLR- and glucocorticoid signaling and upon engagement of C-type lectin receptors SIGN-R1 and Dectin 1. IL-10 receptor triggering by its ligand leads to STAT3 activation, driving the expression of Fc-receptors, chemokines CXCL13 and CXCL4 as well as PRRs: formyl-peptide receptor (FPR1), TLR1, TLR8 and MARCO [102]. Tumor-associated macrophages (TAMs) are induced by tumor-derived factors, such as IL-6, LIF (leukemia inhibitory factor) and MCF (macrophage chemotactic factor), and are hard to categorize to one specific M2 category as they vary depending on the TME [97,103,104]. M-

CSF has also been shown to shift macrophages to an M2 polarization. Contrary to M1, M2 macrophages produce low levels of IL-12 and high levels of IL-10, express Ym1 (Chil3) and Fizz1 (Retnlα) and produce Arginase 1 which inhibits T cell proliferation by depleting arginine from the microenvironment [105]. They promote so called Type 2 immunity which supports wound healing and tumor progression. They express low levels of MHC-II and promote immunosuppression through production of CCL22, which attracts Tregs [106]. They also express programed death ligand 1 (PD-L1), which antagonizes activated T cells by binding to its receptor programed death 1 (PD-1) on their surface and inducing apoptosis [107]. They are considered anti-inflammatory and pro-tumorigenic.

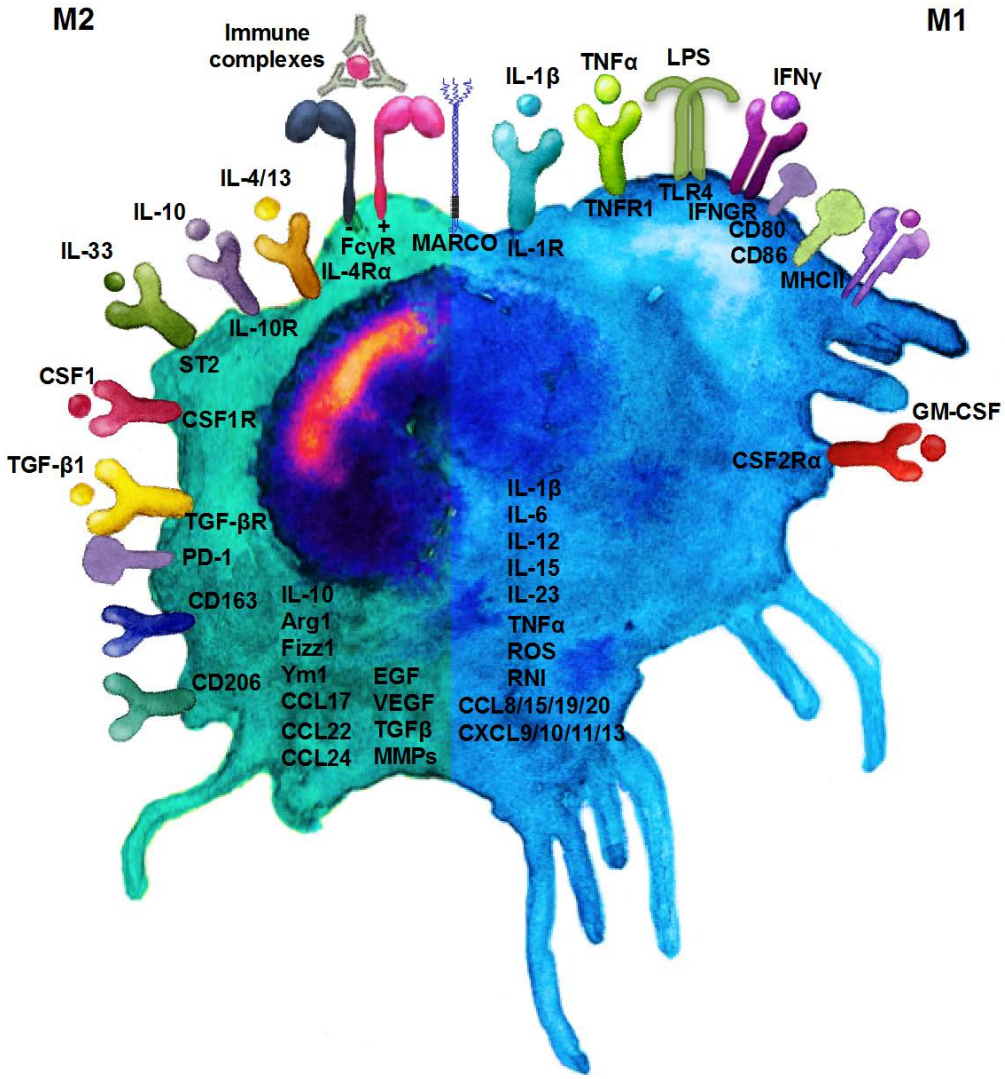

Figure 3. Receptors and ligands involved in macrophage activation and produced mediators
The main receptor/ligand pairs responsible for M1 activation are IFNγ/-R, LPS/TL4 and TNF/-R, leading to the production of pro-inflammatory cytokines and cytotoxic mediators. M2 activation is induced by signaling of IL-4 and IL-13 through IL-4Rα and IL-33 via ST2, inducing anti-inflammatory factors and regulatory enzymes. Additional receptor/ligand pairs are involved in intermediary activation states that can be encountered depending on the microenvironmental context of activation.

Scavenger receptors on macrophages

Scavenger receptors (SR) are PRRS expressed by phagocytes, with wide ligand specificity for both endogenous and foreign molecules. SRs comprise a range of receptors grouped together based on their ability to bind polyanionic (negatively charged) ligands [108] or based on the fact that they contain a highly conserved version of the Ig domain known as the scavenger receptor cystein-rich (SRCR) domain [109]. Based on their multi-domain structure they have been classified into eight different classes (A-H). Due to the wide range of ligand specificity they are involved in both homeostatic regulation as well as in various inflammatory disease settings and have been extensively studied in the contexts of infection, atherosclerosis and autoimmune disease [110-115 116].

However, an increasing number of studies report the involvement of different scavenger receptors also in cancer [117]. For example, Class A scavenger receptor A (SR-A), which is closely related to MARCO and is expressed on macrophages, has been implicated to promote ovarian and pancreatic cancer [118,119]. Moreover, it has been shown to negatively regulate Ag-specific antitumor immunity by limiting Ag cross-presentation [120]. In a separate study, targeted depletion of SR-A-expressing leukocytes inhibited peritoneal ovarian tumor progression [121]. SR-A can signal via the receptor tyrosine kinase (Mertk), which is important in the uptake of apoptotic cells [122]. Mertk was recently associated to M2 polarization and promotion of anti-inflammatory responses in cancer [123]. This could be linked to increased loads of apoptotic tumor cells in the TME. SR-A deficiency, on the other hand, led to a shift in M1 polarization of TAMs and delayed tumor growth in a lymphoma model [124].

Another member of the scavenger receptor family, CD163, has been associated with an M2 macrophage phenotype in cancer [125]. CD163 correlates to negative prognosis and poor patient survival in breast cancer [126,127], adult T cell leukemia/lymphoma [128] and rectal cancer [129].

The various functions of scavenger receptor MARCO are described in detail below, while consequences of its targeting on macrophages through monoclonal antibodies in the context of sterile- and cancer-related inflammation are discussed in Paper I and II.

Scavenger receptor MARCO

Class A scavenger receptor MARCO was cloned by Karl Tryggvasson at Karolinska Institutet in 1995. MARCO is a membrane bound receptor, which structurally consists of a disulphide-bonded trimer with a partly collagenous structure (**Figure 4**) [130]. The 210kDa protein is composed of three glycosylated subunits and consists of five domains; the N-terminal short cytoplasmic domain I, the membrane-spanning domain II, and domains III, IV and V which are extracellular. Domain III is a 75-residue spacer domain that separates the collagenous domain IV from the plasma membrane.

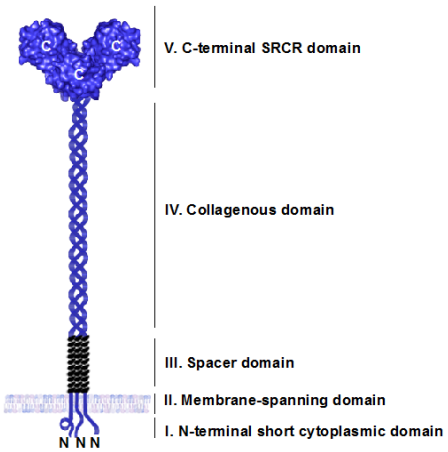

Figure 4. Schematic representation of the five domains of the MARCO structure.
The SRCR domain is adapted from the RCSB Protein data bank. PDB ID: 2OY3 (reference #132)

The collagenous domain contains 270 amino acids forming an overall collagenous structure which is only interrupted at a single site and is involved in trimerization [131]. The interruption by an Ala-Gly-Lys sequence suggests a hinge region in the triple helix of the MARCO molecule. The C-terminal end domain V of MARCO contains six cysteine residues making up the scavenger receptor SRCR domain and proximally the ligand-binding domain [132] **(Figure 4)**.

Although structurally closely related to SR-A, MARCO exhibits different regulation [133]. For example, both SR-A and MARCO bind CpG-ODNs. However, MARCO-binding leads to an activating response in macrophages characterized by TLR9-mediated NO production and IL-12 secretion, while SR-A-binding lead to negative regulation of IL-12 responses [134]. Also, in contrast to SR-A expression, which is inducible but not constitutive, expression of MARCO is dependent on TLR4-signaling [135,136]. MARCO is constitutively expressed on distinct subsets of macrophages restricted to certain tissue compartments. MARCO is expressed on the macrophages of the MZ of the spleen (MZM) and medullary regions of the lymph nodes [130]. Its expression on this highly specialized subset of macrophages, located in strategic proximity to DCs and B cells in the marginal zone, highlights MARCO as an important receptor for the capture and clearance of antigens and apoptotic cells from the blood. Inability to clear apoptotic cells from the circulation is associated with increased risk for developing SLE [137,138]. Also, mice lacking MARCO, have a defective MZ architecture, show impaired responses to T cell-independent (T-I) type 2 Ag, produce elevated levels of autoantibodies to DNA and develop symptoms of SLE [71,139]. Additionally, autoantibodies to MARCO have been found early on in mouse models for SLE before the onset of disease and the receptor is down-regulated on MZMs [140]. Autoantibodies to MARCO have similarly been found in SLE patients however it is unclear whether they are involved in driving the disease [71].

As for most SR, its ligands are negatively charged polyanionic molecules. More specifically, MARCO has been shown to bind low density lipoprotein (LDL) through its SRCR domain [141], as well as bacterial agents such as *Esherichia coli*, *Staphylococcus aureous* [130] and LPS [142], but also un-opsonized particles, such as titanium dioxide (TiO_2), ferric oxide (Fe_2O_3), silica ($CsiO_2$) and latex beads [64,143-145].

The fact that MARCO has a very short intracellular domain suggests that the receptor requires a signaling partner to relay its downstream effects. Although little is known about the exact mechanism of action and interaction partners it is clear that MARCO has a role in modulating inflammatory responses. Several studies have attempted to elucidate this by studying its function on macrophages. More specifically, it was shown that cooperation of MARCO, TLR2 and CD14 induces macrophage cytokine responses to *Mycobacterium tuberculosis* [146], while MARCO co-operation with TLR2 and nucleotide binding oligomerization domain-containing 2 (NOD 2) is important for clearance of *Streptococcus pneumoniae* [147]. In alveolar macrophages, uteroglobin-related protein 1 (UGRP1) was identified as a ligand for MARCO [148]. Moreover, it has been suggested that MARCO is negatively regulated by FcRγ through the recruitment of Src homology region 2 domain-containing phosphatase 1 (SHP-1) during *Esherichia coli* binding to FcγRIII, resulting in

decreased phagocytosis and enhanced production of TNFα [149]. Also, ligand internalization by MARCO decreases surface-sensed TLR4 responses, while simultaneously enhancing intracellular TLR3, NOD2 and NACHT, LRR and PYD domains-containing protein 3 (NALP3, inflammasome) [150]. Similarly, MARCO has been shown to modulate TLR-induced DC activation, possibly providing a bridge between innate and adaptive immunity [151]. While MARCO is important for the clearance of *Pneumococcus pneumoniae* infection [152], it has a deleterious role mediating uptake of the intracellular parasite *Leishmania major* [153] and suppressing early inflammatory responses to *Infuenza A* viral infection [154] as well as facilitating HSV-1 infection and spread [155].

Although primarily a MZM receptor, MARCO can be rapidly up-regulated on other macrophages and DCs after activation by bacterial products [156,157]. LPS binding to TLR4 leads to up-regulation of both MARCO (and SR-A, in both an MyD88-dependent and independent ways, and leads to B cell activation [158]. Up-regulation of MARCO expression is associated with cytoskeletal rearrangements [159], which induce drastic morphological changes to cells, such as the formation of lamellipodia and dendritic-like processes. These result in loss of cell adhesion and decreased migration, processes that are necessary for the engulfment of particles [160]. Another study by Grolleau *et al*. reported that the inducible up-regulation of MARCO on DCs after pulsing with tumor cell lysate is associated with increased phagocytic capacity [161]. Here, targeting MARCO with a monoclonal antibody led to enhanced DC motility and anti-tumor activity in a mouse model of melanoma [162]. In a follow-up study, it is was shown that MARCO deficient tumor-pulsed DCs responded to CCL21 with an increased migratory capacity to draining lymph nodes of B16 melanoma tumors, leading to improved anti-tumor IFNγ T cell responses [163]. Similarly, lack of MARCO led to enhanced DC migration to lymph nodes and increased production of pro-inflammatory mediators in the context of airway inflammation [164].

Human MARCO was recently identified and found to be highly similar to the mouse molecule [165]. Studies on MARCO expression in humans show a wider distribution pattern of the receptor on several tissue macrophage subsets. However it should be noted that expression of the receptor was characterized on samples from septic patients and may therefore not be representative of the steady state [166]. Although extensively studied in infection and autoimmunity, the role of MARCO in cancer still remains poorly understood. In the context of cancer, the role of MARCO is so far contradictory, correlating with better prognosis in follicular lymphomas [167], but with poor prognosis in human breast cancer [168]. MARCO has been suggested to be expressed on a subpopulation of macrophages in the tumor stroma with immunosuppressive activity, however its role is unknown [169]. Paper II describes an attempt to shed light on the so far diffuse role of MARCO in cancer. Data acquired from other physiological or pathophysiological settings so far pinpoint MARCO as an important regulator of responses to both self- and exogenous antigens and systemic inflammation [170].

1.2 THE IMMUNOSUPPRESSIVE TUMOR MICROENVIRONMENT

Cancer is a multifactorial disease where a number of mechanisms converge to initiate, sustain and promote malignancy. The hallmarks of cancer progression include evasion of apoptosis, genome instability and mutation, sustained angiogenesis, limitless replicative potential, self-sufficiency in growth signals, insensitivity to growth inhibitors, avoiding immune destruction, as well as invasion and metastasis. However, tumor cells do not act alone in the processes of tumorigenesis, tumor progression and metastasis. They are rather part of a permissive microenvironment established by the concerted actions of different cell types and stromal components, such as cancer-associated fibroblasts, pericytes, myeloid cells, lymphocytes, endothelial cells and extracellular matrix [171]. Recently, tumor-promoting inflammation was recognized as the 10th hallmark of cancer development [172,173]. It is generally characterized by the presence of inflammatory cells and mediators (e.g. chemokines, cytokines) in the tumor stroma, tissue remodeling, angiogenesis and tissue repair. The key cellular players in cancer-related inflammation are the tumor-infiltrating leukocytes (TILs); cells of the myeloid lineage exhibiting a great degree of plasticity and diversity, such as TAMs and the related myeloid-derived suppressor cells (MDSCs), tumor-associated neutrophils (TANs), mast cells and eosinophils [98]. TILs are present in most tumors to varying degrees. Although initially the presence of TILs was considered beneficial, accumulating evidence suggested that tumor-derived factors imposed phenotypic and functional changes on TILs, which altered them to in fact promote tumorigenesis.

TAMs, TANs and the related MDSCs, but also T regulatory cells (Tregs) are responsible for imposing a generalized immunosuppressive milieu within the tumor stroma [174-176]. Immunosuppression is achieved through a variety of mechanisms, such as up-regulation of cell surface receptors, secretion of cytokines and chemokines, and different enzymatic activities.

Shimon Sakaguchi first identified T_{regs} as a naturally occurring subset of CD4$^+$ T cells. They are characterized as CD4$^+$ CD25^{hi} and rely on the transcription factor FoxP3 for their development. Other surface markers that are typically expressed on T_{regs} include CTLA-4, glucocorticoid-induced TNF receptor (GITR) and lymphocyte activation gene-3 (LAG-3). T_{regs} are recruited to tumors through the CCR4/CCL17, CCL22, CCR8/CCL1 and CCR6/CCL20 chemokine axes to promote suppress anti-tumor immunity [177]. Moreover, tumors promote T_{reg} expansion by producing TGF-β. Studies have shown that large numbers of Tregs in the tumor, but not in the draining lymph nodes, inversely correlate with survival of patients with ovarian carcinoma. Therapeutic approaches targeting Tregs for elimination, such as targeting GITR or CD25, have shown promising results heaving immunosuppression and unleashing anti-tumor CD8$^+$ T cells [178,179].

Although less explored than TAMs in the context of cancer, tumor-associated neutrophils (TANs) are also important mediators of cancer-related inflammation and tumor progression [180]. TANs are attracted to the inflammatory TME by chemokines, such as tumor-derived CXCL8 and epithelial cell-derived CXCL1, 2 and 3. On site, depending on the local cytokine milieu they can be polarized to an N1 anti-tumorigenic phenotype with the ability

to kill tumor cells and block metastasis or to an N2 pro-tumorigenic phenotype [181]. Blockade of TGF-β-signaling inhibits N2 polarization [182]. While primarily thought to originate from granulocytic bone marrow precursors, they have also been shown to accumulate in the spleen of tumor-bearing mice, from where they are later deployed to the tumor [183]. N2 TANs have a variety of immunomodulatory functions, which can have profound implications on anti-tumor immunity. These include but are not limited to, induction of a tolerogenic DC phenotype [184], affecting NK cell development [185] as well as B cell survival and maturation [186].

MDSCs are a group of heterogeneous immature myeloid precursors with profound capacity to suppress effector T cells responses. Under physiological conditions they are present in very few numbers and rapidly differentiate into granulocytes, macrophages and immature DCs. Normally they account for 20-30% of all cells in the bone marrow, and 2-4% in the spleen, while they are absent from lymph nodes. However, pathological inflammation caused by cancer, infection, autoimmune disease or trauma, halts their differentiation and induces expansion of the immature cells with immunosuppressive capacity. They are subdivided into monocytic (Mo-MDSC) and polymorphonuclear (PMN-MDSC), with distinct functions in tumors. Mo-MDSC represent ca 20-30%, while PMN-MDSC are 70-80% of the total MDSCs in a tumor. In mice, Mo-MDSCs are characterized as $CD11b^{+}Gr\text{-}1^{+}Ly6C^{high}$ $Ly6G^{low}$, while PMN-MDSCs are $CD11b^{+}Gr\text{-}1^{+}Ly6C^{low}Ly6G^{high}$. MDSCs employ various mechanisms to impose immunosuppression and these may differ between the periphery and the tumor site. The suppressive capacity of PMN-MDSCs is mediated by Arg1, while for Mo-MDSC iNOS production is more important [187]. They have been shown to produce chemokines, such as CCL3, CCL4, and CCL5, to attract $CCR5^{+}$ T_{regs} in a mouse melanoma model [188]. MDSC can also induce the de novo generation of T_{regs} through production of IL-10 and TGF-β. [189]. In lymphoma MDSCs induced Tregs through Arg1 and Ag presentation [190]. The importance of heaving immunosuppression to achieve a successful immunotherapeutic effect in cancer treatment is becoming more and more apparent. Therefore, strategies to eliminate, block the expansion and recruitment or differentiate MDSCs have become the subject of numerous investigations.

1.2.1 Tumor-associated macrophages

Already in 1863 Virchow described the presence of infiltrating leukocytes in tumors and proposed a link between cancer and inflammation. Indeed, as many as 25% of all cancers are thought to arise as a consequence of chronic smouldering inflammation [191-195]. TAMs are a major component of tumor-promoting inflammation and increased numbers have been associated with poor prognosis in a variety of human cancers, and breast cancer in particular [127,196]. In breast cancer, high infiltration of TAMs has been associated with hormone receptor negative status, including triple negative tumors, which is the type of breast cancer with the poorest clinical outcome [197-199]. However, our understanding of the distinct phenotypes of TAMs, their distribution in the tumor stroma and the connection to pathological outcome is still limited, thus further investigation is warranted.

The origin of TAMs is still disputed and several different sources, known to give rise to different macrophage subpopulations with distinct functions have been suggested over the years (embryonic yolk sac, bone marrow, spleen). In experimental models of mammary carcinoma and lung adenocarcinoma models, most TAM populations found in the tumors originated from $Ly6C^+$ circulating monocytic precursors [200]. However, there is considerable debate regarding the origins of the monocytic precursors, some supporting the idea that they originate from the bone marrow and others suggesting that they arise from the extramedullary hematopoiesis in the spleen [183] that gives rise to an easily mobilized reservoir of pro-inflammatory monocytic precursors. However, the contribution of the latter compartment seems to be minor [201]. Finally, Mo-MDSC and PMN-MDSC are able to differentiate into mature myeloid cells; macrophages and neutrophils respectively. However, in cancer they are skewed to a pathological phenotype, which can give rise to TAMs or TANs [202,203].

TAMs are recruited to the inflammatory TME, where they are sequestered by tumor-derived factors, cytokines and chemokines produced by other inflammatory cells and by hypoxia, all together contributing to a pro-tumorigenic inflammatory microenvironment [204,205]. Tumors secrete pro-inflammatory mediators, such as IL-6 and TNF, which contribute to the conditioning of the inflammatory TME [195]. This induces the production of chemokines (CCL2, CCL5, CCL22, CXCL1, CXCL8, CXCL12 and MCP) by various immune cell types that will attract macrophages to the inflammatory tumor site and promote their production of immunosuppressive cytokines, such as IL-10 and CSF-1, as well as up-regulate the expression of scavenger receptors SR-A and CD163 [99,119]. In addition, tumor cell and TAM crosstalk through CCR2 and CX3CR1 further drives tumor progression and metastasis [206,207]. Moreover, tumor-derived prostaglandin E2 and TGF-β promote a distinct M2 TAM phenotype, the major regulator of which is NF-κB, the downstream signal transducer of the TLR4-MyD88 signaling pathway [208]. This generates a self-propagating circle of pro-tumorigenic inflammation [209].

T_{regs} in particular, are potent inducers of TAMs as they produce abundant amounts of IL-10, which promotes the expression of CD163 and CCL18, suppresses the production of pro-inflammatory cytokines and expression of MHCII [210]. $CD4^+$ T_h2 helper cells have been implicated in the promotion of M2 TAMs through the production of IL-4 [211]. Also, it has been shown, both *in vitro* and *in vivo* that B cells producing IL-10 profoundly affect the phenotype of macrophages shifting them to M2 polarization [212,213]. Moreover, B cells can remotely control macrophages through their production of Abs to tumor-related proteins. These Abs form immune complexes, which are taken up by macrophages through Fc-receptors, resulting in M2 polarization [214]. MDSCs, physically interact with TAMs and suppress macrophage-derived IL-12 in an IL-10-dependent manner, leading to enhanced immunosuppression [215]. As a result, TAMs evolve into different specialized subsets with distinct functions, that ultimately support tumor growth and progression through various mechanisms [119,169,216-218].

Upon recruitment to the inflammatory TME, TAMs produce factors that support tumor cell proliferation, neovascularization and metastasis [219] **(Figure 5)**. They have been found to

assist invasion of the primary tumor into the surrounding tissue but also intravasation and further dissemination of tumor cells. This molecular polarization is reflected in the acquisition of a distinct gene expression signature of M2-like TAMs [220-222]. These have an anti-inflammatory phenotype; suppressing adaptive immunity (TGF-β, IL-10) [174,200], promoting tumor growth (EGF), driving the EMT of invading tumor cells (TGF-β) [223], tissue remodeling (MMP, cathepsin proteases) [224] and angiogenesis (VEGF; vascular endothelial growth factor). TAMs are primarily of M2-like phenotype, but some can have a pro-inflammatory M1-like phenotype, which can elicit potent anti-tumor inflammatory responses. As for macrophages, the M1/M2 polarization axis is however a mere simplification. In reality, tumors are complex heterogeneous tissues and therefore different regions of the tumor may have distinct microenvironments. As a result, the macrophage infiltrate may vary greatly with regards to phenotype and function depending on their localization. Another level of complexity is added depending on the type of cancer and the permissiveness of its microenvironment. Consequently, macrophage plasticity and diversity create a dynamic environment which evolves with tumor progression, where specialized subpopulations of macrophages support different functions [225]. The major functions of the different TAM subsets are described below.

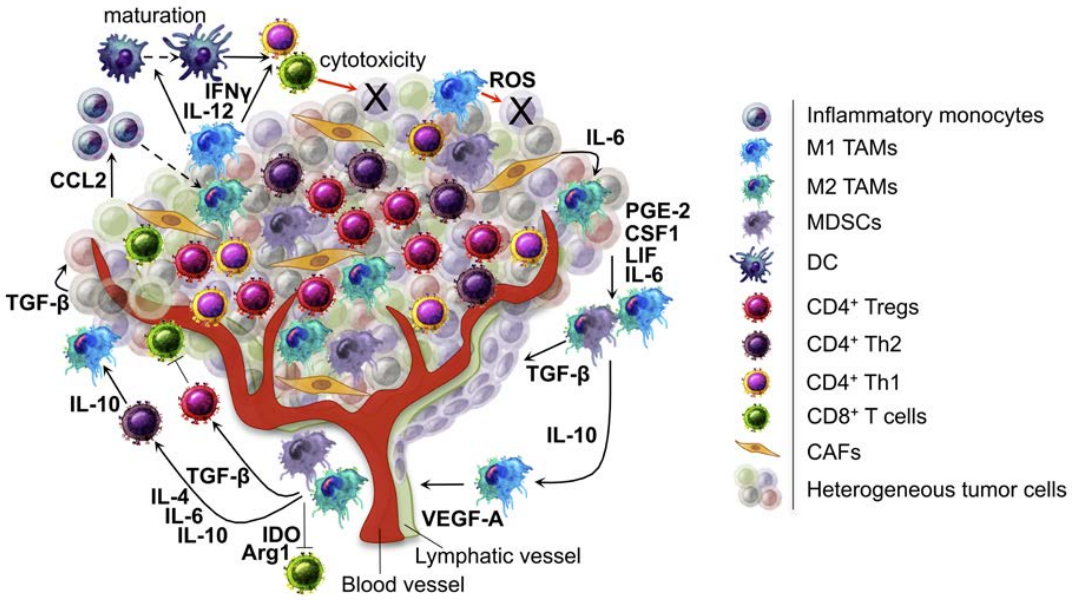

Figure 5. Function of TAMs and MDSCs in the immunosuppressive tumor microenvironment
Heterogeneous tumor cells in the TME produce various factors that promote immunosuppression. For example, tumor-derived IL-6, LIF, PGE-2 and CSF1, drive the differentiation of MDSCs to M2 TAMs and the polarization of M1 TAMs to M2 TAMs. In turn, MDSCs and M2 TAMs secrete TGF-β and pro-angiogenic factors that drive the angiogenesis and migration of metastatic tumor cells. The immunosuppressive phenotype of M2 TAMs are further sustained by tumor-derived and autocrine TGF-β and CAF-derived IL-6. MDSCs and M2 TAMs produce TGF, driving T_{regs} expansion and inhibition of $CD8^+$ T cell anti-tumor responses. They also typically express the enzymes IDO and Arg1, which have a profound inhibitory effect on $CD8^+$ T cells. Moreover, MDSCs and TAMs secrete IL-4, IL-6 and IL-10, which drive T_{h2} T cell differentiation. $CD4^+$ T_{h2} cells augment the anti-inflammatory cytokine IL-10 leading to further M1 to M2 TAM polarization. Additionally, tumor-derived chemokine CCL2 attracts inflammatory monocytes to the TME, providing a replenishing source of TAM precursors. Altogether, these mechanisms establish an immunosuppressive TME and inhibit the production of ROS and pro-inflammatory IL-12 and IFNγ by M1 TAMs, leading to diminished cytotoxic activity against the tumor.

1.2.1.1 TAMs in chemoresistance and angiogenesis

In a recent study, DeNardo *et al*. highlighted the prognostic value of a certain TAM-related tumor signature (high TAM count, high $CD4^+$ T cell count and low $CD8^+$ T cell count) and associated that to poor response to chemotherapy in breast cancer patients [226]. This could be due to chemotherapy-induced CFS1 release by the tumor which leads to macrophage recruitment, as has been shown to be the case in mouse mammary tumors, or it could be related to the immunosuppressive effect of TAMs on $CD8^+$ T cells. Moreover they found that CSF-1-targeting only depleted macrophages in poorly vascularized areas of the tumors. Hypoxia, is known to drive the recruitment of angiopoietin 2 receptor (Tie2) -expressing macrophages [227,228]. $Tie2^+$ macrophages up-regulate transcription factors HIF-1α and -2α, which control VEGF-A production and suppress T cell function [229-231]. TAMs are known to have an angiogenic function, which leads to the formation of poorly perfused leaky vessels [204,225,232,233]. Therefore, the authors speculate that by depleting those macrophages the overall tumor vasculature is normalized, allowing for better delivery of the chemotherapeutic agent [232,234].

1.2.1.2 TAMs in tumor cell invasion and metastasis

The process of metastasis involves several steps, during which tumor cells invade the surrounding normal tissues and extracellular matrix (ECM), intravasate into the blood or lymphatic vessels and travel to distal sites where they extravasate and seed micrometastatic niches. Recent studies demonstrate that TAMs originate from circulating inflammatory monocytes expressing chemokine receptor CCR2, which respond to tumor-derived CCL2 by migrating to the site of tumor inflammation [235]. There they differentiate into TAMs lining the invasive front of tumor cells and assist many of the afore-mentioned processes through the production of a wide array of mediators. It has been shown that colony-stimulating factor 1 (CSF-1), an important factor not only for macrophage differentiation, but also for promoting tumor invasion and metastasis is produced by TAMs, which in turn drives the production of the tumor attractant epidermal growth factor (EGF). Additionally, macrophage inhibitory factor (MIF) promotes tumor cell invasiveness and trafficking. TAMs also produce platelet-derived growth factor (PDGF) to support tumor cell proliferation. Moreover, TAMs produce matrix-degrading enzymes such as matrix metalloproteases (MMPs), cathepsins and proteases, which break down ECM and basement membrane to facilitate the invasion of tumor cells into the surrounding normal tissue. The detrimental role of TAMs in tumor progression has also been shown in macrophage deficient mice, where metastatic disease could be avoided in their absence. Alternatively, metastasis could be abrogated by inducing the polarization of M2-like TAMs to an M1-like phenotype. Finally, it has been shown that macrophages are important for "conditioning" distal sites for the seeding of micrometastases. Thus, targeting M2 TAMs in the TME could limit tumor metastasis (Paper II) [236].

1.2.1.3 TAMS and inhibition of antitumor immunity

In order to inhibit T cell mediated anti-tumor immunity, TAMs up-regulate the expression

of ligands for the inhibitory receptors programmed cell death protein (PD-1 or B7-H1) and cytotoxic T-lymphocyte Ag 4 (CTLA-4), on their surface. Under physiological conditions these molecules function as safety switches expressed on activated T effector cells when the need to resolve inflammation occurs. Engagement of PD-1 and CTLA-4 through their respective ligands (PD-L1/-L2 and CD80/CD86), inhibits effector cells from exerting cytotoxic functions through induction of apoptosis. PD-L1 is constitutively expressed on immune cells and is up-regulated on TAMs by IL-10 and TNFα [107]. In contrast, PD-L2 is exclusively expressed on APCs. Its expression on monocytes and macrophages is induced by CSF-1, IL-4 and IFNγ [237]. Both PD-L1 and PD-L2 are up-regulated by TAMs and MDSCs [238,239]. Another such molecule that is expressed by TAMs and has a suppressive effect on the function of T cells by arresting cell the cycle is B7-H4, which is induced in macrophages by IL-6 and IL-10 [240].

Lack of co-stimulation is another mechanism TAMs employ to render T cells non-responsive. CD86 is constitutively expressed on APCs in low amounts and is up-regulated upon activation, while CD80 is expressed only upon APC activation. CD80 and CD86 are expressed on M1 TAMs [241-243] but are down-regulated on M2 TAMs as a result of hypoxia [244] and by their interaction with MDSCs [245].

As previously mentioned, M2 TAMs are potent producers of IL-10 but have very low levels of IL-12, an essential cytokine for $CD8^+$ cytotoxic T cells activation, NK cell tumoricidal activity [246] and DC Ag presentation [247] **(Figure 5)**. As a result, in the absence IL-12, the TME is skewed towards a pro-tumorigenic T_h2 immune response, where IL-10 and TGF-β impose generalized immunosuppression. IL-10 leads to the expansion of natural T_{regs} and in combination with TGF-β induces the up-regulation of FoxP3 and the generation of induced T_{regs}, which in turn further suppress $CD8^+$ T cell function [248]. TGF-β inhibits $CD8^+$ cytotoxic function in vivo [249,250] and T_h1/T_h2 CD4 functions by interfering with lineage-determining transcription factors [251,252]. Additionally, TAMs secrete chemokine CCL22, which recruits $CCR4^+$ T_{regs} [106] and CCL20 that attracts $CCR6^+$ T_{regs} [253]. TAMs also employ the enzymatic activity of arginase (encoded by the *Arg1* gene), a hallmark of M2 polarization in itself, to suppress T cells by depleting L-arginine, which is essential for the re-expression of the CD3ζ chain after TCR down-regulation upon activation [254,255].

An additional mechanism utilized by TAMs is regulation through the enzyme indolamine 2,3-dioxygenase (IDO). IDO depletes tryptophan from the environment thus leading to cell cycle arrest and cessation of T cell proliferation and effector functions [256]. Alternatively, IDO can be expressed by APCs, which capture and present tumor Ag in draining lymph nodes to naïve T cells, leading to tolerization against the tumor [257,258]. Additionally, tryptophan breakdown by-products, such as kyleneurine, are also cytotoxic to T cells, which might also be a reason for inhibition of T cell proliferation.

1.3 EPITHELIAL-MESENCHYMAL TRANSITION IN TUMOR METASTASIS

Malignant invasion requires tumor cells to undergo EMT, a process recently shown to be assisted by M2 TAMs [259-261]. EMT is a biological process during which a polarized

epithelial cell loses its apical-basal polarity, loses contact to the basement membrane and acquires a mesenchymal phenotype associated with increased migratory, invasive and anti-apoptotic properties. EMT is absolutely essential for several physiological and pathophysiological processes, such as implantation, embryogenesis and organ development (Type 1 EMT), tissue regeneration and fibrosis (Type 2 EMT), as well as tumor growth and cancer progression (Type 3 EMT). EMT is governed by the activation of a highly preserved distinct genetic pattern, including *Snail/Slug*, *Twist, Six1*, *Cripto*, *Tgf-b*, and *Wnt/β-catenin*, across different species [262].

In cancer progression, tumor cells undergoing EMT are found at the invasive front of the tumor and are thought to drive metastasis through intravasation into blood and lymphatic vessels, extravasation and seeding of micrometastases at distal sites, at which point the tumor cells revert to a mesenchymal-to-epithelial transition (MET). However, not all cancer cells can undergo EMT and of the ones that will, not all will survive to seed distal metastases. An interesting conundrum in the case of breast cancer is that, while only a few cancer progenitor cells can give rise to metastases, a high genetic diversity is observed in metastatic breast cancer cells [263]. This discrepancy was explained by the fact that heterogeneity in cancer is not the result of random genetic aberrations, but is rather orchestrated by the evolutionarily conserved genetic programs that make up EMT [264,265].

TGF-β has been implicated as a major inducer of EMT in cancer through at least two pathways. Of importance for this thesis is the p38 mitogen activated protein kinase (MAPK)-mediated autocrine TGF-β-induced EMT, which leads to preferential dissemination of tumor cells through the lymphatics in a targeted manner [266]. Besides being produced by tumor cells, TGF-β can be readily produced by several cell types in the inflammatory TME, including TAMs, tumor-associated DCs, MDSCs and T_{regs} [267] **(Figure 5)**. As previously mentioned TGF-β is considered a master regulator of the processes leading up to the conditioning of the TME and the transitioning of the tumor cells for metastatic spread. An EMT permissive TME is similar to a wound unable to heal, characterized by increased enzymatic activity, matrix remodeling, inflammation, pathological tissue regeneration and scarring [268]. The conditioning effect of TGF-β on the TME relies on imposing immunosuppression by affecting the recruitment, polarization and production of inflammatory mediators by immune cells [269]. Tumor-derived TGF-β is known to attract and polarize macrophages and neutrophils to M2 and N2 activation states respectively. This loop is sustained by the induction of TGF-β production by M2 TAMs. TAMs have been pinpointed as the main drivers of tumor progression, as they assist invasion from the primary tumor into the surrounding tissue by the use of enzymes that specialize in breaking down extracellular matrix (matrix metalloproteases, MMPs), suppress anti-tumor immune responses, produce pro-tumorigenic growth factors and mediators and promote EMT in a NF-κB-dependent manner [223,270]. TGF-β further promotes the overall immunosuppressive milieu by shifting recruited $CD4^+$ precursors to T_h2 responses and by inducing the de novo generation of T_{regs} through the up-regulation of FoxP3 [271,272]. Together, an increasing amount of evidence supports a central role for TAMs

in sustaining and promoting TGF-β-driven EMT and facilitating tumor cell metastasis [273]. During inflammation DCs are capable of migrating to lymph nodes through the lymphatics in response to chemotactic gradients of molecular cues in order to initiate immune responses [274]. In study III we investigate the link between TGF-β-induced tumor cell EMT and the acquisition of an immune cell-like phenotype, which directs lymphatic dissemination and distal metastasis of tumor cells.

1.4 CANCER IMMUNOTHERAPY

The field of cancer immunotherapy was born in 1891, when William Coley, a surgeon at the New York Cancer Hospital (currently Memorial-Sloan Kettering Cancer Center), injected live or inactivated bacteria in order to mimic the spontaneous regression observed in sarcoma patients who had experienced infection by *Streptococcus pyogenes* [275,276]. The theory that "Coley's toxins" activated Metchnikoff's phagocytes to induce inflammation and kill bystander tumor cells was gaining momentum amongst immunologists, albeit not amongst clinical oncologists who turned to the more traditional treatments options of surgery, radio- and chemotherapy.

Harnessing but also unleashing the natural resources of the immune system for the benefit of cancer patients has long been the goal of vigorous research in the field of cancer immunotherapy [277]. The design and combination of sophisticated therapeutics, which overcome tolerance mechanisms and immunosuppression to induce anti-tumor responses incorporating a long-term memory component, is the goal to successful treatment of metastatic disease. Passive immunotherapy approaches consisting of Abs or adoptive transfer of donor effector T cells rely on the induction of an immediate therapeutic effect without the need for prior activation. Active immunotherapy, on the other hand, presents much greater challenges.

Active immunotherapy includes several prerequisites. DCs need to take up tumor-specific Ag against which central or peripheral tolerance has not been established. Upon Ag encounter the DC would also have to receive a second co-stimulatory signal, which would allow it to mature into a professional APC. After maturation DCs migrate to draining lymph nodes where they present the tumor-antigenic peptides in order to elicit Ag-specific T effector responses. DCs may also induce antibody responses, or activate NK and NKT cells. Finally, activated lymphocytes need to exert their function at the tumor site, at which point the suppressive inflammatory tumor environment provides a new level of complexity to the already daunting challenges. Therefore, attempts to simultaneously activate immune effectors and modulate components of the inflammatory TME into a less immunosuppressive state are of great importance for successful immunotherapy.

1.4.1 Immunotherapy using monoclonal antibodies

In 1975 Milstein and Köhler successfully fused B cells from the spleen of an immunized mouse with a myeloma cell line and generated an immortal antibody-producing cell line that produced large amounts of mAbs against the specific Ag [278]. The discovery of the hybridoma

technique was going to revolutionize life science research. To understand the different modes of action and potential of mAbs I have included a brief introduction into the basic molecular aspects of antibodies and Fc receptors.

Abs or Igs are divided into five classes based on the sequence of the heavy chain constant regions; IgA, IgD, IgE, IgG and IgM. Abs are structurally composed of two identical heavy (H) and two identical light (L) chains, which interact through disulfide bonds to form a Y-shaped molecule. They consist of two functional subunits, the Fab (Fragment, Ag binding) domain, which is Ag specific, and the Fc (Fragment, crystallizable) domain, which is responsible for mediating effector functions through its interaction with Fc receptors or complement. The variable region of the Fab domain contains three hypervariable complementarity-determining regions (CDRs), which form the Ag-binding site. The most common antibody class used in cancer immunotherapies is the IgG, which can be further subdivided into subclasses/isotypes IgG1-IgG4 in humans and IgG1, IgG2a, IgG2b and IgG3 in mice. These bind different FcRs with varying affinities and specificities. Abs undergo post-translational modifications during which they are differently glycosylated. This has implications for the effector functions mediated by the Fc part as the glycan alters its binding affinity to FcRs.

There are two types of FcRs, type I FcRs are transmembrane glycoproteins of the Ig superfamily and include FcγRs, while type II FcR belong to the c-type lectin family and include DC-SIGN/SIGN-R1 (CD209) and CD23. Depending on the structural conformation of the Fc, Abs show a particular preference for either Type I or Type II FcRs. Of particular interest for this thesis are type I FcRs, which will therefore be described in more detail. Upon antibody binding, activating FcγRs transduce signals through immunoreceptor tyrosine-based activation motifs (ITAMs) and inhibitory FcγRs through immunoreceptor tyrosine-based inhibitory motifs (ITIMs). In mice there are three activating Fc receptors, namely FcγRI, FcγRIII and FcγRIV and one inhibitory, FcγRIIB. In humans the activating FcγRI (CD64), FcγRIIA (CD32A), FcγRIIC (CD32C), FcγRIIIA (CD16A), FcγRIIIB (CD16B) and the inhibitory FcγRIIB (CD32B) are present. FcγR are expressed on myeloid cells, such as monocytes, macrophages, DCs, basophils and mast cells, which can express both activating and inhibitory FcγRs. More specifically, monocytes and macrophages express all FcγRs, neutrophils mainly express the inhibitory FcγRIIB and the activating FcγRIII and FcγRIV, while DCs express FcγRI, FcγRIIB and FcγRRIII. In the lymphoid lineage, NK cells, express only the activating receptor FcγRIII, whereas B cells only express the inhibitory receptor FcγRIIB. Studies on the therapeutic effects and the mechanisms of action of Abs have become more informative after the development of a mouse model in which murine FcRs have been replaced by their human counterparts, faithfully recapitulating the human FcR-expression profile on hematopoietic cells [279]. Ab-mediated inflammatory signaling through FcRs has been implicated in the physiological processes of Ag presentation and B cell selection, but also in the pathophysiological contexts of infection, autoimmune disease, and cancer. Growing understanding of the underlying mechanisms governing these responses has led to advances in the generation of antibody therapeutics, such as the optimization of Ab Fc-

design, the customized targeting to particular FcR and enhancement of the binding affinity of Abs by introduction of Fc modification, in order to modulate antibody effector functions to clinical benefit. Such approaches have been utilized in vaccinations against infectious diseases and the development of cytotoxic or immunomodulatory Abs for the treatment of autoimmune and neoplastic diseases [280].

1.4.1.1 Monoclonal antibody therapeutics for cancer

The use of monoclonal antibodies in cancer therapy can be direct, targeting molecules expressed by the tumor itself known as tumor Ags, blocking signaling cascades that are important for tumor survival and progression, inducing apoptosis or immunogenic cell death [281]. Tumors have, however, evolved to overcome such blockades, for example by down-regulating the expression of the targeted tumor-associated Ags (TAA) or selecting clones lacking TAA expression [282]. Thus, the need for immunomodulatory Abs targeting the immunosuppressive TME, aiming to evoke long-lasting anti-tumor immune responses emerged. These approaches aim to bridge innate and adaptive effector mechanisms and to establish immunological memory against the tumor [283].

These complex processes boil down to the following basic modes of action for Ab therapeutics used in anti-cancer therapies: i) Abs that recognize TAA and target tumor cells for elimination by Ab-dependent cellular cytotoxicity (ADCC) or Ab-dependent cellular phagocytosis (ADCP), ii) Abs stimulating receptor activity, inducing downstream signaling of receptors expressed on the surface of tumor cells or accessory cells in the TME, iii) Abs blocking receptor signaling, iv) agonistic immunomodulatory Abs targeting activation molecules on immune effector cells to induce anti-tumor responses, and v) antagonistic immunomodulatory Abs blocking inhibitory pathways in order to unleash anti-tumor immune responses. The last are known as checkpoint immunotherapies and have received immense recognition for their therapeutic effects, revolutionizing the field of cancer immunotherapy.

Antibody-dependent cellular cytotoxicity/-phagocytosis

One of the main functions of immunotherapeutic Abs is to exert cytotoxic or phagocytic activity by engaging secondary immune effector mechanisms. This is mediated through ADCC or ADCP, processes during which Abs bound to their ligands interact with activating FcR on innate immune effector cells (most commonly NK cells, eosinophils, monocytes and macrophages) to mediate target cell lysis or engulfment. The cytotoxic capacity of Abs depends on the isotype, which dictates the specificity for different activating FcR [284,285]. More specifically, mouse IgG2a preferentially binds FcγRI with high affinity, FcγRIV with intermediate affinity and FcγRIII with low affinity, while IgG2b binds FcγRIII and FcγRIV. In contrast, IgG1 binds inhibitory FcγRIIb with high affinity and to a lesser extent FcγRIII and thus has low/no cytotoxic activity [286]. Thus, IgG2a and IgG2b isotypes have higher cytotoxic potency than IgG1 and IgG3. A recent promising development in the field of immunotherapeutic approaches utilizing mAb is the engineering of the Fc part of mAbs to enhance or alter their binding affinity or specificity to different FcR and in this way affect their mode of action. ADCC is one of the main modes of action of mAbs in cancer

immunotherapy the efficacy of which can be improved. Enhancement of ADCC could be achieved through the introduction of modifications in the Fc part of mAbs, which lead to increased binding affinity to FcRγIIIA [287]. In fact *in vivo* studies in FcR deficient mice have highlighted the importance of specific Fc-FcR interactions in mediating the anti-tumor effect [288-290]. Moreover, a recent study in FcγR-humanized showed that while FcγRIIIA-binding on macrophages is essential for the ADCC activity of human IgG1 Abs, binding to FcγRIIA on DCs is responsible for the induction of a potent vaccinal effect against the tumor [291]. Moreover, FcR alleles attributing increased binding affinity to activating FcR result in increased ADCC and directly correlate with enhanced clinical responses to mAb therapy in patients [292-296]. There are currently several mAb targeting tumor-associated cell surface differentiation Ag, growth factors or molecules involved in angiogenesis that have been approved for clinical use in the treatment of cancer. These targets are: ERBB2 (trastuzumab), EGFR (cetuximab, panitumumab), VEGF (bevacizumab), CD20 (rituximab, ofatumumab, ibritumomab, tositumomab), CD30 (brentuximab), CD33 (gemtuzumab) and CD52 (alemtuzumab). The anti-CD20 mAb (Rituximab) targets B cells causing their depletion through an FcR-dependent manner. Besides being a useful tactic to eliminate malignant B cells, this mAb can also be used to deplete B cells in tumor settings where they exert a tumor-promoting effect, as has been shown in pancreatic cancer [297]

Immunomodulatory antibodies

Immunomodulatory Abs can target activating molecules on the surface of immune cells. CD40, a member of the tumor necrosis factor receptor (TNFR) family, is a co-stimulatory molecule, which is expressed on APCs such as DCs, B cells, monocytes and macrophages [298]. CD40 triggering by the anti-CD40 mAb Dacetuzumab leads to up-regulation of co-stimulatory molecules, induces cytokine production and enhances Ag presentation. Particularly in B cells, it promotes maturation, GC formation, Ig-isotype switching and affinity maturation. Its mode of action depends on the inhibitory FcγRIIb, and is similar for other members of the TNFR family such as CD95, DR4 (TNFRSF10A) and DR5 (TNFRSF10B) [299,300]. DR3 (TNFRSF25) is expressed on activated $CD8^+$ T cells and interacts with TL1A, a TNF-like cytokine, on $CD4^+$ T cells to promote inflammation and NK cell activation. Crosslinking of DR3 leads to enhanced cytotoxic NK cell activity. Another member of the TNFR family, CD27, is a co-stimulatory molecule expressed on T, B and NK cells. Binding of CD27 to its ligand CD70 is important for the effector functions of these cells. Crosslinking of the receptor by mAb leads to enhanced immune effector functions. Another target receptor is CD137 (4-1BB), which is expressed on activated T cells, T_{regs}, NK cells, NKT cells, DCs, neutrophils and monocytes. Crosslinking of CD137 promotes expansion of T cell populations, $CD8^+$ T cell survival, NK cell proliferation and IFNγ production [301]. Also, targeting activated T cells through their IL-2Rα chain by the anti-CD25 mAb (Daclizuman), promotes T cell proliferation. Moreover, CD25 is highly expressed by T_{regs} which leads to their transient depletion, leading to increased numbers of effector T cells and decreased immunosuppression[178].

A particular sub-category of immunotherapeutic Abs, known as immune checkpoint therapies, has shown great promise, evoking durable clinical responses, in patients with metastatic disease, and has revolutionized the field of cancer immunotherapy in the last decade. Checkpoint inhibitors aim to block regulatory pathways in T cells that inhibit anti-tumor responses leading to enhanced co-stimulation [302]. The first checkpoint therapeutic to be approved by the FDA in 2011 for the treatment of metastatic melanoma was anti-CTLA-4 (cytotoxic T lymphocyte antigen 4) mAb, Ipilimumab. Ipilimumab targets CTLA-4, a negative regulator of T cell activation, which is up-regulated on activated T cells and functions a safety switch to attenuate the response. CTLA-4 is a homologue of CD28 and binds the same ligands, CD80 and CD86, with much higher affinity. Contrary to the co-stimulatory effect of CD28, CTLA-4 cross-linking to its ligands leads to inhibition of the proliferation of activated T cells [303,304]. Validation of the mechanism of action of Abs blocking the interaction between CTLA-4 and CD80 was performed in numerous experimental settings, in combination with various treatments that would expose TAA and make them readily available for uptake by APCs [305-308]. Antibody-mediated blocking of CTLA-4-mediated suppression unleashed effector T cell activation in an FcR-independent manner. At the same time, it induced FcR-dependent elimination of Tregs by intratumoral macrophages [309,310].

Two other immune checkpoint Abs were recently approved by the FDA in 2014. The anti-PD-1 mAb Pembrolizumab and Nivolumab target programmed death receptor 1 (PD-1), which is expressed on activated T cells and B cells and provides a potent inhibitory signal when bound to its ligands PD-L1 and PD-L2 expressed on APCs or tumor cells [311]. Blocking this interaction with mAb interferes with signaling through the TCR leading to T cell inactivation [312].

The ligand for PD-1, PD-L1, can be up-regulated on tumor cells as an immune escape mechanism by inducing T cell death. Targeting PD-L1 with mAb inhibits binding to PD-1 and CD80, rescuing effector T cells. Clinical trials using Ipilimumab and Pembrolizumab/Nivolumab or anti-PD-L1 as a combination therapy are on-going and hold great promise.

There are several inhibitory receptors on lymphocytes that have been explored as potential targets for Ab-mediated immunotherapy. For instance, OX40 a negative regulator of lymphocyte proliferation and cytokine production on activated lymphocytes, GITR, and KIR2DL1/L2/L3 and KIR2DS1/S2) on NK cells which inhibit NK cytotoxic activity [313-316]. New members of the immune checkpoints family are continuously emerging and are being evaluated as monotherapies and in combination with already established therapies. These aim to provide a blockade of inhibitory pathways, such as LAG-3, VISTA, BTLA and co-stimulation of activating pathways ICOS [317-322].

1.4.2 Targeting tumor-associated macrophages

Modulation of the immunosuppressive myeloid compartment has become a crucial factor in determining the success of cancer immunotherapy and is currently the subject of vigorous

investigation. Intensive efforts continue to identify candidate molecules targeting different aspects of the development and function of TAMs. These have three main goals: 1) to decrease the number of precursors recruited to the tumors by inducing apoptosis or maturation or by inhibiting their trafficking, 2) to block molecular mechanisms employed by TAMs to inhibit lymphocyte tumoricidal activity, and 3) to eliminate TAMs or re-program them to an M1 phenotype. The last category is of particular interest for this thesis and some examples are mentioned below.

In a mammary carcinoma model, blockade of the CCL2/CCR2 axis lead to decreased recruitment of inflammatory monocytes and macrophages and as a result inhibited metastatic spread [323]. Combinatorial treatment with clodronate-liposomes and VEGF-neutralizing Abs decreased certain TAM subpopulations and led to a reduction in tumor-associated DC and blood vessel density [324]. Targeting legumain, a stress protein and a member of the asparaginyl endopeptidase family, on TAMs, lead to $CD8^+$ cytotoxic T cell responses against TAMs and decreased pro-angiogenic factors, such as TGF-β, TNFα, MMP-9 and VEGF, thus constricting angiogenesis and metastasis of breast carcinomas [325]. Adenoviral delivery of CCL16, TLR9 stimulation with CpG DNA and anti-IL-10R Abs led to TAM reprogramming from M2 to M1 phenotype leading to tumor de-bulking, followed by DC migration to draining lymph nodes for priming of adaptive responses [326]. Treatment with soluble IL-12 led to a phenotypic switch to M1 TAMs, characterized by a reduction in the levels of anti-inflammatory IL-10, TGF-β and CCL2 and increased levels of TNF, IL-15 and IL-18, and concomitant NK and T cytotoxic cell activation in melanoma, lung and colon carcinoma [327]. Similarly, adoptive transfer of tumor-specific cytotoxic T cells (CTLs) or chimeric-Ag receptor T cells both engineered to release IL-12 shifted the TAM polarization to M1 [328,329]. Targeting of NF-κB, the master regulator of the immunosuppressive phenotype of TAMs, through its downstream signaling kinase IκB re-educated TAMs to the M1 phenotype in ovarian carcinoma [330]. Treatment of mice bearing mammary carcinoma 4T1-Neu with the chemotherapeutic agent docetaxel induced MDSC apoptosis and reprogramming of TAMs to M1 [331]. In another study, HRG (Host-produced histidine-rich glycoprotein) down-regulated PIGF (placental growth factor) and skewed TAM polarization to M1 phenotype promoting anti-tumor responses and vessel normalization [332]. Anti-CD40 Abs in combination with IL-2 reprogrammed TAMs and up-regulated iNOS in TAMs of lung metastases but not in the primary tumor [333]. In patients with pancreatic cancer agonistic anti-CD40 Abs activated macrophages to infiltrate pancreatic tumors and exert tumoricidal activity in combination with chemotherapeutic agent gemcitabine [334]. CSF-1 is an important cytokine for macrophage survival and M2 polarization. Use of CSF-1R antagonist inhibited the expansion of MDSCs and TAMs and their recruitment to lung and prostate tumors [335]. Blockade of CSF-1R and cKIT receptor tyrosine kinase led to reduced TAM recruitment in a mammary carcinoma model [226]. In a mouse model of glioblastoma multiforme, inhibition of CSF-1R altered TAM polarization to M1 and blocked tumor growth [336]. Inhibiting TAMs and their precursor inflammatory monocytes through targeting of CSF-1R and CCR2 reduced the number of tumor-infiltrating TAMs, but also reprogrammed the remaining TAMs, thus reducing metastasis and increased anti-tumor T cell responses in a pancreatic tumor model [337]. This

TAM-reprogramming approach has shown great promise in improving responses to T cell checkpoint immunotherapies using anti-PD-1 and anti-CTLA-4 Abs [338]. In another study, targeting of CSF-1R enhanced anti-tumor responses of adoptively transferred T cells [339]. In Paper II we use mAbs to target scavenger receptor MARCO in order to reprogram M2 TAMs in the TME.

1.4.3 Exosomes in cancer immunotherapy

The release of extracellular vesicles is a way of intercellular communication employed by many different cell types. Exosomes are 30-100nm vesicles consisting of a lipid bilayer membrane that derive from the late endosomal compartment. They are shed from different types of cells, including APCs and tumor cells, during both physiological and pathophysiological conditions. Exosomes can carry membranous proteins on their surface that are involved in membrane transport, fusion, adhesion, Ag-presentation and immune stimulation. They can also contain cytosolic proteins, lipids and RNA molecules. Depending on the cell of origin and its activation state, their composition may vary. As a result, they have various immunomodulatory activities, which also depend on their cargo. These range from cell-cell communication to immunomodulation, and acting as shuttles for Ag-presentation, transfer of proteins, mRNA and microRNA.

The observation that exosomes originating from APCs, such as DCs and B cells, carry MHC-I and -II as well as co-stimulatory molecules on their surface, suggested that they are involved in direct Ag-presentation to T cells. In fact, in a mouse model of melanoma tumor-peptide pulsed DC-derived exosomes exhibited potent immunostimulatory and anti-tumor capacity leading to activation of T cell effector functions and tumor growth inhibition [340]. Alternatively, exosomes can indirectly activate T cell by enhancing the Ag-presenting capacity of DCs. It has been shown that exosomes that are taken up by immature DCs transfer their antigenic load to endogenous MHC molecules, which are then transported to the surface of the DC and present Ag in the classical manner to stimulate T cells [341].

Tumor-derived exosomes have been suggested to both promote and suppress Ag-specific and non-specific anti-tumor responses. For example, tumor-derived exosomes are enriched for receptors that induce T cell apoptosis, such as TNF-related apoptosis-inducing ligand (TRAIL) and CD95, leading to suppression. Additionally, they can promote the immunosuppressive TME as they are enriched in factors such as prostaglandin E2 and TGF-β that drive the generation of MDSCs and M2 polarization of TAMs [342]. Also, as carriers of mRNA and microRNA molecules, exosomes have the capacity to regulate the transcription of different genes in the recipient cells. For example tumor-derived exosomes carrying miR-21 can bind to TLR7 and 8 activating macrophages in the TME to produce TNF, IL-6 and thereby lead to increased tumor growth and metastasis [343]. Tumor-derived exosomes also facilitate tumor invasion and metastatic spread by conditioning the distal locations for the seeding of micrometastases, as has been shown in the case of melanoma-derived exosomes that accumulate in draining lymph nodes [344,345].

However, exosomes carrying tumor-Ags have also been shown to activate DCs to induce potent CTL anti-tumor responses [346]. Moreover, experimental approaches have tried to generate immunogenic exosomes by pulsing APCs in vitro with tumor-derived Ags, aiming to induce Ag-specific immune responses against the tumor. It has been shown that activation of B cells is necessary for the efficient induction of T cell responses to exosome-bound Ags [347]. Additionally, exosomes have also been described as potent inducers of inflammatory cascades and could thus be used as adjuvants as they carry a wide array of stimuli that lead to immune activation. These can be inflammatory mediators (cytokines such as IL-1β), microbial Ags that are ligands for PRRs and TNF-related proteins (FasL, TRAIL, CD40L).

Immunotherapeutic approaches focus on attempts to modulate exosome composition, release and cell targeting capacity, as well as modulating the activity of the APC that they originate from by cytokines, Ag-stimulation, gene transfer and microRNA pulsing, as has been reviewed elsewhere [348,349]. In Paper IV we use DC-derived exosomes loaded with αGC and the model Ag OVA in a vaccination approach to induce Ag-specific anti-tumor responses.

1.5 MURINE MODELS OF HUMAN MELANOMA, BREAST AND COLON CANCER

Breast cancer is the most common cancer in women (second most common cancer overall), with 1.7 million new cases and representing 25% of all cancers in women in 2012. Melanoma accounts for 1.6% of all cancers and colon cancer is the third most common cancer, representing 9.7% of all cancer cases in 2012, (source: www.wcrf.org).

Studies on murine tumor models have shown that metastatic patterns of different cancer cell lines are not random, but rather site specific and depend on the site of injection of the tumors cells, thus taking into account the local tissue-specific microenvironment and supporting the use of orthotopic models in cancer research [350,351]. However, this is not always an easy task. For several reasons the below-mentioned models were assessed as valid to explore the hypotheses described in this thesis.

1.5.1 Mouse models of melanoma (B16)

The B16 melanoma cell line was derived from a spontaneous C57BL/6 tumor and is a widely used model in melanoma studies [352,353]. It is known for its aggressive growth and being difficult to treat. The cells can either be inoculated subcutaneously in the flank of a mouse to generate a primary tumor or intravenously to simulate a metastatic model of melanoma in the lung. B16 exists in many different variants, the difference being their metastatic potential. The most commonly used is the B16.F10, which metastasizes from the primary subcutaneous tumor to the lymph nodes and lungs. B16 is a rather plastic cell line and the degree of pigmentation may vary, however this does not seem to influence the expression of different TAAs. B16 expresses many of the TAAs that human melanomas express, making it a good model system for the human disease. One of the TAAs expressed on the surface of B16 cells is glycoprotein 75 or TRP-1. The surface molecule gp75, is targeted by Abs of the TA99 IgG2a clone (used in Paper II) which when bound trigger its internalization and mediate ADCC by NK cells [354]. This antibody clone is used as a golden standard (positive control) in

many immunotherapy settings tested in the B16 model. The B16 cell line exists in many functional variants genetically modified to express different genes of interest, such as GFP, luciferase, Flt3L, GM-CSF and soluble or membrane-bound ovalbumin. This makes it a very versatile and useful model system. B16 expresses low levels of MHC-I [355] and MHC class II expression can be induced by IFNγ [356]. B16 is a low- or non-immunogenic tumor. The B16 melanoma model is a widely used model with many modified version that allow flexibility in functional read-outs, such as the OVA-expressing and the luciferase-tagged variants that are used in Papers II and IV.

1.5.2 Mouse model of breast cancer (4T1)

The 4T1 mammary carcinoma cell line was derived from a cell line that originated from a spontaneous tumor of a MMTV Balb/c mouse, which was transplanted to a C3H mouse [357,358]. It is a typical triple negative mammary carcinoma cell line with a mesenchymal phenotype ($ER^-/PR^-/HER2^-$), the most challenging to treat type of breast cancer. As shown in Paper II, the MARCO receptor is highly expressed in triple negative human breast cancer, making 4T1 a relevant experimental model to study the effect of MARCO-targeting. 4T1 was selected for being easy to culture, highly tumorigenic when inoculated into mice, resistant to 6-thioguanine and for its capacity to metastasize to distal sites. There are several reasons that make the 4T1 cell line a suitable model for human breast cancer. Firstly, it can be readily introduced orthotopically into the mammary fat pad of mice and can metastasize from the primary tumor site to several sites (lymph nodes, bone, brain liver and lungs) in a pattern similar to human mammary carcinomas. Moreover, after inoculation the disease process progresses over a period of several weeks modeling the human setting. Much like many human cancers, 4T1 tumors are poorly immunogenic, which means that treatment with irradiated tumor cells fails to mount a memory response against a secondary introduction of the same tumor. 4T1 cells express MHC-I but not MHC-II, making them good targets for $CD8^+$ CTLs and NK cells. Finally, as metastatic disease is of great importance to study, a great advantage comes from being able to excise the primary tumor in order to generate a model for metastatic disease simulating the human setting where surgical removal of the primary tumor proceed any other treatment step.

1.5.3 Mouse model of colon cancer (MC38)

The MC38 colon adenocarcinoma cell line was established in a C57BL/6 mouse using dimethyl-hydrazine (DMH). DMH 0.2mg/mouse was injected subcutaneously (s.c.) weekly for 7 moths. Formed colon tumors were excised and passaged and the ones that survived the first passage went on to serial transplants. The MC38 tumor was the only small tumor to survive the first passage and was transplanted by trocar to the axillary region. MC38 was characterized as a grade III adenocarcinoma of the colon, which is highly metastatic to the lung [359]. However, intraperitoneal transplantation gave rise to hepatic metastases, indicating that the site of implantation affects the metastatic pattern. MC38 has been extensively used in studies of checkpoint therapies with anti-CTLA-4, anti-PD-1 and anti-PD-L1 Abs, and thus

was assessed as a relevant model to compare combination therapies of anti-CTLA-4 and anti-MARCO Abs in Paper II.

1.5.4 Mouse model for the study of EMT and metastasis

To study the complexity of the metastatic process, where the evaluation of tumor cell motility and the ability to colonize distal sites is imperative, perhaps the most physiological assay is the injection of tumor cells sub-cutaneously or in the footpad. This route of injection gives direct access into the lymphatics, where the first stop is the popliteal lymph node. This model was used to compare the migratory capacity of cell lines with different EMT profiles in Paper III. EpH4 is a non-invasive non-migratory cell line derived by immortalization of balb/c mammary epithelial cells [360]. EpRas cells were generated by transducing the parental EpH4 cells with a retroviral vector expressing v-Ha-ras [361]. Upon stimulation with TGF-β these cells gain migratory capacity. Finally, EPXT cells are stably in EMT under the influence of oncogenic Ras and autocrine TGF-β-signaling.

2 THE PRESENT STUDY

2.1 AIMS

This thesis aims to identify novel candidates in the tumor microenvironment for targeted immunotherapy of cancer.

Specific aims:

Paper I - To investigate the impact of monoclonal antibodies targeting scavenger receptor MARCO on marginal zone macrophages and adaptive immune responses.

Paper II - To modulate the immunosuppressive tumor microenvironment into induction of anti-tumor immune responses by using monoclonal antibodies to scavenger receptor MARCO targeting tumor-associated macrophages.

Paper III - To investigate the role of TGF-β1-induced epithelia-mesenchymal transition on lymphatic metastasis of cancer cells.

Paper IV - To evaluate the immunomodulatory role of exosomes as cancer vaccines, triggering tumor-specific adaptive immune responses.

2.2 RESULTS AND DISCUSSION

2.2.1 Marginal zone macrophages regulate antigen transport by B cells to the follicle in the spleen via CD21 (Paper I)

The presence of anti-MARCO autoantibodies has previously been reported in SLE patients, however, their role in the pathogenesis of autoimmune disease is still unknown. In this study we investigate the possible regulatory effects induced by the anti-MARCO Abs on other immune cells in responses to foreign Ags. MZMs are strategically positioned at the marginal sinus of the spleen where they capture incoming Ag from the circulation via scavenger receptor MARCO and interact with MZBs. Based on this, we therefore set out to investigate the effect of anti-MARCO Abs on the MZM/MZB interplay.

To study the effects of MARCO-crosslinking on the MZM/MZB interaction, a rat anti-mouse MARCO Ab was injected intravenously (i.v). in wildtype (wt) mice. The Ab selectively bound to MARCO-expressing MZMs in the MZ without depleting them. This resulted in the gradual loss of CD21 (complement receptor 2) on MZBs, and to a lesser extent on other B cell populations, already one hour after administration. The loss of CD21 could not be attributed to local complement activation, blocking of antibody binding, or internalization of CD21. It was also solely targeting CD21, leaving its alternative splice-variant, CD35 on FDCs unaffected. A similar CD21 loss was observed also with polyclonal mouse serum containing Abs against MARCO, thus excluding inter-species cross-reactivity. Since MARCO can be up-regulated in response to LPS-stimulation, we investigated the involvement of MyD88-, TLR2-, TLR4- and TLR9-signalling in mediating CD21 loss on MZBs upon MARCO triggering. However, these pathways were not involved and we could also exclude the involvement of FcγRs.

Due to the speed of the response (observed CD21-loss already 1 hour post administration), we hypothesized that shedding of CD21 from the surface of MZBs could be the underlying mechanism of the CD21 loss. This is known to occur on B cells and soluble CD21 can be detected in several inflammatory and autoimmune conditions. Purinergic enzyme receptors P2X7 and P2Y have the capacity to cleave CD21 in response to extracellular ATP. Based on that, ATP-release after anti-MARCO Ab addition to peritoneal $MARCO^+$ macrophage cultures *in vitro*, was investigated. Extracellular ATP could be detected already 5 minutes post stimulation. Moreover, addition of extracellular ATP to splenocyte cultures led to a decrease in CD21 levels on MZBs. Increased levels of extracellular ATP are associated with pro-inflammatory activation of macrophages, referred to as M1 polarization. This suggests that MARCO-engagement leads to macrophage activation. To further investigate the effect of the released ATP by the macrophages we assessed the expression of CD39, an ectonucleotidase involved in the catabolism of pro-inflammatory extracellular ATP to ADP. This metabolic reaction facilitates the transition of macrophages from a pro-inflammatory to an anti-inflammatory regulatory state, thus functioning as a homeostatic switch during inflammation [362]. Interestingly, 24 hours after stimulation of macrophages with anti-MARCO *in vitro,* CD39-expression increased to levels similar to those induced by the addition of extracellular ATP. This could be regarded as a compensatory mechanism

aiming to regulate macrophage activation in response to immunogenic stimuli, such as ATP.

It has previously been shown that MZBs take up Ag and migrate back and forth to the follicle depositing Ag on the FDCs. CD21 is an important receptor for opsonization of foreign Ags and generation of immune complexes. To assess the impact of CD21 loss in response to anti-MARCO engagement on Ag-shuttling, mice were immunized with high molecular weight dextran, as a model Ag. Dextran is dependent on CD21 for its uptake by MZBs, making it an ideal model-Ag to study complement receptor-dependent Agtransportation. Interestingly, pre-treatment with anti-MARCO Abs significantly reduced binding of dextran to MZBs, due to the decrease in CD21. Importantly, it also decreased the amount of dextran that was deposited on the FDCs. Reconstitution of CD19-deficient mice (lacking MZBs) with splenic B cells made it possible to tract the migration of $CD19^+$ MZBs upon Ag-stimulation. No differences were observed, suggesting that the ability of MZBs to migrate to the follicles of the spleen in response to dextran immunization was not affected by the anti-MARCO Ab treatment. In conclusion, MARCO-crosslinking affects the amount of Ag that can be captured by MZBs due to the loss of CD21, as well as Ag deposition on FDCs in the follicles, while leaving the shuttling capacity of MZBs intact.

Ag deposition on FDCs is important both for the initiation of the GC reaction and consequently also humoral responses. Naturally, we next investigated how the loss of CD21 on MZBs and the consequent reduction of Ag deposition on FDCs affected the subsequent immune response to those Ags. For that, immunization with TI Ag NP-dextran was performed after anti-MARCO treatment, at which point the expression of CD21 was already lost. Analysis of the subsequent humoral response showed significantly lower levels of Ag-specific IgM Abs in mice pretreated with anti-MARCO Abs. This is in line with the fact that dextran is primarily bound by MZBs, which respond best to TI Ags.

Next, we analyzed the humoral response against the TD Ag NP-CGG after anti-MARCO injection. In line with our previous results, anti-MARCO injected mice showed a reduction in GC B cell numbers compared to controls. This was further reflected in the antibody response in treated mice, where lower IgM, IgG1 and IgG3 levels were observed. Thus, anti-MARCO Abs have a pronounced effect on the humoral immune response against both TI and TD Ags. This suggests that the elevated levels of anti-MARCO autoantibodies found in SLE patients could contribute to the increased risk of infection observed in these patients. An additional contribution of anti-MARCO Abs to the pathology of SLE could be that defective complement receptor expression on B cells leads to inefficient clearance of apoptotic cells, which causes a break in self-tolerance.

In summary, this paper reports a novel mechanism through which, resident MZM in the spleen regulate innate and adaptive immune responses to foreign Ags. MZM activation through PRR MARCO by Abs leads to ATP-release, cleavage of CD21 on MZBs and reduced Ag deposition on FDCs. Since MARCO can also bind self-Ags, a similar mechanism could limit their deposition on FDCs. It would therefore be interesting to investigate how this pathway can be manipulated to regulate unwanted adaptive immune

responses to self-Ags or to enhance protective immunity to prevent infection. Finally, crosslinking of MARCO with Abs led to ATP-release, which is associated with M1 macrophage polarization. This knowledge led to the hypothesis that MARCO can be targeted by Abs to induce a switch to a pro-inflammatory phenotype, for example in the setting of cancer.

2.2.2 Reprogramming tumor associated macrophages by antibody targeting inhibits cancer progression and metastasis (Paper II)

Tumors are complex tissues consisting of various types of cells besides the tumor cells. TAMs are a heterogeneous population of macrophages and make up the majority of the tumor-infiltrating inflammatory myeloid cells that account for the immunosuppressive microenvironment of tumors.

Based on the fact that MARCO expression is restricted to certain macrophage subpopulations, we wanted to investigate whether MARCO was also expressed on TAMs in the TME. We screened tumor sections of mammary adenocarcinoma, melanoma and colon adenocarcinoma tumor model and identified the presence of MARCO on macrophages in the tumor stroma. MARCO-expression was only present in a fraction of the $F4/80^+$ cells in the tumor stroma, suggesting that the receptor is restricted to a subtype of macrophages. To further elucidate the nature of the subset of macrophages that expressed MARCO, we sorted the different subsets by flow cytometry. We could identify expression of MARCO on the $CD11b^+$ $Ly6C^{lo}$ $MHCII^{lo}$ subset of tumor infiltrating TAMs. This subset showed characteristic expression of typical M2 markers (*arg1, fizz1,* etc), as well as Cx3cr1 and low expression of MHC-II, as observed by qPCR.

In an effort to identify what is driving the up-regulation of MARCO on TAMs, we set up an *in vitro* system for polarization of bone marrow-derived macrophages by cytokines or tumor supernatant. Interestingly MARCO expression was up-regulated in M2 and tumor supernatant polarized cultures, supporting our in vivo findings.

Based on the fact that, IL-10 and TGF-β are the major immunosuppressive cytokines in the TME and can promote M2 polarization of macrophages, we investigated whether they could also drive the expression of MARCO. In vitro cultures of a peritoneal macrophage cell line were stimulated with IL-10 or TGF-β and indeed, MARCO expression increased in response to both these cytokines.

Having identified MARCO as a specific marker for M2 TAMs, we generated mAbs to MARCO, and evaluated their use in an immunotherapy setting to the three previously mentioned tumor models. Anti-MARCO Abs could infiltrate the tumor and bind to $MARCO^+F4/80^+$ cells. Surprisingly, anti-MARCO treatment led to decreased primary and metastatic tumor growth in the mammary adenocarcinoma model 4T1 **(Figure 6A and B)**. Moreover, we found that anti-MARCO treatment led to an increase in M1 TAMs and a decrease in M2 TAMs. This was accompanied by an increased GC B cell response in the tumor-draining lymph node and an increased $CD4^+h/CD8^+$ T cell ratio in the tumor, suggesting the treatment increased tumor immunogenicity. We did not observe any other

significant difference caused by the anti-MARCO treatment when we examined different lymphocyte populations infiltrating the tumors (NK, B and T cells, DC, neutrophils) or Mo-MDSC and PMN-MDSC in the spleens of tumor-bearing mice.

To further validate the effect of anti-MARCO treatment, we used another tumor model, the B16 melanoma. Also here, treatment with anti-MARCO Abs inhibited tumor growth, to a similar degree as TA99 Ab, an antibody known to induce ADCC to B16. Moreover, the anti-MARCO effect was absent in MARCO-deficient tumor bearing animals, suggesting the observed tumor decrease in the treated wt mice was MARCO-specific. Similar alterations were observed in the different tumor infiltrating leukocyte populations as in the 4T1 model, more specifically a decrease in inflammatory macrophages in the tumor and an increase in $CD4^+$/$CD8^+$ T cells, $CD4^+$/Tregs, OVA-specific $CD8^+$ T cells and OVA-specific IgG2a and b.

Additionally, the effect of anti-MARCO treatment was assessed in combination with other mAb immunotherapies. When combined with TA99 mAbs or immune checkpoint anti-CTLA-4 Abs we observed an additive tumor growth inhibiting effect. In particular, anti-MARCO Ab and anti-CTLA-4 Ab combination treatments decreased tumor growth of B16 melanoma and MC38 colon tumor-bearing mice compared to anti-CTLA-4 Ab monotherapy **(Figure 6C and D)**.

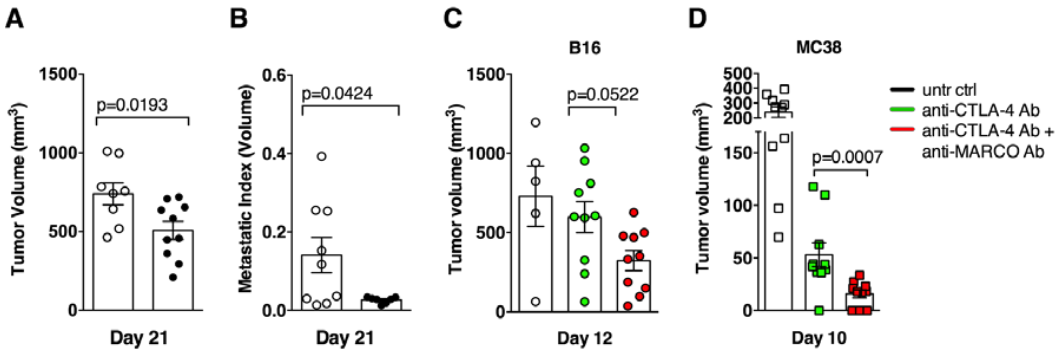

Figure 6. Targeting MARCO by mAbs inhibits *in vivo* tumor growth and metastasis
4T1 (mammary carcinoma), B16 (melanoma) or MC38 (colon carcinoma) cells were injected s.c. in mice followed by Ab treatment. Tumor growth was monitored by manual measurements. (A) Tumor volume of 4T1 mammary carcinoma after anti-MARCO Ab treatment (full circles) compared to untreated controls (empty circles), on day 21 after tumor inoculation (B) Metastatic index (number of lung tumor colonies/primary tumor volume) of 4T1 tumors after anti-MARCO (full circles) compared to untreated controls (empty circles), on day 21 (C) Tumor volume of B16 melanoma untreated, anti-CTLA-4 Ab+Gvax and anti-CTLA-4 Ab +Gvax+anti-MARCO Ab-treated mice, day 12 (D) Volume of MC38 colon cancer untreated, anti-CTLA-4 Ab and anti-CTLA-4 Ab+anti-MARCO Ab-treated mice, day 10. Described also in Paper II: Fig. 3 and 4.

As many immunomodulatory Ab therapeutics require FcγR involvement to exert their function, we generated mouse anti-MARCO Ab variants/mutants with null FcγR-binding and observed that the tumor growth inhibiting effect was FcγR-dependent. Utilizing KO mice deficient for all or only for activating FcγR we could narrow the effect down to the involvement of the inhibitory FcγRIIb. Interestingly, high expression of FcγRIIb correlated

with the macrophage subpopulations exhibiting high MARCO expression, namely the *in vitro* M2 BMDM and tumor sorted M2 TAMs, further strengthening our findings. This suggests that the anti-tumor activity of anti-MARCO Abs is dependent on the ability of the Fc-part of the anti-MARCO Ab to engage the inhibitory FcγRIIB.

To investigate the potential application of the anti-MARCO Ab treatment in the clinical cancer setting we investigated the expression profile of MARCO by gene expression and immunofluorescence on human breast cancer and metastatic melanoma biopsies. In the case of human breast cancer, using the TCGA and KI/Clinseq datasets we found that higher MARCO expression correlated with the basal (triple negative) subgroup of patients. In the case of melanoma, higher MARCO expression was observed amongst in the distal metastases. Thus in both cancers, MARCO expression is associated with more aggressive and metastatic cancer types.

To further interpret MARCO expression with reference to the metastatic cancer profile, we compared MARCO expression relative to an M2 TAM gene signature in the human breast cancer and melanoma datasets. Interestingly, MARCO-expression correlated with many M2 genes as well as with the expression of FcγRIIB, suggesting that MARCO is expressed in tumors with a high TAM content. Furthermore, we analyzed MARCO expression relative to an EMT gene signature, and observed a correlation with the expression of known EMT regulators *mmp9, snail* and *twist*.

Finally, we verified MARCO expression on tumor sections from human breast cancer and melanoma patients by immunofluorescence staining. In human breast cancer, we observed a higher infiltration of $CD68^+$ macrophages in the stroma of the basal triple negative breast cancer compared to the ER^+/PR^+ subgroup. In addition, MARCO was present on $CD68^+$ macrophages and correlated with the presence of the M2 markers CD163, as well as CD206, strengthening our hypothesis that MARCO is a marker for M2 TAMs. These data suggest that MARCO is a clinically relevant marker for distinguishing aggressive metastatic breast cancer and melanoma, and provides a novel candidate for targeted immunotherapy using Abs.

Overall, this study identifies a novel marker for a subset of immunosuppressive TAMs, which correlates with metastatic gene signatures in clinical samples of human breast cancer and melanoma. Pre-clinical models show a promising decrease in primary and metastatic tumor growth, as well as re-programming of TAMs from M2 towards M1 phenotype and accompanied by increased tumor-immunogenicity. Moreover, combinatorial treatment using anti-MARCO Ab and checkpoint therapy using anti-CTLA-4 Ab showed an impressive inhibition of tumor growth and increased overall survival. Altogether, this suggests that immunotherapeutic approaches aiming at repolarizing the immunosuppressive TME offer a promising new approach to cancer immunotherapy.

2.2.3 TGF-β1-induced EMT promotes targeted migration of breast cancer cells through the lymphatic system by the activation of CCR7/CCL21-mediated chemotaxis (Paper III)

Metastatic cancer disease presents the greatest challenge in treating cancer. Tumor cells have the ability to disseminate through lymphatic and the blood circulation during metastatic spread. However, it is still unknown whether lymphatic dissemination is a targeted process. TGF-β1 is an abundant cytokine in the TME and has been associated with metastatic disease in many cancers. TGF-β1-signaling is a known activator of a genetic program known as epithelial-mesenchymal transition (EMT), which induces a migratory phenotype in tumor cells. Based on this, we hypothesized that TGF-β-induced EMT could be connected to lymphatic dissemination of tumor cells.

To investigate whether TGF-β-induced EMT predisposes tumor cells to metastasize through the lymphatics, we used a mouse model frequently used to study DC trafficking to draining popliteal lymph nodes (PLN), by injecting tumor cells into the hind footpad of mice. For this, three versions of a mammary epithelial cell line, with different EMT profiles, were used. EpH4 is non-metastatic in response to TGF-β1, EpRas originates from EpH4 but has constitutive oncogenic Ras-signaling and undergoes EMT in response to TGF-β1, and EpXT is derived from EpH4 but has constitutive expression of oncogenic Ras- and TGF-β1-signaling and is in stable EMT. Fluorescently labeled versions of the cell lines were injected into the footpads of mice to assess the migratory capacity to the draining PLN. EpXT cells formed tumors at the site of injection but also acquired a migratory capacity and were detected in the draining PLN after 2 days. They were primarily located in the subcapsular sinuses, indicating entrance through afferent lymphatic vessels. In contrast, non-migratory EpH4 cells formed small tumors only at the site of injection and could not be detected in lymph nodes. EpRas cells gained migratory capacity only upon pre-treatment with TGF-β1. Thus, induction of EMT by TGF-β1-signaling induced a migratory phenotype on the tumor cells, which led to lymphatic metastasis.

Moreover, immunofluorescence stainings for blood and lymphatic vessels in EpXT footpad tumors showed that blood vessels were evenly dispersed within the tumors, while lymphatic vessels clustered in certain regions. The areas of lymphatic vessel clustering were associated with more invasive/migratory tumor cell morphology and showed evidence of intravasation. This suggested that EMT might induce lymphatic metastasis in a targeted manner. To investigate this, an in vitro 3D matrix co-culture system was used to study migration towards lymphatic endothelial cells (LECs), or vascular endothelial cells (VECs). Interestingly, tumor cells undergoing EMT acquired an elongated morphology and formed clusters of invasive cells that preferentially migrated towards LECs, compared to VECs. Non-EMT cells, on the other hand, did not migrate. Taken together, these results suggest that mammary tumor cells that have undergone TGF-β-induced EMT have an increased migratory capacity and preferentially migrate in a targeted manner towards lymphatic vessels, as opposed to blood vessels.

It is well established that upon activation by inflammatory stimuli in the periphery, DCs use

chemokine receptor CCR7 to respond to a chemokine gradient of its ligand CCL21. The CCR7/CCL21 chemotactic axis guides DC migration through lymphatic vessels in order to ultimately travel to draining lymph nodes where they initiate adaptive immune responses. Based on the fact that breast cancer cells can also express CCR7, we hypothesized that CCR7 could have a similar role in guiding the lymphatic dissemination of EMT cells. Indeed, both TGF-β1-induced EMT models showed up-regulated expression of CCR7, compared to the non-EMT EpH4, suggesting that TGF-β driven CCR7 expression is dependent on EMT. To investigate the CCR7/CCL21 chemotactic axis we set up transwell invasion assays, in which mammary cancer cells that had undergone TGF-β-induced EMT were seeded in the upper chamber and recombinant CCL21, was added in the lower chamber as a chemoattractant. Tumor cells that had been treated with TGF-β1 migrated more efficiently towards the CCL21 gradient, compared to untreated cells. When a neutralizing anti-CCR7 Ab was added to the cultures, the CCR7/CCL21 interaction was blocked leading to reduced targeted migration. Similarly, reduced migration of EpXT cells was observed in the in vitro 3D migration assay towards LECs in the presence of the neutralizing anti-CCR7 antibody, further strengthening our hypothesis. Moreover, in order to assess if this was the case also in vivo, we introduced small interfering RNA to silence CCR7 in EpXT cells and injected these cells into the mouse footpad. Interestingly, CCR7-siRNA-treated EpXT cells expressed lower levels of CCR7 and exhibited reduced migration to the PLN compared to their untreated counterparts. These data demonstrate that the targeted migration of TGF-β1-induced EMT mammary cancer cells to the lymphatics depends on the interaction of CCR7 with CCL21.

TGF-β1-signaling during EMT can be induced through two independent pathways, a Smad-dependent and a P38 MAPK-dependent pathway. In order to elucidate the underlying molecular mechanisms governing CCR7 up-regulation during TGF-β-induced EMT, we utilized molecular inhibitors to those pathways. Surprisingly, both Smad3 and p38 MAPK inhibitors led to decreased CCR7 mRNA levels during TGF-β1-induced EMT in mammary cancer cells. However, when assessing CCR7 protein levels only p38 MAPK had significant inhibitory effect, suggesting that factors operating downstream of p38 MAPK signaling were involved in inducing CCR7 expression in EMT cells. By gene expression analysis we were able to show that AP-1 factors c-Jun and JunB are responsible for the CCR7 overexpression as a result of TGF-β1-induction in EMT in mammary cancer cells. Moreover, induction of JunB is P38 MAPK dependent. These results highlight the P38 MAPK pathway as the underlying mechanism of CCR7-mediated chemoattraction and lymphatic dissemination of mammary cancer cells having undergone TGF-β1-induced EMT. It also demonstrates that P38 MAPK blockade can reverse EMT, thus highlighting it as a novel target for pharmacological inhibition to abrogate lymphatic spread of mammary cancer cells. Interestingly, TGF-β1-induced EMT mammary cancer cells that retain endogenous TGF-β1 production can induce the secretion of CCL21 by lymphatic endothelia vessels, thus potentiating their own metastatic spread. This can be regarded as a feed back loop driving the lymphatic spread of malignant cells. Finally, histological evaluation of human breast cancer tissue sections revealed that CCR7-expression is

associated with tumor cells in invasive areas of the tumors with characteristic low expression of E-cadherin, suggesting they have undergone EMT. Moreover, analysis of microarray data from a cohort of human breast cancers indicated that high expression of CCR7 and CCL21 correlates with an EMT gene signature.

Overall, the results show that tumor cells use a similar mechanism as DCs to gain migratory capacity through the lymphatic system in a targeted manner. Furthermore, it identifies p38 MAPK as a useful candidate for targeted inhibition to limit EMT and lymphatic dissemination of tumor cells. TGF-β1-signaling plays a crucial role in driving EMT and the up-regulation of CCR7. As previously mentioned, TGF-β1 is highly expressed by several cell types present in the suppressive TME, such as TAMs and T_{regs}. Targeting TGF-β-producing cells in the TME for reprograming, with approaches such as the one described in Paper II, may thus provide additional ways to restrict lymphatic dissemination of cancer cells.

2.2.4 Synergistic induction of adaptive antitumor immunity by codelivery of antigen with α-galactosylceramide on exosomes (Paper IV)

NKT cells are potent regulators of innate and adaptive immune responses. Moreover in the context of cancer, they have been implicated in the maturation of DCs facilitating subsequent $CD8^+$ T cell anti-tumor responses, as well as in the killing of immunosuppressive TAMs. Their function is however often compromised in cancer. Thus attempts to re-activate anergized NKT cells are of great interest in cancer immunotherapy.

NKT cells recognize lipid Ags presented by the MHC-like molecule CD1d. α-GalCer is a super-agonist of NKT cell activation, however when administered in a soluble form it leads to NKT cell anergy. Exosomes derived from APCs, and DCs in particular, can express CD1d and have been explored in immunotherapeutic approaches aiming to activate adaptive immune responses towards tumor-Ag.

To assess the capacity of exosomes to activate NKT cells, we generated exosomes from bone marrow–derived DCs from wt and CD1d-KO mice, lacking NKT cells. We next loaded exosomes with α-GalCer and the model Ag ovalbumin (OVA) and assessed NKT cell activation in vitro. OVA-loaded exosomes (OVA/exo) or α-GalCer-loaded exosomes (α-GalCer/exo) were added to splenocytes cultures from Va14 mice, transgenic for the iNKT cell receptor. CD1d-proficient α-GalCer/exo induced greater proliferation of iNKT cells compared to CD1d-deificient α-GalCer/exo, suggesting iNKT cell activation is in part dependent on the presence of CD1d on the surface of exosomes. OVA/exo failed to induce NKT activation, indicating that an activating ligand, in this case α-GalCer, for the NKT cell TCR is necessary for their activation. CD1d-proficient α-GalCer/exo also induced greater IL-4, IFNγ and IL-17A production by splenocytes, compared to the low levels observed in response to CD1d-deficient α-GalCer/exo stimulation and no cytokine production by OVA/exo stimulation. The results suggest, that α-GalCer/exo potently stimulate NKT cell activation, proliferation and cytokine production in part, but not exclusively, through CD1d and require exosomal α-GalCer.

In an attempt to translate our findings to an *in vivo* system, we generated DC-derived exosomes loaded with αGC and OVA (α-GalCer-OVA/exo) or the $CD8^+$ T-cell-specific OVA-peptide SIINFEKL (αGalCer-SIINFEKL/exo). These were injected i.v. in wt recipient mice, and proliferation was assessed after 7 days. Splenic NKT cells proliferation was only observed in response to exosomes loaded with both α-GalCer and the Ag/Ag-peptide, suggesting α-GalCer is required for efficient NKT cell proliferation. Up-regulation of the activation marker CD69 was observed already after 1 day, while proliferation proceeded until day 5 after stimulation and was accompanied by IFNγ production. IL-4 production was observed only during the first 3 days. iNKT cell activation also led to an early activation and proliferation of DCs, NK, and γδ T cells. Our findings suggest that αGC-loaded exosomes are potent inducers of iNKT cell responses, as well as DC, NK- and γδ T-cell activation and proliferation *in vivo*.

α-GalCer/exo stimulation led to increased OVA-specific $CD8^+$ T-cell proliferation, to a greater extent compared to α-GalCer-SIINFEKL/exo, which is in line with previous data where SIINFEKL/exo did not sufficiently stimulate proliferation of OVA-specific $CD8^+$ T cells, due to the lack of B-cell co-stimulation. Moreover, α-GalCer-OVA/exo immunization generated increased numbers of SIINFEKL-specific IFNγ producing cells in a CD1d-dependent manner. These results show that α-GalCer-loaded exosomes boost Ag-specific $CD8^+$ T-cell responses via iNKT cells in vivo in a CD1d-dependent manner. Additionally, α-GalCer-OVA/exo immunization induced proliferation of $CD4^+$ T cells and T follicular helper (T_{fh}) cells in an iNKT cell–dependent manner, as the response was lower in CD1d-deficient mice. In line with the role of T_{fh} in humoral responses, we detected increased numbers GC B cells and plasma cells and increased levels of OVA-specific IgG2c Abs.

Interestingly, when α-GalCer-OVA/exo where compared to soluble α-GalCer and OVA, they were more potent activators of adaptive immune responses as observed by increased activation of γδ T cells, $CD4^+$ T cells, and OVA-specific $CD8^+$ T cells. A second boost injection of α-GalCer-OVA/exo significantly augmented GC B-cell responses as reflected in the increased levels of OVA-specific IgG Abs. These results highlight α-GalCer-loaded exosomes as more potent adjuvants and Ag-delivery systems for the initiation of adaptive immune responses.

An additional advantage to the use of α-GalCer-OVA/exo in vaccination, was the observed lack of NKT cell anergy induction compared to administration of soluble α-GalCer. Importantly, in serial vaccinations only α-GalCer-OVA/exo induced a prolonged second wave IFNγ-response, which also resulted in an increase in OVA-specific $CD8^+$ T cells, when compared to soluble α-GalCer and OVA vaccination. This suggests that α-GalCer-OVA/exo have a more potent long-term immunostimulatory effect, making them promising vaccination vehicles.

Based on the previous finding that exosome co-delivery of glycolipid and protein Ag boosted adaptive immune responses without inducing iNKT-cell anergy, we investigated the potential use of α-GalCer-OVA/exo in tumor immunotherapy. Utilizing an OVA-

expressing B16 melanoma s.c. tumor model we similarly compared the efficiency of α-GalCer-OVA/exo to induce adaptive anti-tumor responses, relative to independent administration of α-GalCer and OVA. A-GalCer-OVA/exo significantly inhibited tumor growth and prolonged the survival of tumor-bearing mice. This was attributed to increased numbers of tumor-infiltrating $CD8^+$ OVA-specific T cells and increased serum levels of OVA-specific IgG in α-GalCer-OVA/exo treated mice compared to α-GalCer plus OVA treated mice. Together these results show that vaccination with α-GalCer-OVA-loaded exosomes can provide a promising immunotherapeutic strategy to induce potent adaptive anti-tumor responses.

2.3 FINAL REFLECTIONS AND FUTURE PERSPECTIVES

Harnessing the inherent potential of the immune system for the therapeutic benefit of cancer patients has long been the goal of intensive research in the field of cancer immunotherapy. As previously mentioned, active immunotherapy poses great challenges but holds greater potential with regards to obtaining durable responses in cancer patients. A plethora of pre-clinical and clinical studies suggest that activation of early innate immune responses appears to be a prerequisite for the induction of potent adaptive immunity with a long-term memory component. Thus, combinatorial treatment approaches targeting multiple components of the TME are more favorable. Therefore, the focus of this thesis has been to identify and evaluate novel targets in the TME, which can be modulated to induce the activation of anti-tumor immune responses and to ultimately constrict tumor growth and metastasis.

Paper I describes a mechanism regulating the interplay between MZMs and MZBs in the spleen, through the use of Abs to scavenger receptor MARCO. This study provides insight into how manipulating this interaction can affect Ag presentation and adaptive immune responses to foreign but also to self-Ags. It also identifies a novel tool to regulate macrophage activation, with implications for future immunotherapeutic approaches aiming to regulate innate immune responses or to increase protective immunity. More specifically, the use of anti-MARCO Ab triggers the release of extracellular ATP by marginal zone macrophages. In the TME, a shift towards a higher extracellular ATP concentration can be crucial as it drives anti-tumor responses. Extracellular ATP is taken up by purinergic P2X7 receptors on DCs, leading to inflammasome activation and increased production of pro-inflammatory cytokines IL-1β and IL-18 [363]. IL-1β together with Ag presentation leads to increased NK cell proliferation IFNγ-production and activation of $CD8^+$ tumoricidal T cells. ATP also induces macrophage maturation and M1 polarization, and increased secretion of IFNγ and IL-17 by T cells. In contrast, adenosine leads to reduced production of IL-12 and increased levels of IL-6, IL-8, IL-10, TGF-β and VEGF by DCs, reduced NK cell cytotoxicity, M2 macrophage polarization, decreased cytotoxic T cell proliferation and effector function, and finally increased generation of T_{regs}.

Paper II identifies scavenger receptor MARCO as a specific marker for the immunosuppressive M2 TAM phenotype. MARCO is expressed on TAMs in the TME of mammary carcinoma, melanoma and colon carcinoma tumor models. Use of Abs to target MARCO significantly inhibited tumor growth and metastasis, and increased survival in combination treatments with checkpoint therapy with anti-CTLA-4 Ab. This effect is attributed to reprograming of TAMs from an immunosuppressive M2 towards an immunostimulatory M1 phenotype characterized by the production of pro-inflammatory cytokines. This is in line with the findings of Paper I, and suggests that anti-MARCO Ab could use the same mechanism of ATP-release to stimulate the polarization of M2 TAMs to M1, thus relieving immunosuppression in the TME. Finally, it identifies MARCO as a clinically relevant target in highly invasive and metastatic subtypes of human breast cancer and melanoma. There, MARCO expression correlates with an M2 TAM gene profile and

increased expression of EMT-associated genes. EMT, as is further discussed in Paper III, is a process involved in the acquisition of migratory properties by tumor cells. This suggests that TAMs, and in particular MARCO-expressing TAMs, can be a link explaining the more invasive phenotypes of those cancers, but also provides a tangible target for immunotherapy. Further studies investigating the relevance of MARCO in other human cancers as well as elucidating the underlying mechanism of action and pathways involved in MARCO-signaling are warranted and will increase our understanding of its role in the TME. These will evaluate new combinatorial immunotherapeutic treatments and provide a solid basis for future translation of these findings into the clinical setting.

Paper III elucidates the mechanism through which TGF-β1, a major immunosuppressive cytokine in the TME and the driving force for EMT, regulates targeted lymphatic metastasis of breast cancer cells. TGF-β controls the CCR7/CCL21 chemotactic axis that guides tumor cells to metastasize through the lymphatic system in an EMT-dependent manner. This study highlights TGF-β1 as a candidate for targeted pharmacological inhibition in an approach to restrict lymphatic metastasis of tumor cells. As was already mentioned TGF-β1 is one of the major immunosuppressive cytokines produced by TAMs. Additionally, macrophages have been implicated in the induction of EMT in pancreatic tumor cells tumor cells and promote invasion and metastasis of mammary carcinoma cells by inducing an inflammatory signature that facilitates metastasis [364]. Although metastasis-promoting TAMs may be a different subtype from the immunosuppressive TAMs, combining the knowledge acquired from Paper II and III to treat cancer with anti-MARCO Ab in combination with molecular inhibitors of TGF-β may significantly abrogate metastatic disease.

Paper IV explores the use of DC-derived α-GalCer-loaded exosomes as a vaccination approach. This study demonstrates that $CD1d^+$ DC-derived exosomes expressing loaded with α-GalCer can be used as adjuvants and are potent inducers of iNKT, $CD8^+$ and $CD4^+$ T and B cell responses that ultimately restrict tumor growth. In a similar manner one could envisage the design of anti-MARCO Ab-carrying exosomes specifically targeting M2 TAMs, containing immunostimulatory receptors or microRNA125, -155 or -378, to induce M1 polarization. Overall, exosomes provide a flexible vehicle for delivery of ”messages” which can be customized to the disease setting and the target of interest.

In conclusion, the immunotherapy approaches described in this thesis provide new insight into mechanisms of innate and adaptive immune regulation, identify novel targets to reprogram the immunosuppressive TME, elucidate genetic programs driving lymphatic metastasis, as well as harness endogenous cellular products and amplify their modulatory capacity. Altogether, this thesis aims to contribute with novel insight into the inner workings of several components of our immune system, which can be used in the design of future approaches to cancer immunotherapy.

3 POPULAR SCIENTIFIC SUMMARY

English

The term cancer was coined by the Father of Medicine, the Greek physician Hippocrates of Kos (460-370 BC), who first used the words "karkinos" and "karkinoma" to describe tumors. In greek, the word karkinos means crab and refers to the similarity observed between the branch-like spread of tumors and the shape of a crab. Although primary cancers can be treated by several different means, it is the metastatic cancer disease that is the ultimate elusive culprit that makes cancer an incurable disease. However, recent advances in the field of cancer immunotherapy, in particular, have lead to new treatment that awaken the patient's own immune system to fight off cancer.

The immune system is a complex network of cells and molecules that are responsible for protecting us against pathogens. In the case of an invasion the immune system recruits specialized cells to the site of invasion in order to mount a defense response to combat the invader. This phenomenon is known as inflammation. Cancers can also mobilize a similar type of inflamamtion, recruiting immune cells to sites of tumor growth. However, immune cells in the cancer microenvironment fail to recognize cancer as a threat and instead are sequestered by the cancer to support its growth and metastasis. Strategies to awaken the body's own immune system to fight cancer are therefore of great importance. This thesis focuses on identifying new targetd to activate the immune system to combat cancer.

Paper I, studies two types of immune cells, the macrophages and the B cells, located in the spleen. Macrophages (form the greek: makros large and phagein eat) are the big eaters of our immune system. They are responsible for taking up and discarding bacteria, dead cells, and metabolic biproducts form our blood circulation which would otherwise harm us if they reached large amounts. They are therefore found in strategy location in our bodies where the blood flows through and can be filtered by macrophages, such as the spleen. This janitorial function is mediated, amongst other, by the so called scavenger receptor MARCO, a molecule found on the surface of macrophages. B cells are a type of immune cells that have the capacity to recognize pathigens and generate specific responses against them by producing target-seeking molecule, antibodies. MARCO can however be falsely attacjed by target seeking molecules, antibodies, as is the case for example in the autoimmune disease SLE. This study shows that, simulating SLE by administering antibodies against MARCO has a profound negative effekt on the function of B cells and restricts the specific responses they generate agains pathogens. This may provide an explanation as to why SLE patients suffer from recurrent infections.

While macrophages have an important defense and janitorial fucntion, they are also the major culprits of inflammation that drives cancer growth and metastasis. In paper II, we found that some macrophages, in the tumor bed of breast and colon cancer as well as melanoma (skin cancer), express MARCO. Thus, we used the same target-seeking molecules, antibodies, as before to target MARCO on macrophages that are present in the tumor, in order to re-activate them against the tumors. Interestingly, antibodies found a subpopulation of macrophages in the tumor bed. Serial treatments with MARCO antibodies, as well as with antibodies that activate other immune cells, lead to the re-education of macrophages. This way they started producing molecules which activated other immune cells against the tumor, instead of suppressing them. This lead to significanlty less tumor growth and metastasis. The presence of MARCO is associated with the most aggressive types of breast cancer and melanoma, which cause metastasis and are hard to treat. This study shows that MARCO is a target that is present in three different types of cancer and that antibodies can be used as a specific tools to target MARCO-macrophages in the tumor. This may provide a useful future cancer therapy.

Cancer cells can grow locally or travel thorugh the blood and lymphatic system to distal sites of the body where they seed metastases. Not all cancer cells become metastatic, but the ones

that do have to activate a particular gene program in order to gain the capacity to migrate. This gene programis normally active during fetal development, when cells need to travel to different site of the developing foetus in order to form different organs, and is known as epithelia-mesenchymal transition (EMT). The wrongfull activation of EMT is attributed to the production of the signaling molecule TGF-β by cancer cells and other sequestered immune cells, such as macrophages, in the tumor bed. In paper III we studies the effect of TGF-β on breast cancer cells. Indeed breast cancer cells that only grew locally, changed form and gained a migratory capacity because of TGF-β. Interestingly the mickicked another type of immune cell, the dendritic cells, whose primary role is to take up pathogens and travel through the lymphatic system to major immune cell hubs, where they present the pathogen and activate specific immune responses against it. In a similar way, TGF-β activating of EMT, lead to the guided entry of cancer cells into the lymphatic system and gave them access to travel to distal sites of the body where they could seed new tumors.

Exosomes are small vesicles that are released by many different type of cells in our bodies. They act as a means of communication, transporting various molecular messages between different cells. Because of this they can be used to load specific/custom molecules which can target specific immune cells and different types of immune repsonses. In paper IV, we used exosomes generated from dendritic cells, which are known to present foregn molecules to activated immune responses. These were loaded with α-GalCer, an activator of a type of immune cell known as NKT cells. Vaccination using exosomes loaded with α-GalCer lead to a more efficient activation of NKT cells, but also several other immune cells, than what α-GalCer alone would have caused. This was particularly important in the tumor setting, where exosomes loaded with α-GalCer as well as a molecule expressed by the tumor, activated immune cells against the tumor leading to less tumor growth.

In summary, this thesis identifies new targets in the tumor microenvironment, which can be used to improve the design of future cancer therapeutics for the benefit of cancer patients.

Svenska

Ordet cancer kommer ursprungligen ifrån läkekonstens fader, den grekiska läkaren Hippokrates av Kos (460-370 f.Kr.), som först använde termerna "karkinos" och "karkinoma" för att beskriva tumörer. Det grekiska ordet karkinos betyder kräfta och hänvisar till likheten som observerats mellan spridningsgrenarna i tumörer och formen på en kräfta. Även om primärtumörer kan behandlas på flera olika sätt, är det den metastaserande cancersjukdomen som är den ultimata skyldige som gör cancer till en obotlig sjukdom. Men de senaste framstegen i forskningsfältet om cancer immunterapi, i synnerhet, har lett till nya behandlingar som aktiverar patientens egna immunförsvar till att kämpa mot cancer.

Immunsystemet är ett komplext nätverk av celler och molekyler som är ansvariga för att skydda oss mot patogener. När en invasion inträffar rekryterar immunsystemet specialiserade celler till platsen för invasionen sker för att att bekämpa inkräktaren. Detta fenomen kallas inflammation. Cancer kan också mobilisera en liknande typ av inflamamtion, dvs rekrytera immunceller till platser av tumörtillväxt. Men immunceller i cancer mikromiljön lyckas inte känna igen tumören som ett hot och istället angrips de av tumören för att stödja dess tillväxt och metastasering. Strategier för att väcka kroppens egna immunsystem för att bekämpa cancer är därför av stor betydelse. Denna avhandling fokuserar på att identifiera nya målmolekyler som kan aktivera immunsystemet för att bekämpa cancer.

I studie I, studeras två typer av immunceller, makrofager och B-celler, som befinner sig i mjälten. Makrofager (från grekiska: makros stor och phagein äta) är storätarna i vårt immunsystem. De är ansvariga för att ta upp och gör sig av med bakterier, döda celler och metabola biprodukter från blodcirkulationen som annars skulle skada oss om de uppnådde stora mängder. Därför befinner makrofager sig i strategiska positioner i våra kroppar där blodet flyter igenom och kan filtreras, såsom i mjälten. Denna vaktmästerifunktion medieras,

bland annat, av den så kallade scavenger receptor MARCO, en molekyl som finns på makrofagernas yta. B celler är en typ av immunceller som har kapaciteten att känna igen patogener och generera specifika svar mot dem genom att producera målsökande molekyler, antikroppar. MARCO kan dock felaktigt bli attackerad av dessa antikroppar, vilket är fallet till exempel i den autoimmuna sjukdomen SLE. Denna studie visar att simulering av SLE genom att administrera antikroppar mot MARCO har en stark negativ effekt på B cellers funktion och begränsar det specifika svaret som genereras mot patogener. Detta kan ge en förklaring till varför SLE-patienter lider av återkommande infektioner.

Medan makrofager har en viktig försvars- och vaktmästerifucntion, de är också de stora skyldiga till inflammationen som driver cancertillväxt och metastasering. I studie II, fann vi att vissa makrofager, i tumörbädden av bröst- och tjocktarmscancer, samt melanom (hudcancer), uttrycker MARCO. Därför använde vi samma antikroppar som tidigare, mot MARCO på makrofager som finns i tumören, för att återaktivera dem mot tumörer. Intressant nog fann dessa antikroppar en subpopulation av makrofager i tumörbädden. En serie behandlingar med MARCO antikroppar, liksom med antikroppar som aktiverar andra immunceller, ledde till omskolning av makrofagerna. På så sätt började de producera molekyler som aktiverade andra immunceller mot tumören, i stället för att undertrycka dem. Detta ledde till significant mindre tumörtillväxt och metastas. Närvaron av MARCO är förknippad med de mest aggressiva typer av bröstcancer och melanom, som orsakar metastaser och är svåra att behandla. Denna studie visar att MARCO är ett mål som finns i tre olika typer av cancer och att antikroppar riktade mot denna kan användas som särskilda verktyg för att omvandla MARCO-makrofager i tumören. Detta kan därför bli en användbar framtida cancerterapi.

Cancerceller kan växa lokalt eller färdas genom blodet och det lymfatiska systemet till avlägsna ställen i kroppen där de utsäder metastaser. Inte alla cancerceller blir metastaserande, men de som gör det måste aktivera ett särskilt genetiskt program för att få förmågan att migrera. Detta genetiska programm är normalt aktivt under fosterutvecklingen, då celler behöver resa till olika platser i det växande fostret för att bilda olika organ, och är känt som epitel-mesenkymal övergång (EMÖ). Den felaktiga aktivering av EMÖ i cancer är beroende av produktionen av signaleringsmolekylen TGF-β utav cancerceller och andra sekvestrerade immunceller, såsom makrofager, i tumörbädden. I studie III, studerar vi effekten av TGF-β på bröstcancerceller. Vi observerade att bröstcancerceller som endast växte lokalt, ändrade form och fick en vandrande kapacitet på grund av TGF-β. Intressant nog, härmade de en annan typ av immunceller, de dendritiska cellerna, vars främsta roll är att ta upp patogener och färdas genom det lymfatiska systemet till stora immuncellsnav, där de presenterar patogener och aktiverar specifika immunsvar mot dem. På ett liknande sätt, leder TGF-β aktiverad EMÖ, till en guidad migrering av cancerceller som ger inträde till lymfsystemet och ger dem möjlighet att färdas till avlägsna ställen i kroppen där de kan utsäda nya tumörer.

Exosomer är små blåsor som frigörs av många olika typer av celler i våra kroppar. De fungerar som ett kommunikationsmedel, som transporterar olika molekylära meddelanden mellan olika celler. På grund av detta kan de användas för att laddas med specifika molekyler som kan riktas mot särskilda immunceller och ge upphov till olika typer av immunsvar. I studie IV, använde vi exosomer som genereras från dendritiska celler, som är kända för att presentera främmande molekyler för att aktivera immunsvar. Dessa laddades med α-GalCer, en molekyl som aktiverar en typ av immunceller som kallas NKT celler. Vaccination med hjälp av exosomer laddade med α-GalCer ledde till en mer effektiv aktivering av NKT celler, men även av flera andra immunceller, än vad α-GalCer enbart skulle ha orsakat. Detta var särskilt viktigt i tumörmiljön, där exosomer laddade med α-GalCer samt en molekyl som uttrycks av tumören, aktiverade immunceller mot tumören och ledde till mindre tumörtillväxt.

Sammanfattningsvis, identifierar denna avhandling nya mål i cancermikromiljön som kan användas för att förbättra framtida cancerterapier till förmån av cancerpatienter.

Ελληνικά

Η λέξη καρκίνος ορίστηκε από τον Πατέρα της Ιατρικής, τον Έλληνα ιατρό Ιπποκράτη τον Κώο (460-370 π.Χ.), ο οποίος χρησιμοποίησε για πρώτη φορά τους όρους "καρκίνος" και "καρκίνωμα" για να περιγράψει όγκους. Ο συμβολισμός βασίζεται στην ομοιότητα που παρατηρείται μεταξύ των διακλαδώσεων εξάπλωσης των όγκων και του σχήματος του καρκίνου. Αν και οι πρωτογενείς όγκοι μπορούν να αντιμετωπιστούν με αρκετούς διαφορετικούς τρόπους, η μεταστατική ασθένεια του καρκίνου είναι ο τελικός ένοχος που καθιστά τον καρκίνο ανίατη νόσο. Ωστόσο, πρόσφατες πρόοδοι ειδικά στον τομέα της ανοσοθεραπείας του καρκίνου, έχουν οδηγήσει στην ανάπτυξη νέων θεραπειών που ξυπνούν το ανοσοποιητικό σύστημα του ασθενούς έτσι ώστε αυτό να καταπολεμήσει τον καρκίνο.

Το ανοσοποιητικό σύστημα είναι ένα πολύπλοκο δίκτυο κυττάρων και μορίων που είναι υπεύθυνα για την προστασία μας έναντι των παθογόνων. Σε περίπτωση παθογόνας εισβολής το ανοσοποιητικό σύστημα στρατολογεί εξειδικευμένα κυττάρα στην συγκεκριμένη τοποθεσία προκειμένου να ενεργοποιήσει μια ανοσοαπόκριση για την καταπολέμηση του εισβολέα. Αυτό το φαινόμενο είναι γνωστό ως φλεγμονή. Ο καρκίνος μπορεί επίσης να κινητοποιήσει έναν παρόμοιο τύπο φλεγμονής, προσελκύοντας κύτταρα του ανοσοποιητικού συστήματος στους τόπους ανάπτυξης του όγκου. Εντούτοις, τα κύτταρα του ανοσοποιητικού συστήματος στο μικροπεριβάλλον του καρκίνου αποτυγχάνουν να αναγνωρίσουν τον καρκίνο ως απειλή και αντίθετα χειραγωγούνται από τον καρκίνο έτσι ώστε να σθνεισφέρουν στην ανάπτυξη και μετάστασή του. Η ανάπτυξη στρατηγικών για την αφύπνιση του ανοσοποιητικού συστήματος του οργανισμού για την καταπολέμηση του καρκίνου είναι, επομένως, ύψιστης σημασίας. Η συγκεκριμένη διατριβή εστιάζει στον εντοπισμό νέων στόχων για την ενεργοποίηση του ανοσοποιητικού συστήματος ως προς την καταπολέμηση του καρκίνου.

Η πρώτη μελέτη, εστίαζεται σε δύο τύπους κυττάρων του ανοσοποιητικού συστήματος, τα μακροφάγα και τα Β λεμφοκύτταρα, που βρίσκονται στο σπλήνα. Τα μακροφάγα (από το: Μακρός και φαγείν) είναι αδηφάγα κύτταρα του ανοσοποιητικού συστήματος. Είναι υπεύθυνα για την ανάληψη και την απόρριψη βακτηρίων, νεκρών κυττάρων και μεταβολικών παραπροϊόντων από την κυκλοφορία του αίματος, που διαφορετικά θα μας έβλαπταν αν έφταναν μεγάλες ποσότητες. Ως εκ τούτου, βρίσκονται σε στρατηγικές τοποθεσίες, όπου το αίμα εισέρχεται και διηθείται από τα μακροφάγα, όπως ο σπλήνας. Αυτή η καθαρτική λειτουργία των μακροφάγων εξασκείται, μεταξύ άλλων, από το λεγόμενη υποδοχέα-καθαριστή MARCO, που βρίσκεται στην επιφάνεια των κυττάρων αυτών. Τα Β λεμφοκύτταρα είναι ένας τύπος κυττάρων του ανοσοποιητικού που έχουν την ικανότητα να αναγνωρίζουν και να παράγουν ειδικές αποκρίσεις ενάντια στα παθογόνα, κυρίως μέσω της παραγωγή αντισωμάτων. Ο MARCO μπορεί ωστόσο να προσδεθεί λανθασμένα από αντισώματα, όπως συμβαίνει για παράδειγμα στην αυτοάνοση ασθένεια του συστηματικού ερυθυματώδους λύκου (ΣΕΛ). Αυτή η μελέτη δείχνει ότι, η προσομοίωση του ΣΕΛ μέσω της χορήγησης αντισωμάτων εναντίον του MARCO, έχει σοβαρές αρνητικές επιπτώσεις στη λειτουργία των Β λεμφοκυττάρων και περιορίζει τις ειδικές ανοσοαποκρίσεις που εξασκούν ενάντια στα παθογόνα. Αυτό μπορεί να αποτελεί μια εξήγηση ως προς το γιατί οι ασθενείς με ΣΕΛ υποφέρουν από υποτροπιάζουσες λοιμώξεις.

Ενώ τα μακροφάγα έχουν σημαντική αμυντική και καθαριστική λειτουργία, είναι και οι κύριοι ένοχοι της φλεγμονής που οδηγεί στην ανάπτυξη και την μετάσταση του καρκίνου. Στη δεύτερη μελέτη, ανακαλύψαμε ότι ορισμένα μακροφάγα, στον καρκίνο του μαστού και του παχέος εντέρου, καθώς και του μελανώματος (καρκίνου του δέρματος), εκφράζουν τον υποδοχέα MARCO. Έτσι, χρησιμοποιήθηκαν τα ίδια αντισώματα όπως και στην προηγούμενη μελέτη για την στόχευση του MARCO σε μακροφάγα εντός του όγκου, ώστε να ενεργοποιήθουν κατά αυτού. Τα αντισώματα στοχεύουν έναν υποπληθυσμό μακροφάγων

στο καρκινικό περιβάλλον. Σειριακές θεραπείες με αντισώματα κατά του MARCO, καθώς επίσης και με αντισώματα που ενεργοποιούν άλλα κύτταρα του ανοσοποιητικού, οδηγούν στην επανεκπαίδευση των μακροφάγων. Έτσι επάγουν την παραγωγή μορίων τα οποία ενεργοποιούν άλλα κύτταρα του ανοσοποιητικού έναντι του όγκου, αντί να τα καταστέλλουν. Αυτό οδηγεί σε σημαντικά μικρότερη ανάπτυξη και μετάσταση του καρκίνου. Η παρουσία του MARCO συνδέεται με τις πιο επιθετικές μορφές καρκίνου του μαστού και του μελανώματος, οι οποίες προκαλούν μετάσταση. Αυτή η μελέτη αποδεικνύει ότι ο υποδοχέας MARCO είναι ένας νέος μοριακός στόχος, σε τρεις διαφορετικούς τύπους καρκίνου και ότι τα αντισώματα μπορούν να χρησιμοποιηθούν ως εργαλεία για την ειδική στόχευση MARCO-μακροφάγων στον καρκίνο. Ως εκ τούτου, η προκείμενη στρατιγική στόχευσης μπορεί μελλοντικά να αποτελέσει μια χρήσιμη θεραπεία κατά του καρκίνου.

Τα καρκινικά κύτταρα μπορούν να αναπτυχθούν σε τοπικό επίπεδο ή να ταξιδέψουν μέσω του αίματος και του λεμφικού συστήματος σε απόμακρα σημεία του σώματος όπου δημιουργούν μεταστάσεις. Τα μεταστατικά καρκινικά κύτταρα, έχουν ενεργοποιήσει ένα συγκεκριμένο γονιδιακό πρόγραμμα προκειμένου να αποκτήσουν την ικανότητα να μεταναστεύσουν. Το συγκεκριμένο γονιδιακό πρόγραμμα ενεργοποιείται υπό κανονικές συνθήκες κατά τη διάρκεια της εμβρυϊκής ανάπτυξης, όταν τα κύτταρα ταξιδεύουν σε διαφορετικές τοποθεσίες του αναπτυσσόμενου εμβρύου, προκειμένου να σχηματίσουν διάφορα όργανα, και είναι γνωστό ως επιθηλιακή-μεσεγχυματική μετάβαση (ΕΜΜ). Η λανθασμένη ενεργοποίηση του ΕΜΜ αποδίδεται στην παραγωγή του μορίου-σηματοδότη TGF-β από τα καρκινικά και από άλλα χειραγωγημένα κύτταρα του ανοσοποιητικού, όπως τα μακροφάγα στο καρκινικό μικροπεριβάλλον. Στην Τρίτη μελέτη, ερευνούμε την επίδραση του TGF-β στα κύτταρα του καρκίνου του μαστού. Πράγματι, τα καρκινικά κύτταρα που αναπτύχθηκαν μόνο σε τοπικό επίπεδο, άλλαξαν μορφολογία και απέκτησαν μεταναστευτικές ικανότητες λόγω του TGF-β. Επίσης, ενστερνίζονται χαρακτιριστικά ενός άλλου τύπου κυττάρων του ανοσοποιητικού, των δενδριτικών κυττάρων, πρωταρχικός ρόλος των οποίων είναι η ανάλυψη παθογόνων και η μετακίνηση μέσω του λεμφικού συστήματος σε κομβικά σημεία του ανοσοποιητικού, όπου παρουσιάζουν τα παθογόνα και να ενεργοποιούν ειδικές ανοσοαποκρίσεις. Με παρόμοιο τρόπο, ο TGF-β οδηγήσει στην είσοδο των καρκινικών κυττάρων στο λεμφικό σύστημα, δίνοντάς τους πρόσβαση σε απόμακρες τοποθεσίες του σώματος όπου μπορούν να συνεισφέρουν στη δημιουργία νέων όγκων.

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