Immunosuppressive Myeloid Cells in Breast Cancer and Sepsis

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2014

Immunosuppressive Myeloid Cells in Breast Cancer and Sepsis

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LUND
UNIVERSITY

DOCTORAL DISSERTATION
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To be defended at the main lecture hall, Pathology building, Skåne University Hospital, Malmö on Friday 7th of March at 9.15 a.m.

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Organization	Document name
LUND UNIVERSITY Faculty of Medicine Department of Laboratory Medicine Malmö Center for Molecular Pathology	DOCTORAL DISSERTATION
	Date of issue: 2014-03-07
Author(s): Caroline Bergenfelz	Sponsoring organization

Title and subtitle: Immunosuppressive myeloid cells in breast cancer and sepsis

Abstract

Immune cells play paradoxical roles in cancer progression. On one hand, the immune system protects us against tumor development by recognizing and eliminating cancerous cells. On the other hand, tumor-associated immune cells can contribute to tumor progression by secreting growth factors as well as immunosuppressive, pro-angiogenic and/or pro-metastatic mediators.

In this thesis we identified a factor (Wnt5a) that may be involved in skewing immune responses towards immunosuppressive, tumor promoting immune cell populations. In a pro-inflammatory setting (*i.e.* in the presence of exogenous pathogen-associated molecular patterns; PAMPs, or endogenous damage-associated molecular patterns; DAMPs), Wnt5a promoted the generation of immunosuppressive monocytes ($CD14^{+}HLA\text{-}DR^{low/-}Co\text{-}receptor^{low/-}$). This was at the expense of generation of pro-inflammatory macrophages (M1). In addition, Wnt5a inhibited monocyte to dendritic cell differentiation (Mo-mDC). When co-injecting monocytes from healthy blood donors with MCF-7 or MDA-MB-231 breast cancer cells (luminal A and basal-like, respectively) into immunodeficient mice, monocytes promoted the generation of an activated tumor stroma and were preferentially recruited to basal-like tumors. Furthermore, monocytes from breast cancer patients were affected early during the disease, gradually becoming reprogrammed towards a novel population of monocytic myeloid-derived suppressor cells (Mo-MDSCs). The gene-expression profile of cancer-derived monocytes was remarkably similar to that of reprogrammed immunosuppressive monocytes from patients with gram-negative sepsis. This suggests that Mo-MDSCs may be generated in a similar manner in cancer and sepsis (by reprogramming of monocytes towards an immunosuppressive phenotype). We finally propose that Mo-MDSCs and granulocytic MDSCs are preferentially induced by different PAMPs.

Altogether, we conclude that myeloid cells are skewed towards an immunosuppressive and tissue remodeling phenotype early during breast cancer. This resembles the situation during severe infections such as sepsis and most likely has a positive impact on tumor progression.

Key words: Monocytes, myeloid-derived suppressor cells, breast cancer, sepsis, tumor microenvironment, tumor stroma, immunosuppression, reprogramming, DAMP, PAMP

Classification system and/or index terms (if any)

Supplementary bibliographical information	Language: English
ISSN and key title: 1652-8220	ISBN: 978-91-87651-47-2
Recipient's notes	Number of pages / Price
	Security classification

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Cover by Charlotte Bergenfelz and Clara Bergenfelz.

Faculty of Medicine, Department of Laboratory Medicine, Lund University, Sweden
ISBN 978-91-87651-47-2
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2014

Till Clara och Charlotte

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

I. Wnt5a induces a tolerogenic phenotype of macrophages in sepsis and breast cancer patients.

Caroline Bergenfelz, Catharina Medrek, Elin Ekström, Karin Jirström, Helena Janols, Marlene Wullt, Anders Bredberg and Karin Leandersson
J Immunol, 2012 Jun 1; 188(11):5448-58

II. Wnt5a inhibits human monocyte-derived myeloid dendritic cell generation.

Caroline Bergenfelz, Helena Janols, Marlene Wullt, Karin Jirström, Anders Bredberg and Karin Leandersson
Scand J Immunol 2013 Aug; 78(2):194-204

III. Cancer associated fibroblast CXCL16 attracts monocytes to promote stroma formation in triple-negative breast cancers specifically.

Roni Allaoui, **Caroline Bergenfelz**[#], Sofie Mohlin[#], Catharina Medrek[#], Sven Påhlman, Lisa Rydén, Janne Malina, Christer Larsson and Karin Leandersson
Manuscript. [#] These authors contributed equally.

IV. Systemic monocytic-MDSCs are generated from monocytes and correlate with disease progression in breast cancer patients.

Caroline Bergenfelz, Anna-Maria Larsson, Kristina Aaltonen, Sara Jansson, Kristoffer von Stedingk, Sofia Gruvberger-Saal, Helena Jernström, Helena Janols, Marlene Wullt, Anders Bredberg, Lisa Rydén and Karin Leandersson
Submitted manuscript.

V. A high frequency of myeloid-derived suppressor cells in sepsis patients, with the granulocytic subtype dominating in gram-positive cases.

Helena Janols, **Caroline Bergenfelz**, Anna-Maria Larsson, Lisa Rydén, Sven Björnsson, Sabina Janciauskiene, Marlene Wullt, Anders Bredberg and Karin Leandersson.
Manuscript.

Paper not included in this thesis

Heterogeneity among septic shock patients in a set of immunoregulatory markers.

Helena Janols, Marlene Wullt, **Caroline Bergenfelz**, Sven Björnsson, Helena Lickei, Sabina Janciauskiene, Karin Leandersson and Anders Bredberg.

Eur J Clin Microbiol Infect Dis. 2013

List of Abbreviations

APC	Antigen presenting cell
ARG1	Arginase 1
BRCA	Breast cancer gene
CAF	Cancer associated fibroblast
CaMKII	Calmodulin-dependent kinase II
CARS	Compensatory anti-inflammatory response syndrome
CCL	Chemokine (C-C) motif ligand
CD	Cluster of differentiation
CIS	Carcinoma *in situ*
CKI	Casein kinase
CREB	cAMP responsive element
CTL	Cytotoxic T lymphocyte
CXCL	Chemokine (C-X-C) motif ligand
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DVL	Dishevelled
ECM	Extracellular matrix
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
FZD	Frizzled
G-MDSC	Granulocytic MDSC
GSK3β	Glycogen synthase kinase-3 beta
HER2	Human epidermal growth factor receptor 2
HLA	Human leukocyte antigen
HMGB1	High mobility group box 1
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRF	Interferon regulatory factor
JNK	c-Jun N-terminal kinase
LN	Lymph node
LPS	Lipopolysaccharide
M	Occurrence of metastases
M1	Pro-inflammatory macrophage
M2	Anti-inflammatory macrophage
MAPK	Mitogen activated protein kinase
MDC	Myeloid DC
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
Mo-M	Monocyte derived macrophage
Mo-mDC	Monocyte derived myeloid DC
Mo-MDSC	Monocytic MDSC
N	Number of axillary lymph nodes
NFκB	Nuclear factor kappa B
NHG	Nottingham histological grade
NK	Natural killer cell
NKT	Natural killer T cell
NO	Nitric oxide
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell

PCP	Planar cell polarity
PDC	Plasmacytoid DC
PKC	Protein kinase C
PLC	Phospholipase C
PR	Progesterone receptor
PRR	Pattern-recognition receptor
ROR	Receptor tyrosine kinase-like orphan receptor
ROS	Reactive oxygen species
RYK	Related to receptor tyrosine kinase
SERM	Selective estrogen receptor modulator
SIRS	Systemic inflammatory response syndrome
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
T	Size of primary tumor
TAA	Tumor associated antigen
TDLU	Terminal duct lobular unit
TDSF	Tumor-derived soluble factor
TGF	Transforming growth factor
Th	T helper cell
TLR	Toll-like receptor
TN	Triple-negative
TNF	Tumor necrosis factor
Treg	Regulatory T cell
VEGF	Vascular endothelial growth factor
Wnt	Wingless-related MMTV integration site

A Brief Introduction to Cancer

"Growth for the sake of growth is the ideology of the cancer cell"
-Edward Abbey, The Journey Home 1977

Cancer. Few diseases have affected and intimidated people more throughout the history. One of the earliest documentations of cancer is an Egyptian papyrus scroll from 3000 BC reporting tumors of the breast and stating "there is no treatment", which, for many millennia, was essentially true.

In the dawn of oncology, cancer was believed to be one malignancy. Today, however, we use "cancer" as a generic term for over 100 distinct diseases, all of which share a common feature: uncontrolled division of abnormal cells. In some respects, the progression from a normal cell to a malignant cancer cell could be compared to a Darwinian selection, in which the common denominator is spontaneous genetic mutations. All cells are subjected to DNA replication errors during cellular division, resulting in mutations and genetic aberrations. The risk of developing mutations is greatly enhanced by environmental factors such as UV light or exposure to chemicals. Most mutations are neutral or "silent", however, some may provide advantage in adapting to a changing environment, and others may contribute to malignant progression if not corrected by DNA repair mechanisms. Cells become cancerous after accumulating mutations that provide a selective growth advantage for the cells [1, 2]. The number of mutations required for malignant progression is not firmly established and most likely varies. In 2000, Hanahan and Weinberg proposed that most, if not all, cancers share at least six essential traits or hallmarks: self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless reproductive potential, sustained angiogenesis and invasive and metastatic potential [1].

In Sweden, the most common forms of cancer include prostate-, breast-, skin- and colorectal carcinomas [3, 4]. Despite advances in understanding the biology of cancer and development of novel treatment strategies, cancer causes approximately eight million deaths annually worldwide and 22 000 in Sweden [3, 5]. It is estimated that one in three will develop some form of cancer during their lifetime [4]. Unfortunately, cancer incidence is increasing which is in part due to the aging population and better screening methods [4]. Although the mortality rate is decreasing, cancer is the second

leading cause of death in Sweden after cardiovascular diseases. This illustrates the grave need for novel and more individualized treatments.

Traditionally, cancer has been regarded a disease of genes, but during the last decades this tumor cell-centric view has shifted towards a more context dependent process involving complex interactions between tumor cells and cells of the tumor microenvironment. In 2011 Hanahan and Weinberg published an updated version of the hallmarks of cancer, including genome instability and mutation, deregulation of cellular metabolism as well as two hallmarks highly associated with the tumor microenvironment: tumor promoting inflammation and evasion of immune cell-mediated destruction [2]. Indeed, non-malignant cells greatly influence tumor progression in multiple ways such as providing scaffolding, mitogenic and/or pro-angiogenic signals to the growing tumor [2, 6]. Many of these tumor-promoting factors are produced by tumor-associated immune cells rather than the malignant cells themselves. *In this thesis*, the overall aim was to further elucidate the intricate interplay between breast cancer cells and cells of the tumor microenvironment, with specific focus on the tumor-associated myeloid cells. It is well known that the immune cells located within the tumor area are either immature or immunosuppressive, thus dampening immune reactions against developing tumors [7]. Many studies have aimed to elucidate the mechanisms behind this phenomenon and although some progress has been made, many factors involved in the tumor-induced immunosuppression are still unknown.

Breast Cancer

"If thou examinest a man having tumors on his breast /---/ There is no cure"
-Edwin Smyth Papyrus 3000 BC

Epidemiology and Etiology

Breast cancer is the most common form of malignancy in women, accounting for 30% of all cancer diagnoses in Sweden [4]. Between 15 and 20 women are diagnosed with breast cancer daily, altogether approximately 8300 cases annually, and the incidence is increasing [3, 4]. Currently, the lifetime risk of developing breast cancer is approximately 10% in Sweden. Despite the increasing incidence, breast cancer related mortality is decreasing and the relative five-year survival rate has improved from 60% to almost 90% since the 1960's [4, 8]. This is generally believed to be due to the employment of new screening techniques, such as mammography, as well as better treatment strategies [4].

As with all cancers, the etiology of breast cancer is multifactorial, including age, hormonal factors, genetic predisposition, infectious agents, smoking and diet. Among these risk factors, hormone exposure is considered of highest relevance. In addition to the previously mentioned factors, it has become apparent that early menarche, late menopause, late first birth, oral contraceptives and hormonal replacement therapy all increase the risk of breast cancer [9].

Although the vast majority of breast cancers arise sporadically it is believed that approximately 5-10% of all cases are hereditary with inactivating mutations of the tumor suppressor genes *BRCA1* and *BRCA2* accounting for 30-40% of these familial cases [10, 11]. Mutations in these genes generally disable DNA repair and render the individual more prone to acquiring additional mutations that may promote breast cancer. In rare cases, hereditary breast cancer may also be due to mutations in the well-known tumor suppressor genes *TP53* and *PTEN* and many are yet of unknown origin [12]. Patients with specifically *BRCA1* mutations often develop triple-negative

breast cancer (*i.e.* negative for estrogen receptor, progesterone receptor and without HER2 amplification, see below) early in life and are correlated with worse prognosis [12, 13].

Breast Cancer Progression

A normal breast consists of glandular tissue as well as surrounding supportive tissue. The supportive tissue is composed of connective- and adipose tissues, which provide scaffolding and structure. The functional entity of the breast (the terminal duct lobular units; TDLUs) is responsible for the production and transportation of milk during lactation [14]. Both ducts and lobules consist of a polarized luminal epithelial cell layer, a surrounding layer of contractile myoepithelial cells and a basement membrane separating the epithelial cells from the supporting stromal cells. Figure 1 shows a schematic illustration of the breast.

The progression from normal epithelial cells to carcinoma cells with invasive potential can be simplified into a sequential series of events, the first of which being benign alterations of the normal duct (Figure 2). The majority of these alterations arise in the luminal epithelia of the TDLUs [15]. Upon acquisition of additional mutations the benign lesion will progress into premalignant states called atypical hyperplasia and carcinoma *in situ* (CIS) [16, 17]. During this phase, abnormal cells accumulate within the lumen of the duct or alveoli yet the cells remain within the basement membrane. Ductal carcinoma *in situ* (DCIS) accounts for approximately 20% of all breast cancers detected by mammography [17, 18]. In some cases, the carcinoma *in situ* further progresses into invasive breast cancer, with the associated disruption of the basement membrane. At this stage, the breast cancer may metastasize to regional lymph nodes or advance to form distant metastases (primarily in the lungs, bone, liver and brain)[17].

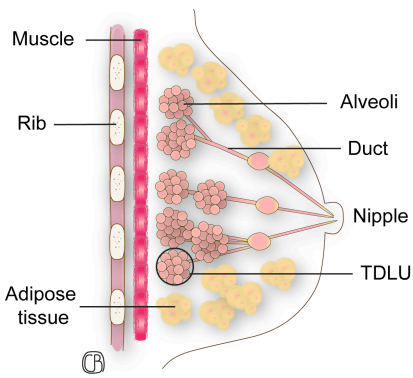

Figure 1. A schematic illustration of the normal breast. The breast gland consists of a branched ductal network extending from the nipple into the breast tissue, ending in the terminal duct lobular units (TDLUs). The TDLUs are composed of alveoli and represent the functional units of the breast.

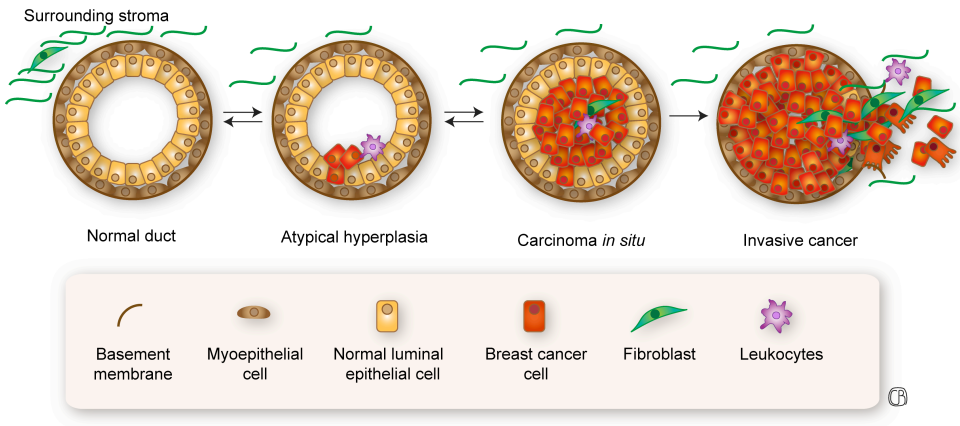

Figure 2. An overview of the main steps in breast cancer progression. The normal ducts are highly organized and surrounded by a basement membrane as well as normal stroma. During atypical hyperplasia and carcinoma *in situ*, neoplastic cells proliferate and begin to fill up the lumen as well as recruit leukocytes to the site. Eventually, the carcinoma *in situ* may progress to invasive cancer, which is characterized by disruption of the basement membrane as well as potential to metastasize to lymph nodes or distant organs.

Breast cancer diagnosis is generally preceded by the patient detecting a solid lump in the breast or by detection of an anomaly during mammography screening. Final diagnosis is subsequently confirmed by an additional mammogram or magnetic resonance imaging (MRI) of the breast and biopsy [11]. Due to the heterogeneity of the disease, combinations of histopathological and immunohistochemical features as well as assessment of grade and stage are evaluated in order to determine the likelihood of progressive disease and response to chemotherapy [19].

Breast Cancer Classification

Histological classification, grade and staging

Based on the growth pattern of the tumor, breast carcinomas can be divided into either ductal or lobular carcinoma, constituting approximately 75 and 15% of all

breast cancer cases, respectively. In addition, several other minor subgroups have been identified, including medullary, mucinous, papillary and inflammatory breast cancer [11, 15]. It should be noted that this histological classification is not intended to reflect the origin of the carcinoma, but is rather an assessment of the morphological and cytological features of the cells [15, 20].

Generally, histological classification has low prognostic and/or predictive significance, however, the *Nottingham Histological Grade* (NHG) is routinely used at diagnosis to predict disease aggressiveness [21, 22]. In essence, poorly differentiated, irregular and proliferating cells correlate with the aggressiveness of the tumor and worse prognosis for the patient [22].

The *TNM staging* system is utilized to assess the progression of the breast cancer and to predict the clinical outcome. By combining three important prognostic factors; tumor size (T), lymph node status (N) and distant metastases (M), breast tumors are classified into stages 0-IV, where stage 0 represents carcinoma *in situ*, stages I-III carcinoma *in situ* with lymph node involvement and stage IV metastatic disease [11]. The combined use of NHG with TNM staging systems constitutes a strong prognostic index, which is of extreme importance with regards to deciding treatment plans.

Hormone receptor status

In modern breast cancer diagnostics, all tumors are assessed for expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) using immunohistochemistry. The presence of these receptors confers both prognostic and predictive information. It has been estimated that ER is expressed in the majority of all invasive breast cancers, while in normal breast tissue ER expression is limited to a minority of individual cells [23]. The ER-positive breast tumors are dependent on estrogen and proliferate in response to the hormone [23]. Interestingly, estrogen has also been suggested to promote tumor growth in ER-negative tumors by recruiting ER-positive myeloid cells, highlighting the complexity of estrogen signaling in breast cancer pathogenesis [24]. Furthermore, overexpression of the receptor tyrosine kinase HER2 (encoded by *ERBB2/NEU* gene) may also promote tumor cell proliferation and is correlated with poor prognosis [11, 13].

Molecular subclassification

In the early 2000's, a novel classification system for breast cancer based on gene expression patterns was proposed [25-27]. Similar to the earlier classification protocols, gene expression patterns allowed for classification of tumors into two distinct clusters

with ER-positive and ER-negative features, respectively [25, 26, 28]. Furthermore, five to six major molecular subgroups could be identified within these clusters: Luminal A, luminal B, HER2-enriched, basal-like, claudin-low and normal breast-like [25, 26]. These subgroups are evident during early tumor progression, display distinct patterns of metastases and are highly associated with clinical outcome for the patient [26, 29-32]. Table 1 summarizes the main characteristics of the intrinsic subgroups of breast cancer.

Table 1. Characteristics of molecular breast cancer subtypes. [13, 15, 25, 26, 29, 31, 33]

Molecular subtype	ER, PR, HER2	Frequency	Characteristics	Outcome
Luminal A	ER+(high), PR+, low HER2	50-60 %	Low proliferation and histological grade. Express genes associated with luminal epithelial cells.	Good
Luminal B	ER+ (low), PR+, Low/variable HER2	12-20 %	High proliferation. Intermediate or high histological grade.	Intermediate (or poor)
Basal-like[1]	ER-, PR-, HER2-	10-20 %	High proliferation and histological grade. Aggressive clinical behavior. Express genes present in normal breast myoepithelial cells.	Poor
HER2	ER-, PR-, HER2+	10-15 %	High proliferation and histological grade. Aggressive clinical behavior.	Poor
Normal-like	ER-/+, PR unknown, HER2-	5-10 %	Low or intermediate proliferation and histological grade. Similar expression patterns as normal tissues.	Intermediate
Claudin-low	Most are ER-, PR-, HER2-	12-14 %	High proliferation and histological grade. Low expression of genes involved in tight junctions (e.g. claudins). Enrichment of EMT[2], immune response and stem cell-like features.	Poor

[1.] May occasionally also be called triple-negative breast cancer due to the lack of ER, PR and HER2 in 77% of cases [33].

[2.] EMT; Epithelial to mesenchymal transition.

Breast cancer recurrence and metastasis

Although local breast cancer can be cured, the risk of developing recurrence may persist for up to 20 years, however, the majority of breast cancer recurrences occur within 5 years of primary diagnosis [11]. Lymph node involvement as well as presence of distant metastases is currently the most important predictive factor for assessing the risk of recurrence [11]. There is also evidence that increased tumor size and histological grade may be indicative of disease recurrence [11]. Once the disease has disseminated to the lung, liver or bone, the prognosis is dramatically worsened and the 10-year survival for patients with distant recurrence is merely 9%, (56% for patients with local recurrence) [34]. This observation is in agreement with the estimation that over 90% of cancer mortality is due to metastases.

Breast Cancer Treatment

As previously stated, the most important prognostic factors used to evaluate treatment strategies are a combination of age, histological grade, TNM status, and hormone receptor status. Currently, treatment strategies include conventional surgery, radiotherapy and chemotherapy as well as endocrine therapies (e.g. selective estrogen receptor modulators; SERM, such as tamoxifen for ER-positive tumors) and targeted therapies (e.g. trastuzumab for HER2-enriched tumors) [11].

During the last decade, several novel targeted treatments and immunotherapeutic strategies against various forms of cancer have been developed. Some of these will be discussed in later sections.

The Tumor Microenvironment

Stroma. From Late Latin; "a mattress"

The Tumor Stroma

Today, it is well recognized that tumors are not homogenous entities of malignant cells, but rather consist of a mixture of neoplastic cells, entrapped or recruited normal (*i.e.* non-transformed) cells and extracellular matrix (ECM). In some tumors, cancerous cells comprise only 30% of the tumor mass [35]. Apart from the tumor cells, all surrounding cells and structures are referred to as the tumor stroma. Typically, the tumor stroma consists of a specific type of ECM as well as fibroblasts, endothelial cells, pericytes, adipocytes and immune cells [36]. These non-malignant cells of the tumor stroma were originally considered passive by-standers in tumor development and progression. This notion persisted throughout the 20th century, as highlighted by the omission of the tumor microenvironment from the widely cited "Hallmarks of cancer" [1]. However, apart from providing scaffolding to the growing tumor, an accumulating body of evidence acknowledges the importance of stromal cells in tumor progression. Indeed, many of the "hallmarks of cancer" can be attributed to the non-malignant cells of the tumor stroma [2, 7, 36].

While considered "normal", the stromal cells are generally affected by the growing tumor and display altered phenotype when compared to their counterparts in non-malignant tissues [37]. Whereas a normal microenvironment has been suggested to restrain malignant progression despite activation of oncogenes in epithelial cells, tumor- or environmentally-induced alterations in the stroma compartment have been shown to directly contribute to tumorigenesis [38-40]. Indeed, an activated or "reactive stroma" has been identified in several human malignancies including breast, colon and prostate cancer [37]. This reactive stroma involves activated non-malignant cells, such as fibroblasts and leukocytes, which promote tumor cell proliferation, invasiveness and metastatic potential [37, 41]. Furthermore, recent studies have indicated

that the molecular profiles of the tumor stroma, similar to the molecular subgroups defined for several tumors (based on the entire tumor tissue), correlate with tumor progression and outcome [26, 42-46].

Cancer Associated Fibroblasts

Fibroblasts are spindle shaped cells with extended processes embedded in the ECM and are the principal cell type involved in the ECM homeostasis [47]. In addition, activated fibroblasts, or cancer-associated fibroblasts (CAFs), are the predominant cell type in the tumor stroma [36, 47, 48]. Apart from producing ECM components, CAFs produce growth factors that affect the differentiation or proliferation of adjacent epithelial cells, regulate inflammatory processes and are involved in wound healing [47-52]. Studies on normal- *versus* tumor-associated stroma have suggested that normal fibroblasts would inhibit tumor growth whereas the activated fibroblasts present at the tumor site would promote tumor progression [37, 39, 47, 53].

CAFs have been suggested to resemble the fibroblasts associated with wound healing with regards to that they produce more ECM components and proliferate to a higher extent than normal fibroblasts [39, 47, 54]. In addition, CAFs as well as wound-associated fibroblasts regulate epithelial cell plasticity (e.g. induction of epithelial-to-mesenchymal transition; EMT and an invasive phenotype) and may induce angiogenesis as well as recruit leukocytes (Figure 3) [2, 39, 47, 50, 55-57]. Furthermore, several tumor-promoting factors are produced by CAFs and co-cultures with primary CAFs have been shown to induce proliferation and invasion of cancer cells *in vitro* [47, 51, 52, 55, 58, 59].

Today, CAFs are generally defined as α-smooth-muscle actin (αSMA), fibroblast-specific protein-1 (FSP-1), fibroblast-activated protein (FAP), neuron-glial antigen-2 (NG2) and PDGFβ-receptor expressing cells [39, 47, 54, 58, 60, 61]. However, many of these markers may be expressed by other cell types as well [47]. In addition, recent reports have highlighted the heterogeneity of CAFs and several subgroups of CAFs, with overlapping expression of markers, have been proposed [62, 63]. Consequently, as of yet, no common CAF marker has been identified.

One likely explanation to the heterogeneity of CAFs is that it may reflect variability in cellular origin. In murine models, CAFs have been suggested to originate from several sources (Figure 3): 1) Activation of resident fibroblasts, 2) transdifferentiation of epithelial cells, endothelial cells or cells of mesenchymal origin such as pericytes or adipocytes, and 3) recruitment and subsequent differentiation of precursors including bone marrow derived progenitor cells or mesenchymal stem cells [56, 64, 65].

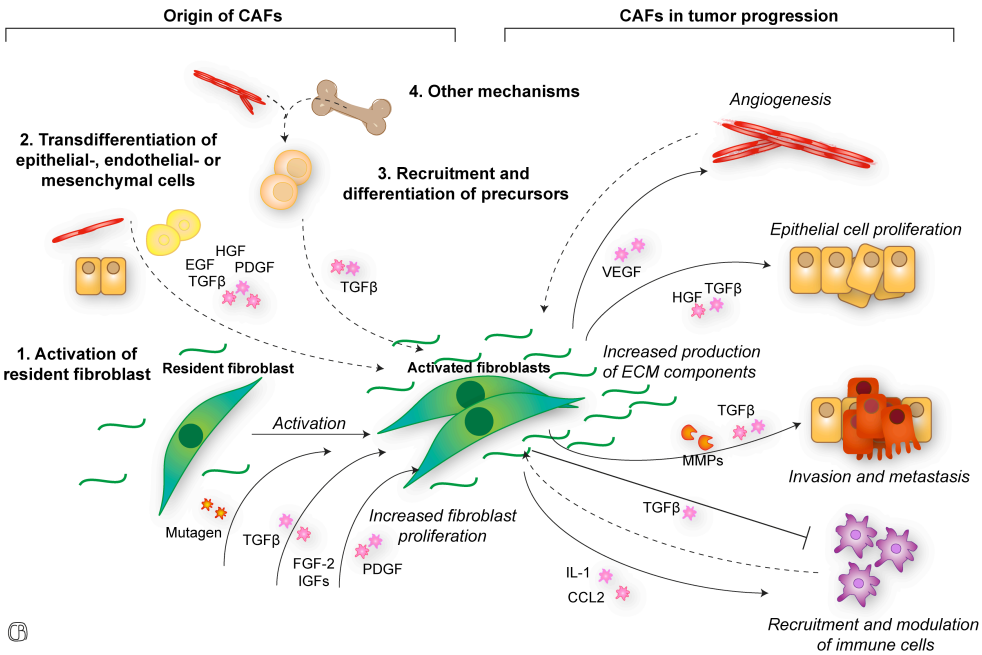

Figure 3. The origins and functions of CAFs in tumor progression. CAFs can be derived from several sources in response to either tumor-derived factors or mutational events (*left panel*). Compared to their normal counterparts, CAFs display increased production of ECM components and higher rate of proliferation. In addition, CAFs produce factors that may induce angiogenesis, tumor cell proliferation, invasion and metastasis as well as recruit leukocytes to the site (*right panel*).

The History of Tumor Immunology in Brief

Similar to CAFs, the leukocyte populations present in the tumor area are generally tumor promoting, but also immunosuppressive. In combination with CAFs, recruited tumor-associated leukocytes and malignant cells comprise a vicious cycle enhancing tumor progression.

Already in 1863, Rudolph Virchow noticed an abundance of inflammatory cells in human tumors and hypothesized that cancer might originate at sites of chronic inflammation. Since then, scientists have been struggling to understand the relationship between inflammation, immunity and cancer. Among the pioneers of

early tumor immunology, Paul Ehrlich was first to propose that the immune system has a critical role in protecting the host from cancer. He suggested that immune cells could recognize and eradicate transformed cells, before a tumor could form [66]. Following in his footsteps, Burnet and Thomas further developed this immunosurveillance hypothesis in the 1950s [66, 67]. However, mid-20th century studies were contradictory and the majority failed to support this theory and thus the controversies continued. The debate was finally put to rest in the 1990's when knock out mice verified central roles of immune cells and their mediators (e.g. B-, T-, natural killer- and natural killer T cells, interferons; IFNs, and perforin) in tumor immunity [68-77]. In 2002 Dunn *et al* postulated a refinement of Burnet and Thomas' cancer immunosurveillance theory, which they termed the *cancer immunoediting hypothesis* [68]. This hypothesis will be discussed in more detail below. Figure 4 summarizes some of the most important milestones in tumor immunology research.

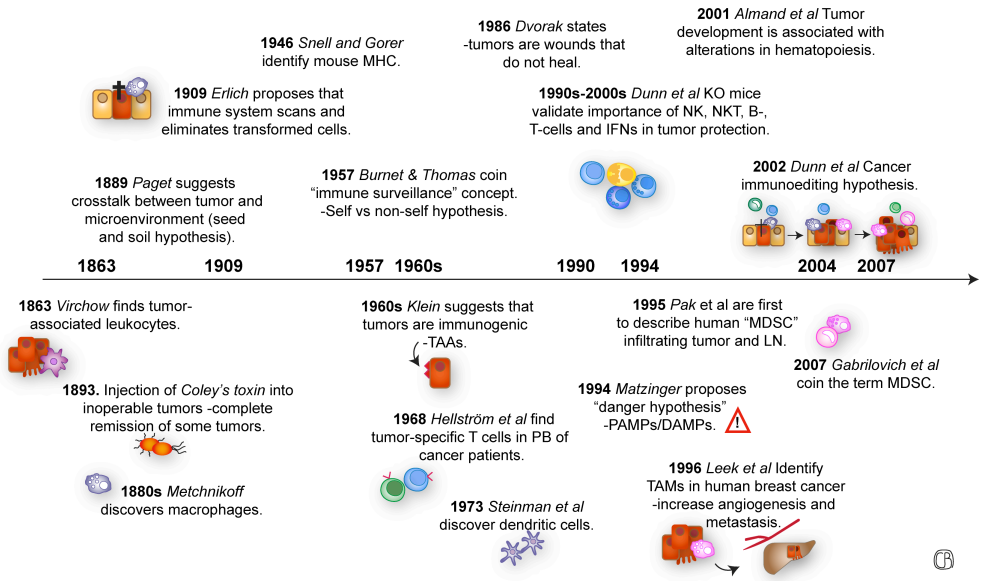

Figure 4. A summary of some of the most important milestones in tumor-immunology research. Abbreviations: MHC; major histocompatibility complex, NK; natural killer cells, NKT; natural killer T cells, TAA; tumor-associated antigens, MDSC; myeloid-derived suppressor cells, PAMPs; pathogen-associated molecular patterns, DAMPs; damage-associated molecular patterns and TAMs; tumor-associated macrophages.

Inflammatio. Latin for "to set on fire"

Inflammation and Cancer

Ever since the days of Virchow, cancer has been strongly associated with inflammation, which led to the notion that "tumors are wounds that do not heal" [78]. In many malignancies, inflammation precedes the malignant transformation and contributes to tumor development. In other malignancies, however, oncogenic changes may create an inflammatory microenvironment that augments malignant progression [79, 80]. Therefore, the relationship between inflammation and cancer is generally described as a two-parted process triggered either by *extrinsic* factors (inflammation elicited by infections, environmental pollutants or irritants, or dietary factors) or *intrinsic* factors (inflammation elicited by mutational events) (Figure 5) [79-81].

Inflammation-induced cancer

Several pathogens have been shown to be associated with increased risk of cancer and it has been estimated that approximately 15-20% of all cancers are related to chronic inflammation in response to infectious agents [54, 80, 82]. Among these, the most common as well as most studied, are cervical carcinoma (human papilloma virus), hepatocellular carcinoma (hepatitis B and –C) and gastric carcinoma (*Helicobacter pylori*) [54, 80, 82]. In addition, infectious agents as well as chemical irritants or injuries result in recruitment and activation of innate immune cells that may contribute to cancer development by releasing several soluble mediators and triggering a chronic inflammatory condition (Figure 5) [54]. Indeed, many cancer risk factors induce NFκB and/or STAT3 signaling: the major pathways activated during inflammatory conditions [80, 83]. However, far from all chronic inflammatory diseases increase the risk of cancer. Some, such as psoriasis, may even reduce the risk [84].

Cancer-induced inflammation

Immune cells and inflammatory mediators are present even in cancers without causal relationship to inflammation. The importance of the immune system in cancer development is supported by several clinical studies where it was described that tumors with no known pathogenic etiology arise more frequently in

"immunosuppressed" individuals (transplant recipients as well as neonates and elderly when the immune system is not fully functional) [38, 68, 85]. Furthermore, non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to reduce the risk of developing certain forms of tumors [54, 79, 81, 86-88]. These observations led to the discovery of the intrinsic inflammatory pathway in which the tumor itself triggers a "sterile" inflammatory microenvironment. Several oncogenes have been shown to induce a response similar to that seen during infections and wound healing [79, 89-92]. Moreover, constitutive activation of NFκB or STAT3 is frequently found in tumor cell lines and specimens [83, 93]. Activation of NFκB and/or STAT3 signaling ultimately results in products involved in many aspects of tumor progression including inflammation, survival, proliferation, angiogenesis, invasion and metastasis [79, 80, 83, 86, 93-97]. It is tempting to speculate that the potential of tumor cells to induce a sterile inflammation, or to shape the surrounding microenvironment, is what determines whether a tumor is formed or not.

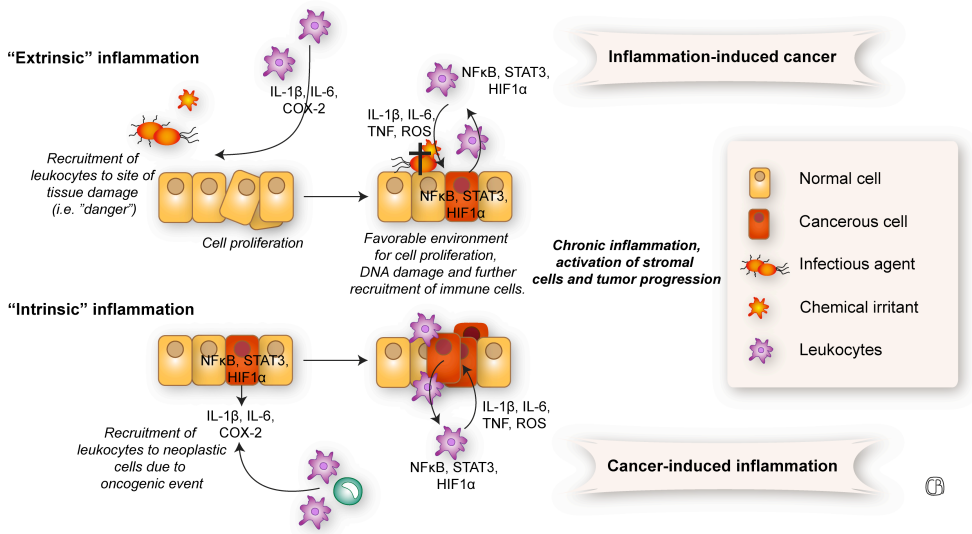

Figure 5. The relationship between inflammation and cancer. (*Upper panel*) Various exogenous pathogens and irritants can trigger inflammation by recruiting leukocytes. These leukocytes produce pro-inflammatory and antimicrobial factors, which may induce DNA damage as well as trigger cell proliferation. (*Lower panel*) Several tumor-intrinsic factors such as activation of oncogenes, NFκB or STAT3 induce production of pro-inflammatory mediators. This results in recruitment of leukocytes, which augment the local inflammatory process.

Tumor-Associated Immune Cells

The 7h hallmark of cancer –the inflammatory microenvironment
-Alberto Mantovani, 2009

A Brief Overview of the Immune System

The word immunity derives from Latin *immunis*, which means "exemption from" and encompasses all the mechanisms used by the individual as protection against potentially harmful agents. The immune system is an astonishing network of interconnected cells, mediators and physical barriers and is generally divided into two branches: the innate and the adaptive. *Innate immunity* involves physical barriers, rapidly mobilized phagocytes (such as granulocytes, monocytes and macrophages) and soluble mediators including interferons and components of the complement system. Although conferring a rapid and forceful response, innate immunity is nonspecific and holds no memory of previous encounters. In contrast, *adaptive immunity* is both specific (respond only to unique entities, albeit a vast variety) and has the capacity to recall previous contacts and respond accordingly (so called immunological memory). Adaptive immune responses are slower and involve presentation of antigens by antigen-presenting cells (APC, e.g. dendritic cells) to B- and T lymphocytes, leading to the subsequent activation and clonal expansion of antigen-specific lymphocytes and production of antibodies.

The Immune Cell Paradox in Cancer

Leukocytes play a paradoxical role in tumor development and progression. On one hand, leukocytes act as sentinels, continuously scanning the body for signs of "danger" and eliminating nascent transformed cells [66, 68, 81]. On the other hand, tumor-associated immune cells contribute to tumor progression by secreting mitogenic, pro-angiogenic and/or pro-metastatic factors [86, 98].

Virtually all solid tumors are infiltrated by leukocytes [54]. The tumor-infiltrating leukocytes are comprised of essentially all known immune effector cells and can be broadly divided into two groups; 1) Immune cells promoting anti-tumor immune responses and 2) tumor-promoting immune cell populations with immunosuppressive, pro-angiogenic and/or pro-metastatic phenotypes (Figure 6) [99, 100]. Depending on the relative frequency and activation status as well as location and density of these cells within the tumor mass, the balance between anti-tumor and pro-tumor immune responses may be tilted (Figure 6) [99]. During the last decades, the crosstalk between cancer cells and the immune system has been studied extensively and today it is well known that tumors actively modulate the immune system.

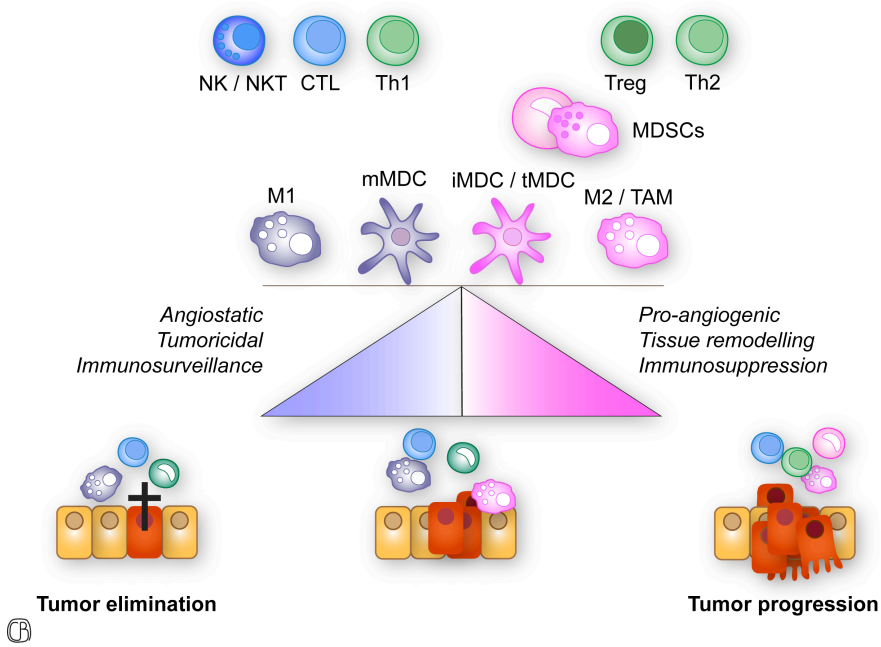

Figure 6. Immune cell balance in tumor elimination *versus* tumor progression. Tumor-infiltrating leukocytes can be divided in two branches. Angiostatic, tumoricidal leukocytes involved in immunosurveillance and tumor elimination (*left*), and pro-angiogenic, tissue-remodeling and immunosuppressive leukocytes involved in tumor progression (*right*). Abbreviations: NK; natural killer cells, NKT; natural killer T cells, CTL; cytotoxic T lymphocyte, Th; T helper cells, Treg; regulatory T cell, MDSCs; myeloid-derived suppressor cells, M1; pro-inflammatory macrophages, mMDC; mature myeloid dendritic cells; iMDC; immature myeloid dendritic cells, tMDC; tolerogenic myeloid dendritic cells, M2; anti-inflammatory macrophages, TAM; tumor-associated macrophages. Adopted from DeNardo *et al* 2010 [99].

Cancer Immunoediting Hypothesis

The cancer immunoediting hypothesis, as proposed by Dunn *et al*, is a three-phase model of tumor-immune cell interactions during tumor development (Figure 7) [66, 68, 101]. The first phase, *elimination*, refers to leukocytes recognizing nascent transformed cells as something dangerous and destroying them, before a tumor has been formed. Apart from potential tumor-associated antigens (TAAs), early alterations of stromal cells and release of endogenous alarmins from neoplastic cells result in activation of anti-tumor immune responses [102, 103]. Several mediators and immune cell populations have been documented to be involved in this phase, see Figures 6 and 7 [66, 68, 70-77]. In essence, this process comprises the immunosurveillance phase proposed by Burnet and Thomas.

During the *equilibrium* phase, the surviving tumor cells are under constant pressure from the recruited immune cells [66, 68]. Although the tumor is not growing, it is not removed either. Similar to a Darwinian selection, tumor cells with high immunogenicity are recognized and eliminated whereas tumor variants with poor immunogenicity may escape the immunosurveillance. This phase is most likely the longest one and may extend over several years, or even decades.

Due to the intrinsic genetic instability of neoplastic cells, some tumor cells may acquire traits that render them resistant to the immune attack, either by becoming "invisible" or resilient to the immune cells. This results in *escape* of tumor variants, which may develop into clinically detectable tumors. Several mechanisms have been suggested in this phase: loss of TAAs or induction of tolerance towards TAAs, modulation of antigen presenting machinery and induction of anti-apoptotic signals [85, 102, 104-106]. In addition, many tumor- and stromal cells produce immunosuppressive mediators such as IL-10, VEGF and TGFβ [66, 85, 104, 105]. The establishment of this immunosuppressive microenvironment has several severe consequences on the innate and adaptive immune cells recruited to the tumor site. Macrophages are skewed towards an anti-inflammatory and tissue remodeling phenotype (M2 macrophages) and dendritic cell activation and maturation is inhibited, both of which result in inhibition of tumor-specific T cell responses [101]. In addition, accumulation of other immunosuppressive populations, such as myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Treg), contribute to ablation of anti-tumor immune responses [101, 107]. In essence, the tumor-associated leukocytes are induced to create an environment that is permissive for further cancer growth. During this co-evolution of cancer cells and leukocytes, a local state of immunosuppression is induced.

Furthermore, it is possible that the local suppression eventually may generalize to involve reduced systemic immunity against tumors, thereby facilitating the metastatic processes [108]. Some studies have suggested that immune cells are crucial in preparing

the pre-metastatic niche. In 1889, Paget postulated that tumors (seed) require a proper microenvironment (soil) in order to establish a secondary tumor [109]. This could explain the preferential homing of breast cancer metastases to liver, lung and bone. Indeed, in addition to creating a favorable environment for primary tumor growth, as described above, leukocytes are able to promote angiogenesis and the metastatic process (via secretion of e.g. VEGF, IL-8 and matrix metalloproteinases; MMPs)[110]. Upon intravasation into the blood stream, tumor cells frequently aggregate with myeloid cells, which confer physical protection as well as a possible mean for "directed metastasis" [111]. Moreover, hematopoietic progenitors have been shown to accumulate at distant sites and promote recruitment of tumor cells to these sites, thus creating a pre-metastatic niche and a favorable "soil" for the metastasis [112, 113].

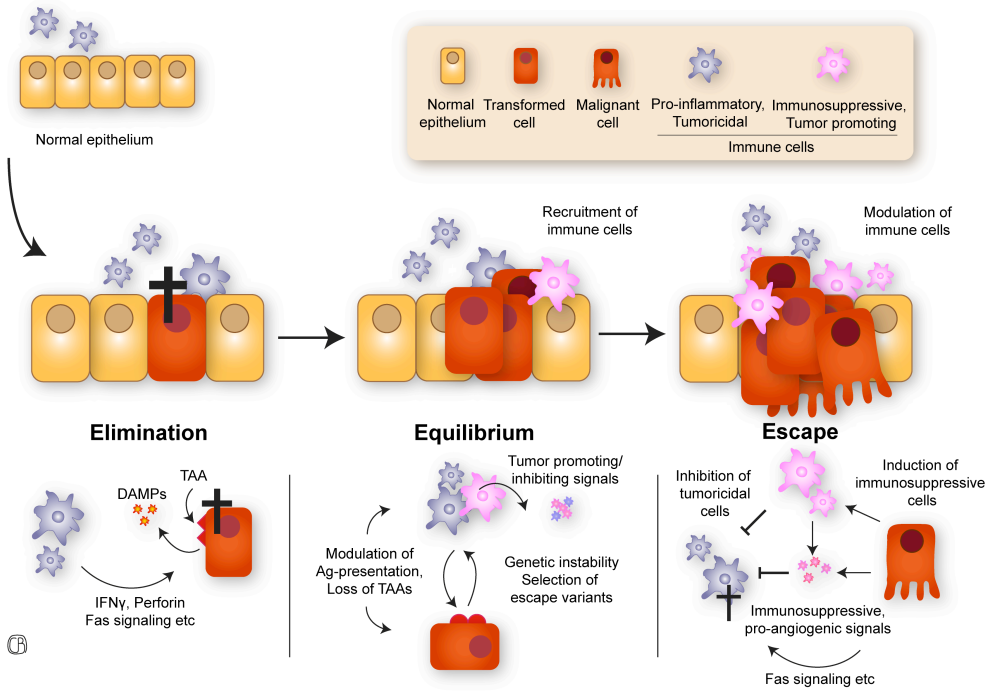

Figure 7. The three "Es" of cancer immunoediting. *Elimination.* Transformed cells are recognized due to expression of tumor-associated antigens (TAAs) or endogenous alarmins (DAMPs) by tumoricidal leukocytes. The neoplastic cells are subsequently eliminated via various pathways. *Equilibrium* is the phase in which the tumor is neither growing nor removed. *Escape* of cancerous cells due to various tumor cell-intrinsic mechanisms as well as induction of tolerance of immune cells. Adapted from Dunn *et al* 2002 and Swann and Smyth 2007 [68, 114].

Myeloid Cells in Cancer

"The only constant is changes"
-Heraclitus, 500 BC

Myeloid cells constitute the predominant immune cell population in the peripheral blood as well as in tumor tissues. These innate immune cells are very versatile and are among the first cells to be recruited to "sites of danger", be it an infection or a tumor [115]. During non-malignant conditions, myeloid cells play a critical role in defense against pathogens as well as in tissue homeostasis and repair. In addition, myeloid cells participate in tumor initiation, progression and metastasis and have been suggested to confer resistance to various therapies [7, 116]. Myeloid cells are recruited to the tumor site continuously (from early to advanced stages) by tumor-derived factors such as CCL2, CSF-1, CXCL12, IL-8 and VEGF [98, 117, 118]. Several of these factors may also affect the functionality of the recruited cells [118]. Indeed, unarguably the most important feature of myeloid cells is their inherent plasticity [119]. Consequently, it is no surprise that tumors can have profound effects on myeloid cell differentiation, activation and overall function.

Monocytes

Monocytes are heterogeneous populations of innate immune cells and generally comprise 3-10% of all peripheral blood leukocytes. This versatile population plays a crucial role in fighting pathogens as well as controlling inflammation, tissue repair and inducing angiogenesis [120]. Monocytes continuously patrol the blood, scanning for signs of infection and inflammation. Apart from possessing direct antimicrobial properties via production of antimicrobial factors and pro-inflammatory cytokines, monocytes may also promote adaptive immune responses by presenting antigens to T lymphocytes [121-124].

Originally, monocytes were defined based on their morphology such as irregular cell shape, oval- or kidney-shaped nucleus and high cytoplasm-to–nucleus ratio [121, 125]. With the introduction of flow cytometry, monocyte identification is now based on light scatter properties, and expression of cell surface markers such as the receptor for lipopolysaccharide (LPS) CD14. During the early 21st century, three monocyte subgroups were identified based on their expression of CD14 and the low affinity Fc receptor CD16 (Fcγ receptor III): 1) The predominant *classical $CD14^{++}CD16^{-}$ monocytes*, 2) the patrolling *non-classical $CD14^{+}CD16^{++}$ monocytes* and 3) the *intermediate $CD14^{++}CD16^{+}$ monocytes* [125]. Although the complexity of the monocyte heterogeneity is only beginning to be unraveled, these subpopulations have been suggested to differ not only in expression of receptors but also at a functional level [120, 121, 123]. Figure 8 summarizes the main characteristics of the monocyte subpopulations [121, 123, 125-133].

Monocytes in cancer

Apart from their role in innate immunity, monocytes are also emerging as key players in several forms of malignancies. Although the functions of monocytes in cancer patients are relatively unexplored, studies have shown that some monocytes may induce angiogenesis ($Tie2^{+}$ monocytes) as well as augment the invasive and metastatic potential of breast cancer cells [134-140]. This contradiction with regards to the inherent cytotoxic potential and antigen-presenting cell function of monocytes may be explained by the plasticity of myeloid cells, as tumor cells induce deactivation and polarization of monocytes [141, 142]. In addition, some tumor-derived factors may result in specific enrichment of peripheral blood $CD14^{+}CD16^{+}$ monocytes early during breast cancer formation [143].

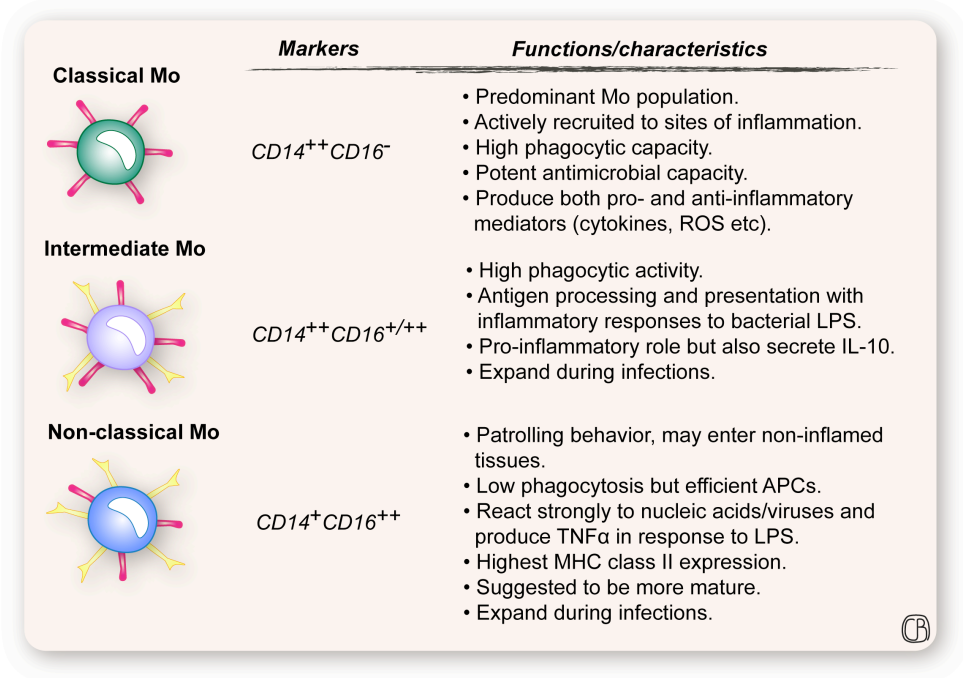

Figure 8. A summary of the characteristics of the three monocyte (Mo) subpopulations [121, 123, 125-133].

Several tumor-derived factors, as well as the pro-inflammatory stimuli during an infection, lead to recruitment of monocytes. Upon extravasation into tissues, monocytes readily differentiate into macrophages or dendritic cells (DCs) [120, 144, 145]. *In vitro*, this can be mimicked by addition of cytokines such as GM-CSF and IL-4 [120, 146, 147]. Indeed, the most studied, and possibly also most important, function of monocytes is that they act as a systemic reservoir for some populations of macrophages or DCs [121, 122].

Macrophages

As the name implies, macrophages (literally Greek for "big eaters") are crucial phagocytes residing in tissues. Many are recruited during infection (*i.e.* monocyte-derived macrophages) while others are strategically dispersed in specific organs (*i.e.* tissue-resident macrophages such as Kupffer cells in the liver, microglia in the central nervous system and osteoclasts in the bone) [120]. Since their discovery over a century ago, macrophages have been attributed a plethora of functions including elimination of pathogens and tissue remodeling during wound healing. As with all myeloid cells, macrophages are very plastic by nature. Depending on environmental cues, macrophages can become either pro-inflammatory (M1 macrophages) or anti-inflammatory (M2 macrophages) (Figure 9) [103, 120, 148].

The *classically activated M1 macrophages* are induced by several inflammatory cytokines such as IFNγ, tumor necrosis factor (TNF) as well as conserved pathogen-associated factors such as LPS [119, 148, 149]. M1 polarized macrophages are the principal effector macrophage population generated during immune responses. As such, they are responsible for the microbicidal or tumoricidal properties of macrophages. Generally, M1 macrophages are characterized by high secretion of pro-inflammatory mediators as well as having a high capacity to phagocytose and present antigens, leading to T cell activation [98, 150]. Given the crucial role of macrophages in host defense, it is easy to forget that macrophages also possess vital roles in homeostatic processes. Possibly the most important function of macrophages is their role as "housekeepers", removing cellular debris and apoptotic cells [103, 151].

The umbrella term "*alternatively activated macrophages*" (*M2*) comprises macrophage populations with tissue remodeling, wound healing, angiogenic and/or immunosuppressive attributes [103, 150]. M2 macrophages may be induced by several factors including anti-inflammatory cytokines (IL-4, IL-10, TGFβ, PGE_2), glucocorticoids, immune complexes and even some ligands for Toll-like receptors (TLRs) [149, 150, 152, 153]. Although many of these factors confer M2 macrophages with higher phagocytic capacity, due to increased expression of scavenger receptors, they also lead to suppressed macrophage secretion of pro-inflammatory cytokines [150, 153].

It should, however, be noted that the M1-M2 dichotomy is an oversimplification as macrophage polarization most likely comprises a continuum of various polarization states, with M1-M2 being the two extremes.

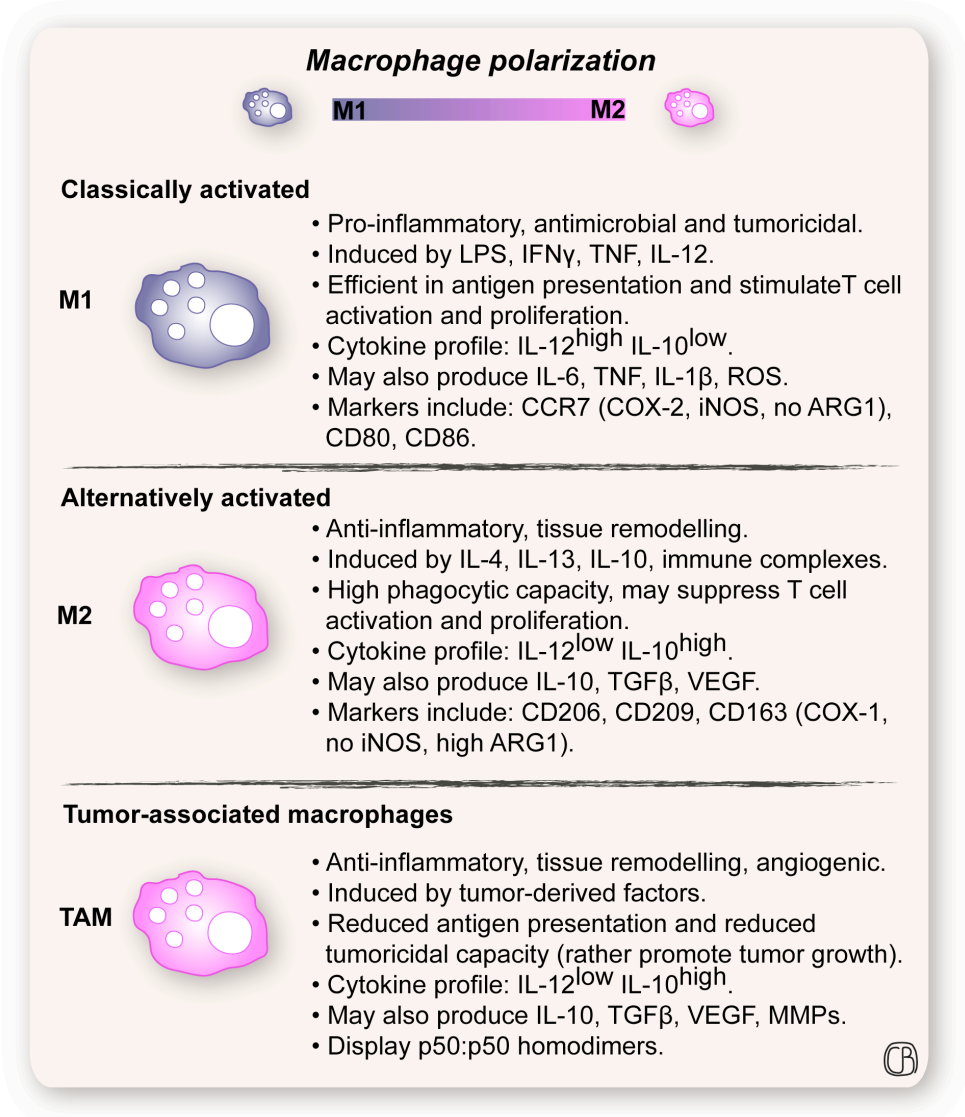

Figure 9. A summary of the characteristics of M1 and M2 macrophages as well as tumor-associated macrophages [103, 119, 120, 148, 150].

With regards to some diseases, macrophages (or monocytes) are characterized by a preferential polarization into alternatively activated monocyte-macrophages, most notably the endotoxin tolerant monocyte in sepsis patients and the tumor-associated macrophages (TAMs) in cancer patients [148, 154, 155].

Tumor-associated macrophages

A vast number of studies have emphasized the role of tumor-associated macrophages (TAMs) in tumor progression. TAMs are generally the most abundant leukocyte population in solid tumors and may comprise up to 50% of the total tumor mass [98, 156]. TAMs are generally believed to display low capacity to present antigens, low cytotoxicity for tumors and an IL-10^{high}IL-12^{low} immunosuppressive cytokine profile, strongly resembling M2 macrophages [98, 148, 154, 157]. Although the biological effects of TAMs vary depending on the local cytokine and chemokine profile, they are believed to be involved in tumor progression, inhibiting anti-tumor immune responses, inducing angiogenesis, invasion and metastasis formation (Figure 10) [98, 148, 156, 158, 159]. In addition, ablation of macrophages in a mouse breast cancer model has been shown to reduce tumor growth and progression as well as inhibit angiogenesis and metastasis formation [160, 161]. Thus, TAMs generally correlate with poor outcome in breast cancer as well as other forms of malignancies [98, 156, 158, 160, 162]. Figure 10 summarizes the most important functions of TAMs.

Several tumor- and immune cell-derived factors have been implicated in the recruitment and induction of TAMs such as CCL2, CSF-1, VEGF, IL-4, IL-10 and TGFβ [98, 140, 162, 163]. Many of these factors promote homodimerization of the inhibitory NFκB family member p50 [164]. Indeed, TAMs generally display overexpression of nuclear p50, and the accompanying defective NFκB activity, with associated reduced production of pro-inflammatory mediators and increased production of IL-10 [164-166]. This can at least in part explain the TAM-phenotype observed in cancer patients. Similar observations have also been made regarding immunosuppressive monocyte-macrophages in infectious diseases [154, 167].

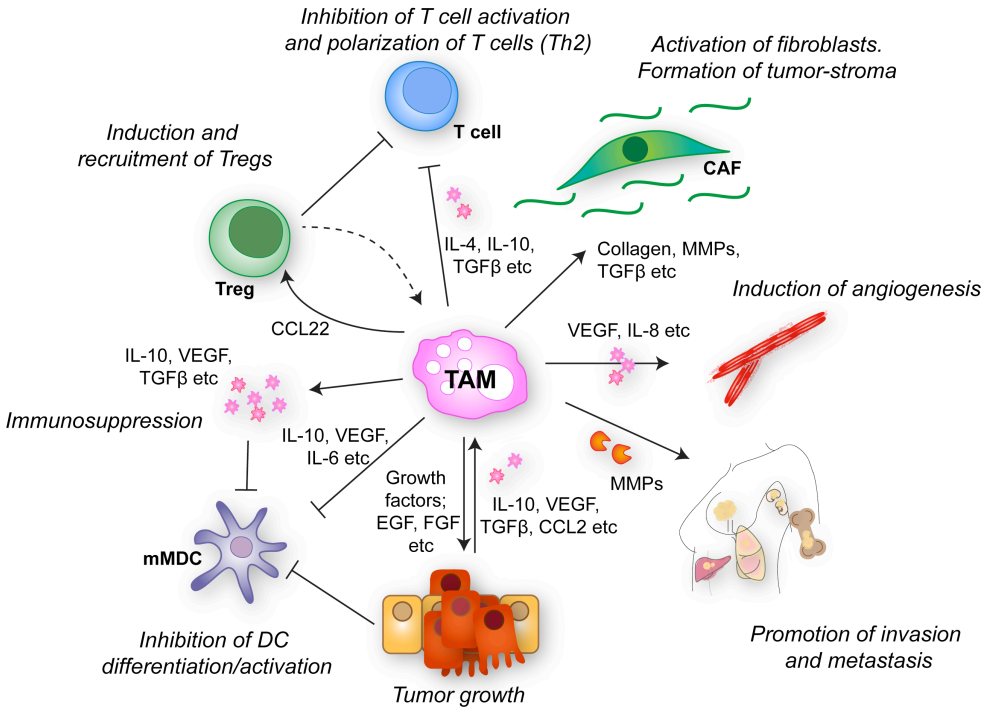

Figure 10. The role of tumor-associated macrophages in tumor progression. Tumor-associated macrophages (TAMs) affect tumor progression in several ways. TAMs may promote tumor growth directly via secretion of growth factors or indirectly by inhibiting differentiation of DCs and inducing Tregs, which suppress tumor-specific T cell responses. In addition, TAMs may produce collagen and factors that activate fibroblasts thus contributing to the formation of a reactive stroma. Several factors secreted by TAMs are also involved in angiogenesis as well as in promoting invasion and metastasis.

Dendritic Cells

Dendritic cells (DCs) were first identified by Ralph Steinman based on their morphology with extensive dendritic processes [168]. The phrase "professional antigen presenting cells" (APCs) is commonly used to describe DCs. This statement emphasizes the crucial role of DCs to capture, process and present antigens to T cells (naïve and memory T cells, Figure 11). Immature DCs are very efficient in the phagocytosis of antigens, however they display a low capacity to present those antigens, and has been suggested to play a role in peripheral tolerance (Figure 11) [169-171]. Some studies have termed these immature DCs that induce tolerance, tolerogenic DCs, although some tolerogenic DCs may display markers of maturation [171, 172].

In response to pathogens, damage or pro-inflammatory cytokines, DCs mature leading to an up-regulation of co-stimulatory molecules, chemokine receptors and cytokine production, which may activate other innate immune cells in the proximity (Figure 11) [172-174]. The most important function of these mature DCs is, however, to migrate to lymph nodes where they induce antigen-specific T cell activation, proliferation and polarization [171, 172, 174]. This incomparable ability of DCs to stimulate T cell responses serves as a crucial link between innate and adaptive immunity.

Apart from being present throughout tissues, DCs have also been identified in the circulation [125]. Three peripheral blood DC populations have been identified which together constitute less than 1% of all mononuclear cells [175]; two myeloid DC populations (MDCs; MDC1 and MDC2) and plasmacytoid DCs (PDC; formerly also called lymphoid DCs) [125]. These populations express different patterns of cell surface markers and respond to different stimuli, see Figure 11 [125, 175-177].

Tumor-associated dendritic cells

Dysfunctional DC populations have been proposed to be an important mechanism for tumor escape [178, 179]. Several tumor- and stroma-derived factors may inhibit DC differentiation and activation including VEGF, IL-10 and IL-6 [179-183]. Reduced levels of circulating DC populations have been observed in breast cancer as well as other forms of malignancies [181, 184]. Furthermore, the tumor-associated DCs (TADCs) within breast tumors are generally immature or tolerogenic, and thus poorly immunogenic, whereas more mature DCs are located in peritumoral areas [179, 185, 186]. In breast cancer tissue, high numbers of mature MDCs tend to correlate with longer relapse-free and overall survival times, whereas infiltration with PDCs tend to correlate with poor survival [187-189].

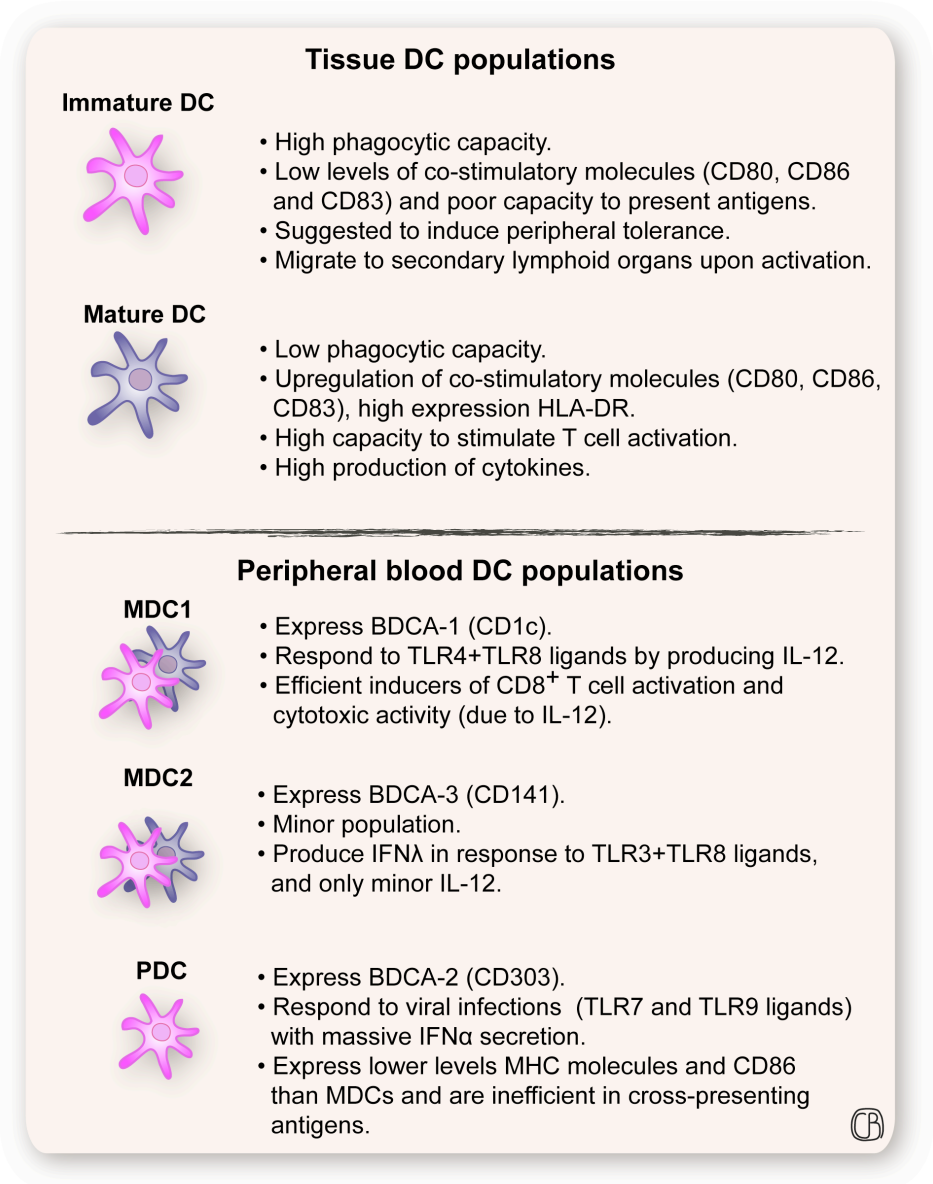

Figure 11. A summary of the characteristics of tissue- and peripheral blood dendritic cell populations [125, 171, 175-177].

Myeloid-Derived Suppressor Cells

During the last decade, tumor immunologists have put a lot of effort in studying myeloid-derived suppressor cells (MDSCs) in the process of tumor immune escape. MDSCs are commonly described as immature cells of the myeloid lineage that possess potent immunosuppressive properties [190, 191]. While rare in healthy individuals, immature myeloid cells are known to accumulate in the peripheral blood, lymphoid organs as well as in tumors in response to tumor-derived factors such as GM-CSF, VEGF, IL-10, PGE_2 and IL-6 [190-194]. These cells acquire suppressive attributes upon activation by IL-4, IL-13, PGE_2 and/or ligands of Toll-like receptors (TLRs) [191, 194-196]. The underlying mechanism of generation of MDSCs as well as establishment of their phenotype and immunosuppressive functions may vary depending on the environmental signals, however these processes remain poorly understood.

At least two subsets of MDSCs have been identified in humans; polymorphonuclear or "granulocytic MDSCs" (PMN-MDCs or G-MDSCs) and the recently discovered "monocytic MDSCs" (Mo-MDSCs). G-MDSCs are usually characterized as immature $CD33^+$ and/or $CD11b^+$ cells that lack all lineage markers (Lin^-), although expression of CD15 may be observed in some subpopulations of G-MDSCs (Figure 12) [111, 194]. Mo-MDSCs, on the other hand, are believed to be more mature and are characterized as $CD14^+HLA\text{-}DR^{low/-}Co\text{-}receptor^{low/-}$ cells [194]. Both MDSC populations potently suppress T cell-, NK cell- and antigen-presenting cell activity and function [194]. On the other hand MDSCs may also induce Tregs and produce pro-angiogenic factors [194]. Figure 12 summarizes the main mechanisms used by MDSCs to perturb innate and adaptive immune responses against the tumors [191, 194, 197, 198].

Most published studies on MDSCs have focused on G-MDSCs, which seem to accumulate in most forms of cancer. In general, G-MDSC accumulation correlates with tumor progression, angiogenesis and poor prognosis [111, 191, 199]. In breast cancer, accumulation of G-MDSCs is associated with clinical stage, metastatic burden and poor overall survival [181, 195, 199, 200]. Mo-MDSCs, on the other hand, are far less studied yet have been reported to be enriched in patients with melanoma [201, 202], prostate cancer [203], bladder cancer [204], hepatocellular carcinoma [205], non-Hodgkin lymphoma [206] and glioblastoma [207]. In some of these studies, the presence of Mo-MDSC correlated with more active [201] or aggressive disease [206] as well as increase tumor size and grade [204]. In this thesis, we show for the first time that Mo-MDSCs also increase in breast cancer peripheral blood (see *Paper IV*).

Apart from accumulating in tumor bearing hosts, MDSCs are also increased in non-malignant conditions such as during trauma and acute infections. In these situations, MDSCs function to dampen excess immune responses that may cause tissue damage [194, 197].

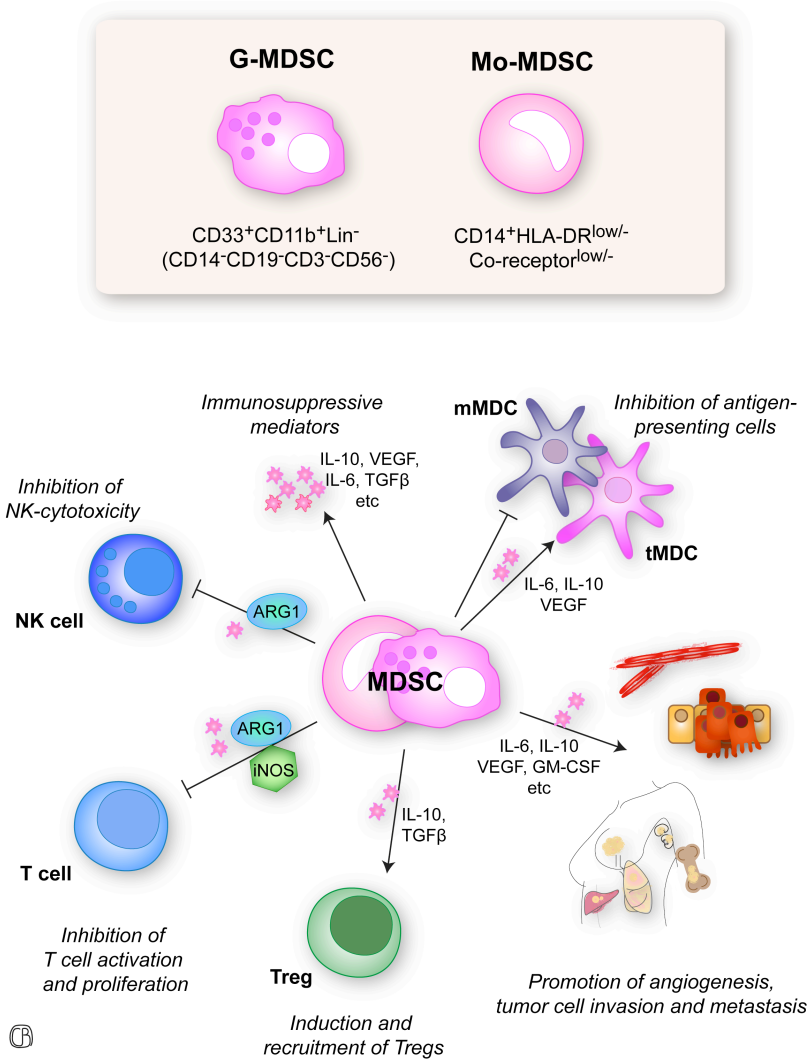

Figure 12. MDSC-mediated suppression of immune responses and role in tumor progression. MDSCs inhibit tumor-specific immune responses in several ways. The enzymes ARG1 and iNOS catalyze the amino acid L-Arg, which is essential for T- and NK cell activity and function. In addition, MDSCs produce immunosuppressive cytokines, which induce Treg and M2 polarization as well as inhibit MDC maturation, thus inhibiting the APCs capability to elicit T cell responses. Furthermore, some of these factors may promote tumor growth, induce angiogenesis as well as contribute to the metastatic process.

Lymphocytes in Cancer

Various lymphocyte populations, in addition to myeloid cells, have been reported to infiltrate solid tumors. Lymphocytes are generally divided into four populations: T- and B-lymphocytes, natural killer (NK) and natural killer T (NKT) cells, all of which may be present at the tumor site and affect tumor progression (Figure 6). The most studied cell population in the adaptive immunity is unarguably the $CD3^+$ *T lymphocytes*, which can be grossly subdivided into $CD8^+$ cytotoxic T cells (CTLs), $CD4^+$ T helper cells (Th) and the minor population of γδ T cells.

CTLs are involved in recognition and elimination of infected or malignant cells by IFNγ, perforin or Fas-signaling, and their presence is generally correlated with a favorable prognosis in multiple malignancies including breast cancer [43, 99, 208, 209]. Similarly, an anti-tumor function has been ascribed γδ T cells, especially in cutaneous carcinomas [73, 210]. *Th cells*, on the other hand, orchestrate immune responses and are generally divided into Th1 and Th2 cells as well as the novel Th17, Th22 and Th9 [211, 212]. Among these, the Th1 (characterized by secretion of IFNγ, TNFα and IL-12) and Th2 (secrete IL-4, IL-6 and IL-10) are by far the most studied [99, 213]. In tumorigenesis, Th1 cells induce immunosurveillance by promoting CTL activation whereas Th2 cells inhibit T cell-mediated cytotoxicity and promote M2 macrophages [99, 213]. Tumors are generally associated with a shift from a Th1 to Th2 pattern [178, 214]. Accordingly, a low density of $CD8^+$ T cells or a high density of $CD4^+$ T cells correlates with reduced overall survival in breast cancer [209]. Normally, the activation, amplitude and quality of the T cell response are tightly regulated by antigen recognition by T cell receptors and additional co-stimulatory (e.g. via CD28) and co–inhibitory signals (e.g. via CTLA-4). This balance between stimulation and inhibition may be altered in cancer-associated lymphocytes [215]. Furthermore, lymphopenia is a common paraneoplastic complication, which can be worsened following chemotherapy. Reduced peripheral blood T cell count ($CD3^+$) as well as both $CD4^+$ and $CD8^+$ T cell subpopulations has been observed in breast cancer patients [216].

Regulatory T cells (Tregs) also belong to the Th cells. Tregs are critical for maintaining peripheral tolerance in healthy individuals and dampening immune responses during persistent inflammation [217, 218]. In addition, Tregs may be recruited to tumors where they inhibit anti-tumor immune responses by secreting immunosuppressive factors or inducing apoptosis of effector cells [218-220]. In breast cancer, Tregs are enriched in the

peripheral blood as well as within the tumor and their presence correlates with higher tumor grade and shorter overall survival time [221, 222].

The role of *B lymphocytes*, however, is far less studied and remains controversial. On one hand, B cells may contribute to anti-tumor immune responses by producing tumor-specific antibodies, while on the other hand they have also been proposed to promote tumor progression [223].

Natural killer (*NK*) *cells* are "innate lymphoid cells" that are capable of recognizing missing-self (loss of MHC class I molecules) as well as stress-induced self [224]. NK cells were originally identified as a naturally occurring lymphocyte population exhibiting spontaneous cytotoxic effects on leukemia cells [225]. The presence of NK cells, as well as the functionally related *NKT cells*, generally correlates with a better prognosis in cancer patients due to their roles in the process of tumor surveillance [75, 224]. In breast cancer, markers indicative of NK cells or CTLs was proposed to correlate with good prognosis for the patients [42].

Figure 13 summarizes the local and systemic effects of distinct leukocyte populations in breast cancer.

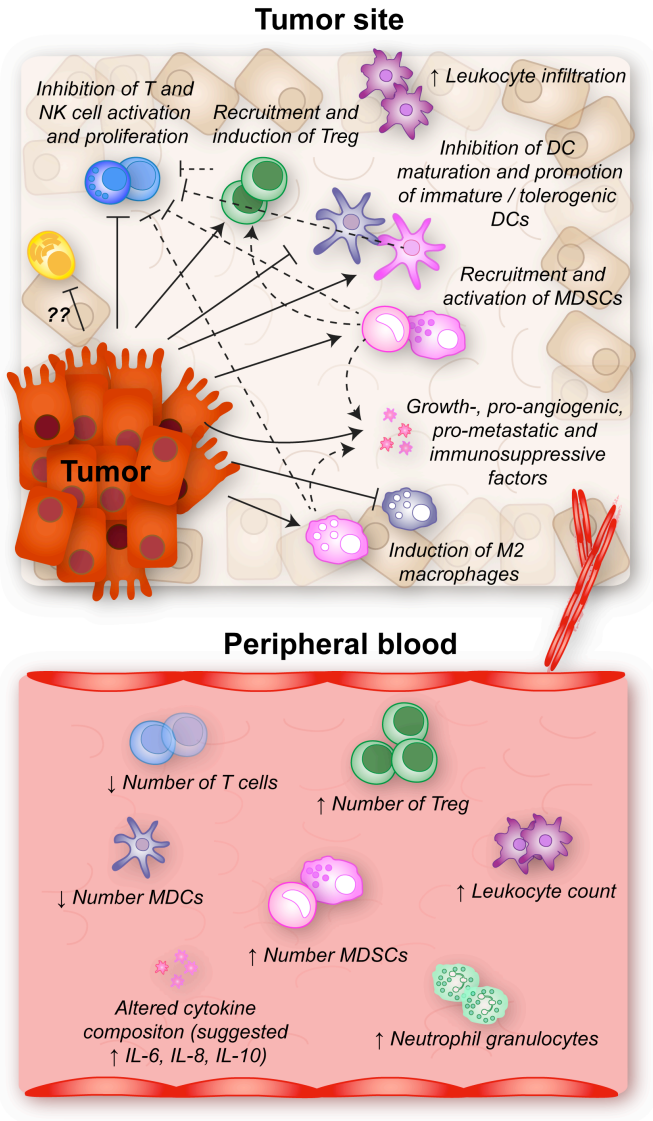

Figure 13. A summary of the local and systemic leukocyte alterations observed in cancer patients. Tumors induce several defects in the immune response, locally as well as systemically. *Upper panel;* Local defects include inhibition of T and NK cell activation, inhibition of DC maturation and function, inhibition of M1 polarization, as well as inducing Treg, MDSCs, tolerogenic DCs and TAMs. This is accomplished via production of immunosuppressive mediators, amongst other factors. Dotted lines represent effects of immune cells on other immune cells. *Lower panel;* Systemic alterations in the peripheral blood involve lymphopenia, leukocytosis and neutrophilia as well as increased numbers of Tregs and MDSCs.

Targeting the Microenvironment

"I can't understand why people are frightened of new ideas. I'm frightened of the old ones."
-John Cage, 1988

The vast majority of currently used cancer therapeutics has been developed with the goal of targeting the malignant cells of the tumor. Acquired resistance is still one of the most challenging obstacles in cancer treatment. Targeting the tumor microenvironment is an exciting possibility to circumvent this problem. Stromal cells are thought to be genetically stable and may provide a better target, however the risk of disturbing normal homeostatic processes may pose another obstacle.

As of today, combination of various cytotoxic chemotherapeutics is the standard treatment for invasive neoplasia. Some of these approaches may also have clear immunomodulatory side effects including inducing apoptosis of MDSCs, depleting/inactivating Tregs or activating macrophages and NK cells [226, 227]. In addition, drugs targeting the microenvironment specifically are currently being developed. Some of these drugs, such as various angiogenesis-inhibiting drugs, are currently used in the clinic. These include the VEGF-neutralizing antibody bevacizumab, which was the first anti-cancer drug targeting the tumor microenvironment to be approved for clinical use [228].

Cancer Immunotherapy

The exploitation of immune cells as a treatment strategy for cancer forms the essence of cancer immunotherapy. The first evidence of this appeared already in the 1890's when William Coley injected cancer patients with bacterial extracts, thus eliciting a powerful inflammatory response ultimately resulting in tumor regression [229]. Although resulting in severe side effects, and even death in some patients, this provided evidence that it is indeed possible to activate the patient's own immune system against the tumor. In addition, as many tumors are immunogenic,

development of specific cancer immunotherapeutic regimens could revolutionize cancer treatment.

Tumor-associated antigens

Numerous studies have reported the presence of immune responses against tumors, however, these responses are usually weak due to the immunosuppressive tumor microenvironment [178, 230-232]. Tumor-specific T cells and auto-antibodies have been demonstrated in patients with several cancer types [231-234]. This provides evidence for the existence of tumor antigens that are capable of eliciting an immune response, as was proposed by Burnet and Thomas. The first study regarding tumor-associated antigens (TAAs) was conducted already in the 1960's and, today, approximately 100 different TAAs have been identified [233, 235, 236]. These antigens include mutated proteins (e.g. p53, Ras, Bcr-Abl), over- or aberrantly expressed proteins (e.g. HER2/neu or melanoma-associated antigen; MAGE family, respectively) or viral antigens (e.g. HPV-associated proteins E6 and E7) [237]. Specific targeting of many of the identified TAAs by monoclonal antibodies is currently used in the clinic. These include e.g. trastuzumab (Herceptin; a monoclonal antibody targeting HER2/neu and is used in treatment of HER2 over-expressing breast tumors) and imatinib mesylate (Gleevec; a compound targeting the Bcr-Abl fusion protein in chronic myelogeneous leukemia) both of which dramatically increase the survival of these patients [226, 237]. Some of the anti-tumor effects elicited by these drugs may be mediated by immune cells [227].

Early studies in cancer immunotherapy

The discovery of TAAs formed the basis of cancer immunotherapy research. Traditionally, cancer immunotherapy has been divided into either "passive", *i.e.* injection of preformed effector immune cells, cytokines or antibodies, or "active" *i.e.* activation of the patient's own immune system [237, 238]. Early attempts at cancer immunotherapy include injections of potentially anti-tumorigenic cytokines (type I IFNs, IL-12) and adoptive transfer of activated T cells [237]. In addition, active vaccination by treating immune cells (generally treatment of DC *in vitro*) with either lysates from cancer cells, specific tumor antigens or DNA vaccines was evaluated [237]. Although many appeared promising, only limited efficacy was observed in humans. However, in the 1990's, a monoclonal antibody targeting the B cell-associated antigen CD20 was proven effective against B cell lymphomas and is currently used in the clinic [237].

Recent advancer in cancer immunotherapy

The main types of novel cancer immunotherapeutics that are currently used include DC-based therapeutic vaccines, novel monoclonal antibodies and non-specific immunotherapies. *DC-based treatment* strategies have proven successful in patients with metastatic castration-refractory prostate cancer. Activation of autologous DC *ex vivo* and subsequent transfer into the patient significantly enhanced the overall survival (Sipuleucel-T) [239]. Moreover, activation of cytotoxic T lymphocytes may be an alternative. Ipilimumab is a *monoclonal antibody* directed against the inhibitory co-receptor CTLA-4 on T cells, which was recently approved for treatment of patients with metastatic melanoma [215, 240]. Blockade of CTLA-4 enhances the endogenous anti-tumor immune response by T lymphocytes and thus induce tumor regression [215]. Furthermore, specific targeting of the inhibitory co-receptor PD1 is also currently in clinical trials [215]. DC based vaccines as well as targeting of CTLA-4 or PD1 may also be of interest in patients with breast cancer and is currently under investigation.

DAMPs and PAMPs…

"I don't like you because you're dangerous"
-Iceman to Maverick, Top Gun 1986

… connecting inflammation, wound healing and cancer?

For decades, it was believed that the function of the immune system relies upon distinguishing "self" from "non-self". In 1994, however, Matzinger proposed that the immune system is designed to recognize and respond to "danger" [115, 241, 242]. This implies that inflammation is elicited both by pathogens and in response to sterile tissue injury.

The chronic inflammation associated with tumors as well as the concurrent necrotic cell death causes release of endogenous alarmins -so called damage-associated molecular patterns (DAMPs) [103, 243]. Similar events occur during normal tissue damage. These endogenous danger signals are generally intracellular proteins or factors such as HMGB1, heat-shock proteins, uric acid and S100 proteins, which are released upon non-programmed cell death [151, 243, 244]. DAMPs generally recruit and activate innate immune cells and are implicated in promoting tissue remodeling and angiogenesis [244]. In addition, some DAMPs such as S100A8/A9 may regulate MDSC accumulation in tumors as well as promote tumor homing to pre-metastatic niches [243]. Hence, the presence of DAMPs in the tumor microenvironment may promote tumor progression and metastasis, although this is likely dependent on cell type as well as other signals present in the vicinity.

Pathogen-associated molecular patterns (PAMPs) are classical "non-self" molecules referring to exogenous signals from pathogens [244]. These include bacterial endotoxin (lipopolysaccharide; LPS) from gram-negative bacteria, flagellin and lipoteichoic acid from gram-positive bacteria, and double stranded RNA from viruses [245]. PAMPs and DAMPs are mutually recognized by pattern recognition receptors (PRRs), which are expressed on virtually all innate immune cells. PRR stimulation ultimately results in

activation of the NFκB, mitogen-activated protein kinase (MAPK) and/or interferon regulatory factor (IRF) pathways and subsequent production of pro-inflammatory cytokines and type I IFNs (Figure 14) [243, 245-247]. Rather than recognizing *one* specific epitope (which is the case with adaptive immunity), these receptors recognize a broad range of conserved molecular moieties associated with "danger". Thus, the innate immune system is capable of recognizing a vast array of PAMPs and DAMPs using a limited set of receptors. This forms the basis of the rapid response elicited by innate immune cells.

The Toll-like receptor family (TLR) is regarded as the archetypical PRR, which recognizes a vast array of PAMPs as well as DAMPs (Figure 14) [243, 244]. Interestingly, TLR signaling is emerging as one of the most important links between chronic inflammation, tumorigenesis and tumor progression.

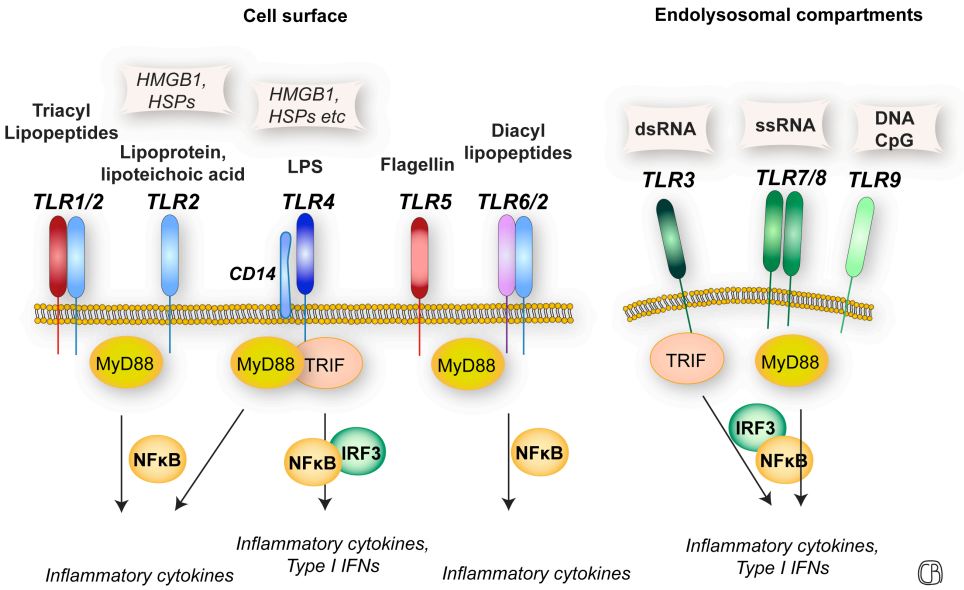

Figure 14. An overview of the mammalian Toll-like receptors and their ligands. TLRs can be divided into extracellular (TLR1, TLR2, TLR4, TLR5, TLR6) and intracellular (TLR3, TLR7, TLR8, TLR9). Extracellular TLRs recognize various bacterial and fungal moieties as well as extracellular DAMPs (*boxes*) whereas intracellular TLRs recognize viral patterns and intracellular DAMPs. Downstream signaling is mediated via signaling through adaptor proteins MyD88 or TRIF. MyD88-dependent pathway induces inflammatory cytokines via activation of NFκB whereas TRIF-dependent pathways induce type I IFNs (via IRF3) or pro-inflammatory cytokines (via NFκB).

Sepsis

"Ignorance is not bliss where microbes are concerned"
-Bruce Beutler, 2007

The link between chronic inflammation and cancer is firmly established. However, despite displaying different kinetics, there are also striking similarities between acute infections with subsequent resolution of the inflammatory response and the tumor-induced immune alterations. During normal immune responses, challenge with pathogens induces a vigorous pro-inflammatory reaction with associated removal of the threat. Although essential, this response may cause tissue damage if not tightly regulated. Therefore, a homeostatic mechanism is activated in parallel with the pro-inflammatory response, with the main aim to limit the inflammation, and initiate tissue regeneration and angiogenesis. It is tempting to speculate that it is this homeostatic mechanism that is triggered in tumor-associated immune cells and exploited by the neoplastic cells.

What is Sepsis?

Activation of TLR signaling in innate immune cells may initiate a widespread systemic inflammatory response syndrome (SIRS) if the pathogen is not immediately contained and eliminated. During this hyper-inflammatory state, monocytes-macrophages and neutrophils release massive amounts of pro-inflammatory cytokines (TNFα, IL-6, IL-8, IL-1β and IFNγ) that augment recruitment and activation of leukocytes. SIRS can be caused by several pathological events including trauma, pancreatitis (*i.e.* DAMP-triggered) and infection (*i.e.* PAMP-triggered). A systemic inflammatory response to infection is diagnosed as *sepsis* [248]. Originally, sepsis was thought to be the result of pathogens spreading in the blood stream (hence the layman's term "blood poisoning"). However, many patients died even after successful treatment with antibiotics. This led to the hypothesis that it is the host's response to the pathogen that is driving the pathogenesis of sepsis, rather than the actual pathogen itself.

Etiology, diagnosis and treatment

The most common cause of sepsis is infection with gram-negative or –positive bacteria, although fungal and viral infections have also been reported to cause sepsis [249-251]. The clinical manifestations are highly variable due to different causative organisms (pathogen load and virulence), site of infection (most commonly lungs and urinary tract) as well as general health status of the patient [250, 252]. Depending on the severity of the clinical symptoms, the syndrome is classified as sepsis, severe sepsis (sepsis with additional organ dysfunction) or septic shock (severe sepsis with persistent hypotension) (Table 2) [248]. In the United States, approximately 750 000 cases of severe sepsis are diagnosed annually, with a 20-30% mortality rate [250, 252]. Early treatment is of paramount importance and includes fluid replacement and immediate control of pathogens using antibiotics [250]. Despite adequate treatment, however, patients with septic shock often succumb to the disease due to multi-organ dysfunction. Altogether, this makes sepsis mortality one of the leading causes of morbidity and mortality worldwide [250, 252].

Table 2. Clinical definition of sepsis [248].

Clinical criteria for SIRS
Two or more of the following symptoms: Body temperature: >38°C or <36°C Heart rate: > 90 beats/minute Respiratory rate: > 20 breaths/minute or $PaCO_2$ <4.3 kPa White blood cell count: >12x10^{9}cells/L, < 4x10^9 cells/L or >10% of immature cells (*i.e.* left shift)
Clinical criteria for sepsis
SIRS + suspected/clinically verified infection
Clinical criteria for severe sepsis
SIRS + infection Either hypotension, hypoperfusion or organ dysfunction
Clinical criteria for septic shock
SIRS + infection Hypotension despite adequate fluid replacement Either hypoperfusion or organ dysfunction

SIRS and CARS

Although a crucial entity in host defense, inflammation may also pose a severe threat to the patient by causing collateral tissue damage, multiple organ failure and even death. Consequently, tight regulation of inflammatory processes is essential. Oversimplified, sepsis can be regarded a two-wave process (Figure 15). First, the above-mentioned pro-inflammatory response in order to eliminate the pathogen and leading to SIRS if the threat is sufficiently severe. This phase is believed to be responsible for the organ dysfunction or failure observed in many sepsis patients as well as early sepsis-related death [251, 253]. The second phase, termed compensatory anti-inflammatory response syndrome (*CARS*), is elicited as an attempt to restore homeostasis by counteracting the pro-inflammatory response [253, 254]. CARS can be equally detrimental as SIRS, since excessive immunosuppression (sometimes called immune paralysis) renders the patient either unable to clear the primary infection or susceptible to secondary infections for which no immune response is possible [253, 255].

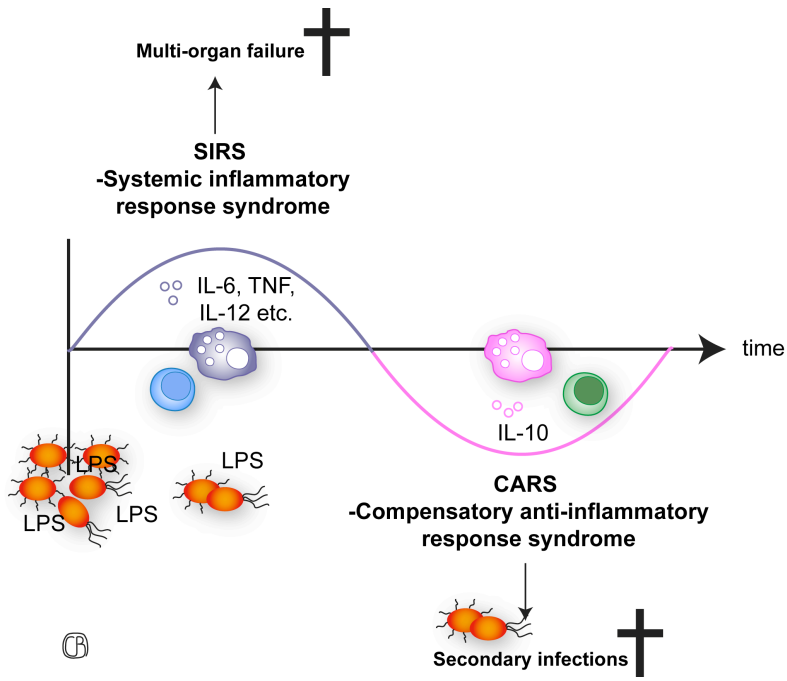

Figure 15. Simplified course of disease in sepsis. Upon infection, a massive pro-inflammatory response is elicited. This may result in a severe systemic syndrome (SIRS) as well as multi-organ failure or death. In order to dampen this response, a compensatory anti-inflammatory response (CARS) is activated. This immunosuppressive state may also result in death due to inability to clear primary or secondary infections.

Although generally described as two separate phases, CARS most likely occurs in parallel with SIRS in patients with severe infections [256]. If the balance and thus homeostasis is not restored, organ dysfunction (predominantly due to SIRS) or immunosuppression (due to CARS) ensues.

Endotoxin Tolerance –the reprogramming of monocytes

Several immune dysfunctions have been described in sepsis patients. These include apoptosis of lymphocytes [257, 258], decreased number of DCs [259, 260], decreased production of pro-inflammatory cytokines by PBMCs in response to LPS *ex vivo* [261] and reduced capacity of APCs to present antigens [262]. On the other hand, the frequency of circulating Treg and MDSCs has been proposed to increase [255, 263, 264].

The most studied sepsis-induced immune alteration is that of monocytes. As previously stated, monocytes are rapidly mobilized to sites of infections and respond to the danger and an enrichment of $CD16^+$ monocyte populations is frequently observed in the peripheral blood of sepsis patients [265]. However, during persistent exposure to inflammatory substances, a homeostatic program, called *endotoxin tolerance*, is triggered in monocytes. This describes a state of transient hypo-responsiveness to subsequent challenges with endotoxin (*i.e.* LPS) [266]. Endotoxin tolerant monocytes are characterized by a diminished capacity of monocytes to produce pro-inflammatory cytokines (e.g. TNFα, IL-1β, IL-12 and IL-6) and concomitant enhanced production of IL-10 or TGFβ in response to TLR ligands (Figure 16) [261, 266-268]. Apart from this shift in cytokine profile, the main hallmark of endotoxin tolerance is considered to be the down-regulation of the MHC class II molecule HLA-DR on monocytes, which has been shown to correlate with decreased survival [262, 267, 269]. Although the monocyte's capacity to ingest antigens is retained, the loss of HLA-DR expression in combination with the reduced expression of the co-receptor CD86, renders the endotoxin tolerant monocytes unable to stimulate T cell activation and proliferation [267, 270]. Endotoxin tolerant cells also frequently up-regulate genes associated with wound healing (including VEGF and MMPs) and have therefore been described to resemble M2-polarized macrophages [271].

This reprogramming of monocytes from a pro-inflammatory phenotype into an immunosuppressive state is often described as the classical example of how the host avoids excessive immune reactions (*i.e.* switches from SIRS to CARS). *In vitro*, this state of tolerance can be mimicked by pre-treatment of monocytes-macrophages with TLR ligands (PAMPs; e.g. LPS or DAMPs; HMGB1) followed by subsequent treatment with LPS [270, 272, 273]. The importance of this process becomes evident when considering that mice pre-treated with a low dose of LPS and subsequent pre-defined

lethal dose of LPS have a marked increase survival rate when compared to mice without pre-treatment [266, 267].

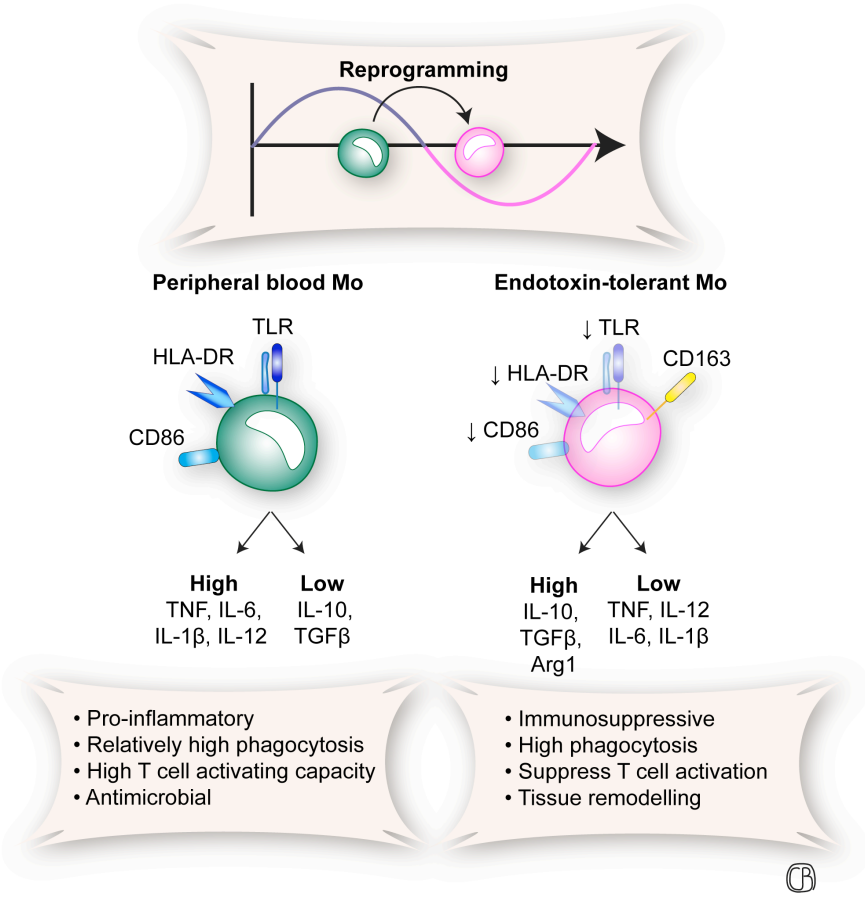

Figure 16. Reprogramming of monocytes towards an endotoxin tolerant state. Mirroring SIRS and CARS, monocytes (Mo) can be reprogrammed by sequential or prolonged exposure to endotoxin. Characteristics of a normal, pro-inflammatory peripheral blood monocyte (*left*) and an immunosuppressive, endotoxin-tolerant monocyte (*right*) are illustrated.

Proposed molecular mechanisms to endotoxin tolerance

Although the underlying molecular mechanism is not fully elucidated, down regulation of TLR4, altered recruitment or activation of TLR signaling mediators as well as accumulation of nuclear p50 homodimers have all been implicated in endotoxin tolerance [167, 272, 274]. The NFκB family member p50 lacks the transactivation domain and may thus be responsible for the reduction in pro-inflammatory cytokines observed in endotoxin tolerant monocytes [272, 274]. This is in accordance with studies implicating p50 homodimers as inhibitors of M1 and promoters of M2 polarization in macrophages as well as the accumulation of p50 homodimers in TAMs [164]. Anti-inflammatory cytokines such as IL-10 have been proposed to induce p50 homodimers, preferentially [164]. Furthermore, p50 homodimers may, in turn, induce transcription of IL-10 and/or Th2-related cytokines, thus favoring immunosuppression [166, 167].

It is interesting to note that these endotoxin tolerant monocytes exhibit immune profiles similar to those associated with Mo-MDSCs. Both of these populations display reduced expression HLA-DR, CD86 as well as diminished production of pro-inflammatory cytokines and capacity to stimulate T cell activation. On the other hand, they retain the capability to produce anti-inflammatory cytokines. In addition, TAMs (with similar cytokine profile and reduced capacity to elicit T cell responses as tolerant monocytes and Mo-MDSCs) frequently display nuclear accumulation of p50 homodimers. It is therefore tempting to speculate that the mechanisms involved in tumor-induced modulation of the immune cells are indeed similar to those observed in sepsis.

Wnt

A Wnt-Wnt situation?

Wnt proteins are a family of conserved glycoproteins implicated in developmental processes. The first mammalian Wnt was identified when investigating the underlying mechanism by which the mouse mammary tumor virus (MMTV) causes mammary carcinoma [275, 276]. Integration of MMTV into the host genome was shown to be associated with activation of the proto-oncogene *int1*, which was later termed *Wnt1* (Wingless-related integration site) due to the homology to the *Drosophila* gene *Wingless* [276]. As of today, 19 mammalian Wnt family members have been identified, all of which share a cysteine rich sequence and a hydrophobic sequence [275, 277, 278]. In addition, many Wnts require post-translational modifications including glycosylation and palmitoylation that is important for secretion and binding to receptors, respectively [279, 280]. Their receptors and co-receptors include the Frizzled (FZD) family of seven transmembrane G-protein coupled receptors, the low-density lipoprotein receptor-related proteins LRP5 and LRP6, the ROR1 and ROR2 receptor tyrosine kinases (RTK) and the RTK-like receptor RYK [281-283].

Wnt proteins are divided into canonical and non-canonical Wnts. The canonical Wnt proteins, such as Wnt1 and Wnt3a, induce β-catenin activation and can transform C57MG mammary cells [277, 284, 285]. The β-catenin-dependent signaling ultimately results in transcriptional activation of genes involved in cell proliferation (e.g. cyclin D) and oncogenesis (e.g. c-Myc) [283]. See Figure 17 for a schematic overview of canonical Wnt signaling.

Non-canonical Wnt signaling, on the other hand, is in essence an umbrella term for all β-catenin-independent pathways. In contrast to canonical Wnts, non-canonical Wnts (such as Wnt5a) are non-transforming and have been proposed to be involved in several processes, many of which are linked to polarized cellular movements [277, 283-286]. Figure 18 summarizes the main β-catenin-independent Wnt signaling pathways. The specific mechanisms by which Wnts signal via canonical or non-canonical pathways is dependent on which distinct Wnts and corresponding receptors are involved (e.g. LRP5/6 preferentially in canonical signaling and ROR2 or RYK in

non-canonical signaling) as well as cellular context and densities of the ligands and receptors [281, 287]. In addition, it should be noted that some Wnts might signal via both β-catenin-dependent and –independent mechanisms, highlighting the complexity of Wnt signaling [281].

Wnt5a

As previously mentioned, Wnt5a is a non-transforming Wnt that signals predominantly through β-catenin-independent pathways [277]. Wnt5a is expressed in a highly regulated pattern during development and tissue homeostasis. The importance of Wnt5a can be exemplified by Wnt5a knockout mice, which die shortly after birth and show several developmental abnormalities such as dwarfism, shortened and deformed limbs and tails, as well as facial abnormalities [277, 288, 289].

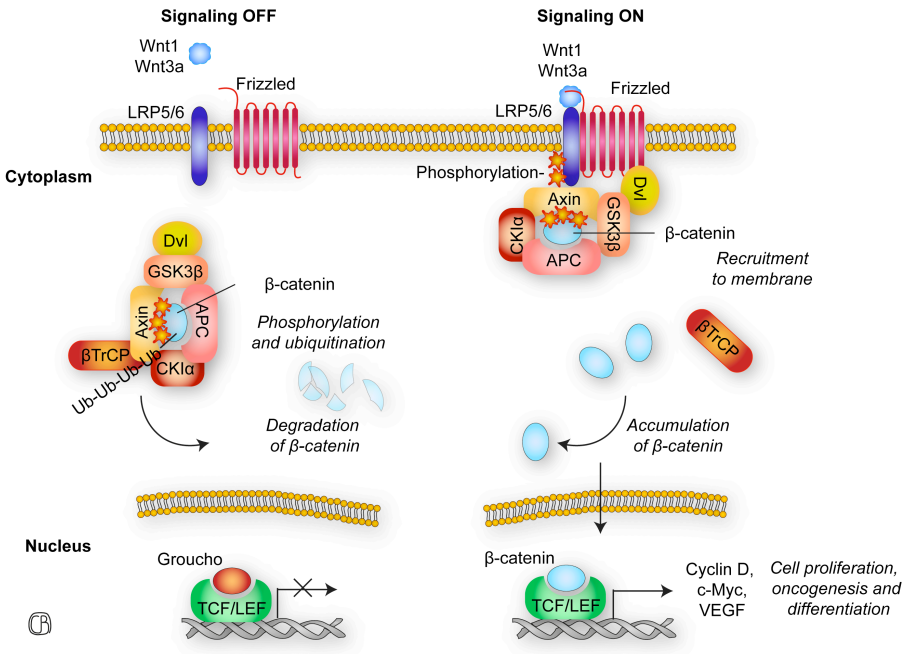

Figure 17. Canonical Wnt signaling. In the absence of canonical Wnts, β-catenin is targeted for degradation via sequential phosphorylation by CKIα and GSK3β and ubiquitinylation by βTrCP. Upon ligation of Wnt to FZD and LRP5/6, the degradation complex translocates to the plasma membrane and newly produced β-catenin is free to translocate to the nucleus and activate TCF/LEF-dependent transcription of target genes, ultimately resulting in cell proliferation, amongst other cellular responses.

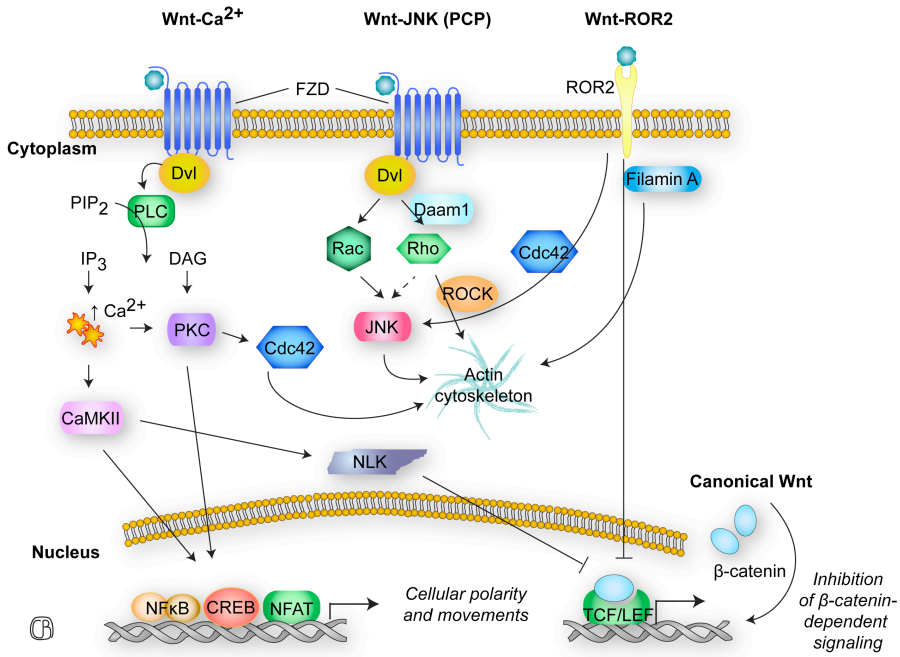

Figure 18. Non-canonical Wnt signaling. Non-canonical Wnts can activate several signaling pathways including Wnt-Ca^{2+}, Wnt-JNK and Wnt-ROR2, all of which regulate cellular polarity and movements. This is generally performed via activation of calcium-dependent kinases (PKC, CaMKII and calcineurin) as well as activation of several regulators of the cytoskeleton (Rho, Rac and filamin A).

Apart from eliciting β-catenin-independent pathways such as Wnt-Ca^{2+}, Wnt-JNK (or Wnt-PCP) and Wnt-ROR2 signaling (Figure 18), Wnt5a may also inhibit β-catenin-dependent Wnt signaling [277, 286]. Given the role of β-catenin-dependent pathways in oncogenesis, it has been suggested that Wnt5a might function as a tumor suppressor. This was further supported by experiments where transfection of mammary epithelial cells with antisense Wnt5a could mimic the effect of Wnt1-mediated transformation [290]. While this may hold true in some tumor models, the opposite has also been observed.

Wnt5a in cancer

Indeed, the role of Wnt5a in tumorigenesis is paradoxical. Down-regulation of Wnt5a has been correlated with poor outcome in colon cancer [291] and breast cancer [292]. In addition, loss of Wnt5a is correlated with early relapse of invasive ductal breast cancer [293] and a Wnt5a mimicking hexapeptide could reduce the formation of breast cancer metastases in a mouse model [294]. In contrast, increased expression of Wnt5a is associated with disease progression and/or reduced survival in melanoma [295, 296]. Similarly, up-regulation of Wnt5a correlates with poor outcome in gastric cancer [297] and non-small cell lung carcinoma [298]. The reason for this discrepancy is unknown, but likely involves the expression of distinct receptors and co-receptors, varying concentration of Wnt5a as well as the presence other mediators and signaling pathways.

Wnt5a in infectious diseases and immune cells

The importance of Wnts in development is well known. However, less is known about the contribution of Wnts in haematopoiesis and regulation of immune cells as well as their function during infectious diseases. Canonical Wnts have been implicated in self-renewal of hematopoietic stem cells as well as being important for T- and B cell development and DC maturation [299]. Wnt5a, on the other hand, has been suggested to inhibit T cell development [300]. With regards to myeloid cells, Wnt5a was significantly up-regulated in monocyte-derived DCs, but not monocyte-derived macrophages [301]. The role of Wnt5a in monocyte differentiation was, however, not investigated. Nevertheless, Wnt5a may be expressed in breast cancer TAMs and it has been suggested to be of importance in macrophage-induced invasiveness of breast cancer cells [302]. In addition, stimulation of macrophages with various microbial stimuli resulted in up-regulation of Wnt5a [303, 304]. This is further supported by the presence of Wnt5a expressing macrophages in the granulomatous lesions of patients with pulmonary tuberculosis [303] as well as in atherosclerotic lesions [305]. Both of these pathological events are inflammatory diseases associated with accumulation of macrophages. Furthermore, although Wnt5a is generally considered to exert its functions in a paracrine or autocrine manner (due to its hydrophobicity), high levels of Wnt5a have been observed in the sera of sepsis patients [304]. Altogether this would suggests that Wnt5a may affect myeloid cells in a pro-inflammatory manner, however, *in this thesis* we show that Wnt5a also has an anti-inflammatory effect during certain conditions.

"The most exciting phrase to hear in science,
the one that heralds new discoveries,
is not 'Eureka!', but 'That's funny…'"
-Isaac Asimov

The Present Investigation

Aims

The general objective of this thesis was to investigate the local and systemic induction of immunosuppressive myeloid cells and their functional impact in breast cancer and sepsis patients.

The specific aims were:

I. To study the effects of Wnt5a on monocyte to macrophage differentiation and polarization in breast cancer and sepsis patients.

II. To study the effects of Wnt5a on monocyte to dendritic cell differentiation in breast cancer and sepsis patients.

III. To study the effects on human monocytes in tumor stroma formation in breast cancer xenograft models.

IV. To study the presence and generation of systemic myeloid suppressor cell populations in the peripheral blood of breast cancer patients.

V. To study the myeloid-derived suppressor cell populations present in the peripheral blood of sepsis patients caused by different microbial agents.

Paper I –Wnt5a induces tolerogenic Mo-M

Background and results

Wnt5a is a secreted glycoprotein that previously was shown to increase in human monocyte-derived macrophages (Mo-M) in response to LPS [303, 304]. However, knowledge about the role of Wnt5a in hematopoiesis is limited. We therefore set out to study whether Wnt5a may affect monocyte to macrophage differentiation or polarization.

Freshly isolated monocytes from healthy donors were differentiated *in vitro* into either pro-inflammatory M1 macrophages or anti-inflammatory M2 macrophages with or without rWnt3a or rWnt5a treatment. For M1 macrophages, two different stimuli-scenarios were used: a typical PAMP (LPS) and a typical DAMP (HMGB1). No effect of Wnt5a was seen in M2 differentiation cultures as assessed by flow cytometry. However, in wells where rWnt5a was added, we observed a significantly reduced M1 yield ($CD14^{+}HLA\text{-}DR^{+}CD80^{+}CD86^{++}$ cells) with a concomitant increase in $CD14^{+}HLA\text{-}DR^{low/-}Co\text{-}receptor^{low/-}$ cells. When performing functional assays on rWnt5a treated M1 differentiation cultures, these cells produced more ROS, IL-10 and TGFβ. In addition, we observed suppressed T cell proliferation as well as decreased cytotoxicity towards human breast cancer cells. Although these were traits of M2 macrophages, these cells did not express CD163 and we concluded that Wnt5a induced a distinctive immunosuppressive monocyte-macrophage population.

NFκB is the predominant mediator of pro-inflammatory signaling. Therefore, we next investigated whether the anti-inflammatory effect of Wnt5a on Mo-M was mediated via NFκB. We observed a significant reduction in NFκB activity and induction of p50 homodimerization in rWnt5a treated macrophages when cultured in a pro-inflammatory environment. The activity could be restored when using an antibody blocking the IL-10R. Similarly, blocking the IL-10R restored the M1 differentiation in rWnt5a treated cultures. Altogether, this suggests that Wnt5a is an anti-inflammatory mediator when in a pro-inflammatory setting and that the effects are executed via IL-10 production and p50 homodimerization, resulting in inhibition of NFκB signaling.

As Wnt5a had previously been suggested to be a pro-inflammatory factor in sepsis [304], we next investigated the effects of Wnt5a on monocytes from sepsis patients. In line with our previous finding, we observed a significant enrichment in peripheral blood $CD14^{+}HLA\text{-}DR^{low/-}$ monocytes. In addition, a $CD14^{+}CD163^{+}$ anti-inflammatory monocyte population was evident, and re-stimulation of monocytes *ex vivo* with

rWnt5a and LPS further increased this population. When looking at a breast cancer tissue microarray, we observed a significant correlation between Wnt5a and $CD163^+$ TAMs, supporting the idea that Wnt5a promotes anti-inflammatory monocyte-macrophages in breast cancer and sepsis patients.

Discussion

Cells of the myeloid lineage are very plastic by nature. Upon entering tissues, monocytes can differentiate into either M1 or M2 macrophages, depending on the character of the environment [148]. The macrophage population present in human tumors is generally skewed towards an M2 phenotype by tumor- and/or stroma-derived soluble factors [116, 154, 157]. Wnt5a is a factor that can be secreted from tumors and that is highly enriched in sera of patients with sepsis [304].

During severe inflammatory conditions, such as sepsis, PAMPs elicit a potent pro-inflammatory response that, if not constrained, may cause tissue damage (SIRS). Therefore, a homeostatic antagonistic mechanism is activated (CARS) in order to limit the inflammatory response and initiate tissue regeneration. The typical example of this is the reprogramming of monocytes during sepsis towards an immunosuppressive state known as endotoxin tolerance upon sequential challenges with LPS [267]. Tumors, on the other hand, are usually rich in DAMPs, especially at necrotic regions [243]. DAMPs have previously been shown to signal through the same receptors as PAMPs (*i.e.* Toll-like receptors; TLRs).

In this paper we show that non-canonical Wnt5a induces a tolerogenic phenotype in human Mo-M in a pro-inflammatory setting (be it exogenous PAMPs or endogenous DAMPs). This was in sharp contrast to previous findings implicating Wnt5a as a pro-inflammatory mediator in Mo-M [303-305]. The molecular mechanism behind this induction (IL-10 production and inhibition of NFκB signaling) was identical to that previously described for endotoxin tolerant monocytes [267]. This indicates that the up-regulation of Wnt5a mRNA seen in monocytes upon LPS treatment may be a homeostatic response destined to limit the inflammatory processes elicited by PAMPs or DAMPs [303, 304]. In addition, this would be in line with a previous report showing that the *Drosophila* WntD acts as a feedback inhibitor of the NFκB homologue Dorsal during embryonic development as well as during infection [306].

Wnt5a has previously been attributed both tumor suppressive and promoting functions in several tumor forms [277]. The tumor cell specific functions of Wnt5a include regulation of cellular polarity, migration and adhesion. The effect of Wnt5a on the tumor microenvironment is, however, unknown. Here we give one possible role for Wnt5a in regulation of tumor-associated immune cells. Wnt5a in combination with other signals within the tumor, such as DAMPs, affects the

leukocyte populations present in an anti-inflammatory manner. Indeed, the correlation between Wnt5a and $CD163^+$ TAMs in breast cancer tissues suggests that Wnt5a may be involved in generation of this tumor-promoting macrophage population. As we did not see any difference in the M2 macrophage generation, it is plausible that this generation occurs in DAMP-rich areas of the tumors, which may otherwise induce generation of M1 macrophages.

Paper II –Wnt5a inhibits Mo-mDC generation

Background and results

In *Paper I*, we showed that Wnt5a inhibits M1 generation from human monocytes. Whether Wnt5a may affect monocyte to dendritic cell differentiation was, however, unknown. A previous study using gene expression profiling of monocyte-derived dendritic cells (Mo-mDC) reported that Wnt5a is highly up-regulated during Mo-mDC differentiation [301]. We therefore hypothesized that Wnt5a could promote Mo-mDC generation *in vitro*.

Firstly, we corroborated the increased expression of Wnt5a in human Mo-mDC compared to human monocytes previously observed [301]. However, when freshly isolated monocytes were induced to differentiate into DCs *in vitro*, rWnt5a inhibited the generation of DCs ($CD14^{+/low}CD209^+$). In addition, a significant enrichment of $CD14^{++}CD209^+CD16^+$ monocytes was observed in Mo-mDC differentiation cultures stimulated with rWnt5a. Stimulation with LPS resulted in a further increase in the $CD14^{++}CD1a^-CD206^+$ monocyte population. In addition, the cells generated in the Wnt5a-Mo-mDC differentiation culture displayed inefficient pinocytosis, relatively good T cell stimulatory capacity and produced both anti- and pro-inflammatory cytokines. This is the phenotype of $CD14^{+/++}CD16^+$ monocytes rather than DCs.

Since high levels of Wnt5a have been reported systemically in sepsis patients [304], we decided to investigate the systemic monocyte- and DC populations present in the peripheral blood of sepsis patients. We noted significant increases in $CD16^+$ non-classical- and intermediate monocytes with a concomitant decrease in circulating myeloid DC populations. Together, this strongly indicates that Wnt5a induces enrichment of monocytes while inhibiting DC generation. In order to further elucidate the underlying mechanism, we analyzed the early effects of Wnt5a on monocytes from healthy donors. When compared with untreated and rWnt3a treated monocytes, rWnt5a-stimulated monocytes produced significantly more IL-6 and

displayed delayed and weakened ERK1/2 activity in response to LPS. Previous studies have shown that IL-6 inhibits, while ERK1/2-P promotes, Mo-mDC differentiation [183, 307]. In line with this, inhibition of IL-6 in Wnt5a-Mo-mDC differentiation cultures using an IL-6 blocking antibody partially restored the Mo-mDC differentiation. Similar results were obtained when monocytes were differentiated into DC in the conditioned media (CM) from the Wnt5a-negative, IL-6 producing breast cancer cell line MDA-MB-231. An enrichment of $CD14^{++}CD1a^{-}CD206^{+}$ monocytes was observed in MDA-MB-231 CM. This population was even more evident in rWnt5a-stimulated MDA-MB-231 CM. Furthermore; neutralization of IL-6 restored the Mo-mDC differentiation, suggesting that Wnt5a may affect Mo-mDC differentiation in breast cancer patients.

Discussion

Dendritic cells are crucial mediators of innate and adaptive immunity. Their principal role is processing and presentation of antigens to T lymphocytes and subsequent activation of antigen-specific T cells. Human peripheral blood DCs are principally divided into plasmacytoid DCs (PDCs) or two types of myeloid DCs (MDC1 and MDC2) [125]. Sepsis is a severe systemic disease, which is associated with reduction of MDCs and enrichment of $CD16^{+}$ monocyte populations [260, 265]. Corroborating these results, we observed a slight reduction in MDC1 and significant enrichment of both intermediate- and non-classical monocytes in the peripheral blood of sepsis patients. We could show that rWnt5a can induce these alterations *in vitro* as we observed a slight decrease in Mo-mDC differentiation with concomitant increase in $CD14^{++}$ monocytes in rWnt5a treated differentiation cultures. Similar to sepsis patients, cancer patients also display altered levels of both MDCs and $CD16^{+}$ monocytes [143, 181]. We therefore investigated whether Wnt5a may induce alterations in Mo-mDC differentiation also in the context of breast cancer. Indeed, Wnt5a augmented the effect of MDA-MB-231 CM with regards to enrichment of monocytes.

Upon extravasation into tissues, monocytes differentiate into either macrophages or DCs [144]. In cancer patients, macrophages are subsequently skewed towards an immunosuppressive and pro-angiogenic phenotype whereas DCs generally are maintained in an immature stage [148, 179]. In breast tumors, the presence of mature DCs correlates with increased overall survival [187]. In this study, we found indications that, in breast cancer patients, Wnt5a may inhibit differentiation of Mo-mDCs. During the work of this project, a similar study was published in which Wnt5a also was shown to inhibit Mo-mDC generation, thus strengthening our findings [308]. This would imply that Wnt5a is involved in maintaining pro-tumorigenic immature myeloid cells while inhibiting the development of anti-tumorigenic mature DCs. Similar to the observations of *Paper I,* Wnt5a promoted the generation of monocytes.

However, due to different microenvironmental signals (Th1 *versus* Th2 signals), the monocytes acquired different phenotypes ($CD14^+HLA\text{-}DR^{low/-}$ compared to $CD14^{++}CD209^+CD16^+$). Again, this indicates that Wnt5a may have different effects depending on the local environment.

Based on the findings described above, we suggest that Wnt5a plays a role in modulating the systemic immune cell populations in sepsis (decrease MDC populations and increase $CD14^+CD16^+$ monocyte populations) as well as in tumor-induced alterations of myeloid cells in breast tumors.

Paper III –Myeloid cells induce tumor stroma formation

Background and results

During the work in *Paper I*, we noticed that in a subset of breast cancer patients, $CD163^+$ TAMs were preferentially located in the tumor stroma. Further analyses showed that the presence of these TAMs in the tumor stroma (but not in the tumor nest) correlated with increased grade, tumor size and reduced breast cancer specific survival [309]. Intriguingly, $CD163^+$ TAMs were associated with basal-like/triple-negative breast cancer (TNBC) and granulin (a factor suggested to activate fibroblasts) while, in contrast, were inversely correlated with Luminal A breast cancer [309, 310]. We were fascinated by this preferential location of the $CD163^+$ TAMs in basal-like tumors and also by the correlation to granulin. Activation of fibroblasts is an important event in the formation of a reactive stroma in cancer patients. Is it possible that $CD163^+$ TAMs are specifically recruited to TNBC and contribute to stroma formation?

In order to further elucidate this, we co-transplanted monocytes (which are proposed to be precursors of $CD163^+$ cells) with either Luminal A (MCF-7) or TNBC (MDA-MB-231) cancer cells into severely immunodeficient mice (NSG; NOD-*scid* IL-$2R\gamma^{null}$ mice) and evaluated the xenotransplants using immunohistochemistry. Firstly, we confirmed that monocytes were indeed present at the tumor site (by detection of $CD11b^+$ and $CD163^+$ human cells). Some of these cells were positive for the pan-macrophage marker CD68 whereas no MDCs ($CD208^+$ cells) were observed, indicating that the majority of the cells were immature monocytes. In addition, both Luminal A and TNBC xeno co-transplants displayed enhanced stroma formation as well as angiogenesis, as assessed by Sirius Red and the mouse endothelial marker

CD34 respectively. However, considerable differences in the cellular composition in the xeno co-transplants were apparent.

Luminal A xeno co-transplants displayed a slight increased expression of αSMA (marker of activated fibroblasts) as well as the proposed EMT marker vimentin (in myeloid cells, but also in non-myeloid cells). TNBC xeno co-transplants, on the other hand, displayed a dramatic increase in αSMA^+ cells (predominantly mouse fibroblasts, but also some human myeloid cells) and recruitment of mouse-derived cells (mouse $\beta 2$-microglobulin$^+$ cells). In addition, the myeloid cells present specifically in TNBC xeno co-transplants expressed the immunosuppressive marker S100A9. Interestingly, monocytes cultured in conditioned media (CM) from two TNBC cell lines exhibited higher survival and proliferative capacity than monocytes cultured in CM from two Luminal A breast cancer cell lines. Accordingly, increased secretion of the myeloid survival factor GM-CSF (as well as the pro-angiogenic cytokine IL-8) was observed exclusively in monocyte co-culture with TNBC cells.

In order to investigate whether monocytes may be specifically recruited to TNBC tumors, we performed trans-well migration assays using CM from Luminal A or TNBC breast cancer cell lines as well as CM from primary human cancer-associated fibroblasts (CAFs) derived from ER^+ or ER^- breast tumors. Interestingly, monocytes preferentially migrated in response to MDA-MB-231-CM and CAF-CM from ER^- breast tumors. Using an angiogenesis protein array on supernatants harvested from ER^- and ER^+ CAFs, mRNA expression data from primary breast cancers (The Cancer Genome Atlas) as well as trans-well migration analyses, we identified CXCL16 as a mediator for the preferential recruitment of monocytes into TNBC tumor stroma.

Discussion

Tumor-associated macrophages (TAM) are the predominant myeloid cell population in solid tumors [98, 156]. The presence of TAMs generally correlates with enhanced angiogenesis, invasion and metastases [148, 158]. However, information regarding the effect of myeloid cells on the formation of the tumor stroma is limited.

Here we showed that monocytes are preferentially recruited into TNBC. Increased recruitment of murine cells to the tumor site as well as enhanced expression of αSMA in xeno co-transplants indicates that myeloid cells participate in the formation of a reactive tumor stroma. αSMA is considered a marker for activated fibroblasts and is frequently expressed on CAFs [58]. A reactive stroma as well as presence of CAFs has been suggested to promote tumor cell proliferation and metastatic potential [41, 47]. Thus, the presence of myeloid cells may affect tumor progression directly and indirectly via recruitment and activation of CAFs.

This is in agreement with our previous study in which $CD163^+$ myeloid cells were located in the tumor stroma and correlated with TNBC/basal-like tumors specifically [309]. TNBC tumors are generally rich in reactive stroma and are associated with a poor outcome for the patient. The finding that myeloid cells participate in the formation of the reactive stroma may open up for new forms of treatment. In addition, the induction of immunosuppressive ($S100A9^+$) cells in TNBC specifically suggests that TNBC may be more potent in inducing an immunosuppressive environment, which in turn favors tumor progression.

CXCL16 is a chemokine previously reported to recruit lymphocytes. Here we also identify CXCL16 as a chemo-attractant for monocytes. The presence of CXCL16 producing ER^- CAFs in the tumor stroma could explain the stromal location of $CD163^+$ myeloid cells in TN tumors [309].

Altogether, this study suggests that monocytes are recruited to the tumor site by CXCL16, which may affect angiogenesis as well as the formation of a reactive tumor stroma in TNBC/basal-like tumors specifically.

Paper IV –Mo-MDSCs increase in breast cancer patients

Background and results

Myeloid-derived suppressor cells (MDSCs) are known to expand in several forms of malignancies [194]. In *Papers I-II* we showed that myeloid cells are affected locally and systemically in breast cancer and sepsis patients respectively. We also reported that a tumor-derived factor (Wnt5a) induces immunosuppressive monocytes ($CD14^+$HLA-$DR^{low/-}$Co-$receptor^{low/-}$) *in vitro* and that this population resembles the reprogrammed, endotoxin tolerant, monocyte population observed in the peripheral blood of sepsis patients [267]. In *Paper IV* we aimed to investigate the presence of systemic myeloid suppressor populations in the peripheral blood of breast cancer patients. Specifically, we studied the occurrence of monocyte subpopulations and monocytic MDSCs (Mo-MDSCs; $CD14^+$HLA-$DR^{low/-}$Co-$receptor^{low/-}$). In addition, we where intrigued by the possibility that these cells might be generated by similar mechanisms in breast cancer as in sepsis (*i.e.* by reprogramming of monocytes).

We therefore enriched for peripheral blood mononuclear cells (PBMCs) from patients with early (primary) and advanced breast cancer (patients with locoregional recurrence or metastatic breast cancer; LRR/MBC) as well as healthy donors using Ficoll density centrifugation. The leukocyte populations present were analyzed using

flow cytometry. Several populations were affected in breast cancer patients including reduced numbers of $CD3^+$ T cells, $CD3^-CD56^+$ NK cells, $CD3^+CD56^+$ NKT cells and myeloid dendritic cells (MDCs). Although we could not detect any significant differences in the monocyte subpopulations investigated, we noticed that the monocytes enriched from breast cancer patients (early as well as advanced) were affected functionally; they displayed reduced production of pro-inflammatory cytokines, increased production of anti-inflammatory and pro-angiogenic cytokines, inhibition of T cell proliferation and reduced spontaneous tumoricidal properties.

Although the monocytes were affected early at a functional level, the typical surface profile of Mo-MDSCs appeared as the disease progressed. Indeed a significant enrichment of Mo-MDSCs was apparent in patients with LRR/MBC. The presence of Mo-MDSCs significantly correlated with metastatic disease (number of metastatic sites, metastases to lymph nodes and a borderline significance to visceral organ metastases). No correlation to age, previous adjuvant therapy or tumor size was observed, however, patients with ER-negative tumors were overrepresented in the patient group with high frequency of Mo-MDSCs.

In *Papers I-II* we noticed striking similarities between the leukocyte profiles in breast cancer and sepsis patients. In this paper, we corroborated these results and could further show that the gene expression profile of monocytes from breast cancer patients was similar to that of monocytes from sepsis patients.

Discussion

Human MDSCs are immature cells of the myeloid lineage with potent immunosuppressive properties [194]. In humans, two populations have been characterized; granulocytic MDSCs (G-MDSCs; $CD33^+CD11b^+Lin^-$) and Mo-MDSCs ($CD14^+HLA\text{-}DR^{low/-}Co\text{-}receptor^{low/-}$). G-MDSCs have previously been shown to be enriched in the peripheral blood of breast cancer patients and to correlate with adverse outcome [199]. Whether Mo-MDSCs are also enriched is currently unknown. In addition, the underlying mechanism to systemic MDSC generation is relatively unexplored.

In this paper we expanded our previous hypothesis that immunosuppressive monocytes (or Mo-MDSCs) from cancer patients may be generated by similar mechanisms as endotoxin-tolerant monocytes from sepsis patients (see *Paper I*). Firstly, we found that, similar to G-MDSCs, Mo-MDSCs are indeed enriched in breast cancer peripheral blood. Furthermore, Mo-MDSCs correlated with disease progression and exhibited an immunosuppressive profile (as judged by cytokine release, reduced capacity to elicit T cell proliferation and reduced spontaneous tumoricidal properties).

In sepsis, the immunosuppressive reprogramming that constitutes the endotoxin tolerance process is known to be induced by sequential stimulation with LPS (the typical PAMP), or even pre-treatment with the typical DAMP; HMGB1 [272, 273]. This results in an immunosuppressive phenotype characterized by down-regulation of TLR4 (receptor for LPS as well as HMGB1), inhibition of NFκB signaling and concomitant decreased production of pro-inflammatory cytokines and increase of IL-10 production [267]. Similarly, monocytes from breast cancer patients displayed an immunosuppressive phenotype. PAMPs and DAMPs elicit close to identical signaling pathways in monocytes and macrophages. As tumors are rich in DAMPs, it is likely that Mo-MDSCs are generated in a similar manner as the sepsis-associated endotoxin-tolerant monocytes. By means of gene expression microarray, a remarkable similarity was apparent between breast cancer- and sepsis-derived monocytes. This would indeed suggest that the mechanism of generation is similar between these diseases.

Paper V –MDSC populations vary in sepsis patients

Background and results

MDSCs have long been thought to expand in cancer patients specifically. However, recent studies have proposed that MDSCs may also have a role during severe infections [194]. Whether functional MDSCs, similar to those seen in cancer patients, exist also in sepsis patients is, as of yet, largely unexplored. In *Paper V* we aimed to investigate the presence of Mo-MDSCs and G-MDSCs in patients with sepsis in response different causative agents.

Peripheral blood from patients with gram-negative and gram-positive sepsis was collected and subjected to Ficoll-density centrifugation. Using flow cytometry, we found that $CD14^{+}HLA\text{-}DR^{low/-}$ monocytes (similar to the Mo-MDSCs seen in cancer patients) were preferentially enriched in patients with gram-negative sepsis, although also present in gram-positive sepsis, but to a lesser extent.

During the work in *Paper II* and *IV*, we noticed an enrichment of cells in the granulocyte region in Ficoll-enriched PBMCs in patients with breast cancer and sepsis. This low-density granulocyte fraction has previously been shown to harbor MDSCs [192]. Therefore it was also possible to study the G-MDSC population in Ficoll-treated blood. In contrast to Mo-MDSCs the "classical G-MDSCs" ($CD11b^{+}CD33^{+}Lin^{-}CD15^{+/low}$), were significantly increased in patients with gram-

positive sepsis when compared to healthy controls and patients with gram-negative sepsis. In addition, a population of $CD14^{low}CD64^{low}$ cells was enriched in patients with gram-positive sepsis. This population was, however, difficult to detect in whole blood samples (non-Ficoll enriched samples). In order to characterize this population, $CD33^{+}CD14^{low}CD64^{low}$ cells and $CD33^{+}CD14^{+}CD64^{+}$ cells were sorted using FACS and subjected to further functional and morphological analyses. Similar to "classical G-MDSCs", $CD14^{low}CD64^{low}$ cells inhibited T cell proliferation as measured by ^{3}H-incorporation of CD3/CD28 stimulated allogeneic T cells, strongly suggesting that these are also G-MDSCs (although with slightly elevated CD14 expression). The inhibition was not due to production of immunosuppressive cytokines by G-MDSCs, as these cells released only minor amounts of all cytokines investigated. The relative IL-10:TNFα release was, however, increased, indicating that they are immunosuppressive.

In contrast to breast cancer G-MDSCs ($CD14^{low}CD64^{low}$ cells), the G-MDSCs derived from patients with gram-positive sepsis were morphologically very heterogeneous. Hematoxylin/eosin stained cytospins revealed that $CD14^{low}CD64^{low}$ cells from gram-positive sepsis contained more blast-like G-MDSCs, but also some cells with the ring-shaped nuclei typical for cancer-associated G-MDSCs. Altogether, our data suggest that $CD14^{low}CD64^{low}$ cells are immature myeloid cells with immunosuppressive properties.

Discussion

During severe infections such as sepsis, the massive pro-inflammatory response (SIRS) following the insult must be contained as soon as the threat is eliminated in order to prevent tissue or organ damage. Therefore, an antagonistic anti-inflammatory response (CARS) is induced to dampen excessive immune reactions. Although a monocyte population similar to Mo-MDSCs has been characterized in the peripheral blood of sepsis patients, little is known about MDSCs in this disease. However, as MDSCs frequently produce high amounts of antimicrobial reactive oxygen species (ROS), it is tempting to speculate that these populations may suppress the pro-inflammatory response while still capable of eliminating the bacteria.

Gram-positive and gram-negative bacteria elicit pro-inflammatory signals through partially overlapping sets of TLRs (TLR1, -2, -5 and -6 for gram-positive bacteria and TLR1, -2, -4, -5 and -6 for gram-negative bacteria). It is possible that these signals trigger generation of MDSCs in different manners. This is supported by the preferential enrichment of Mo-MDSC in gram-negative sepsis and G-MDSCs in gram-positive sepsis samples.

Traditionally, G-MDSCs have been characterized as negative for all lineage markers. However, in this study we found functionally immunosuppressive G-MDSCs expressing low levels of the monocyte-macrophage marker CD14. Thus, excluding CD14 in the analysis could lead to underestimations of the number G-MDSCs in patient peripheral blood. In addition, this population was enriched in the low-density granulocyte fraction of Ficoll-treated blood, whereas it was more difficult to detect in whole blood samples. As non-Ficoll treated samples are commonly used in the clinic, it is possible that this G-MDSC population is overlooked when analyzing MDSC populations. By adding a Ficoll-density centrifugation step, it is possible to enrich for G-MDSCs in patient peripheral blood and thus to better assess the immune cell profiles of these patients.

Conclusions

I. Wnt5a induces tolerogenic human Mo-M in a pro-inflammatory environment via inhibition of NFκB signaling and IL-10 induction.

II. Wnt5a inhibits the generation of Mo-mDC in an IL-6 dependent manner, while promoting generation of $CD14^{+/++}CD16^{+}CD209^{+}$ monocytes.

III. Monocytes are selectively recruited to triple-negative breast tumors by CAF-derived CXCL16 and may augment the formation of an activated stroma.

IV. Functional Mo-MDSCs arise early during breast cancer. The typical Mo-MDSCs surface profile correlate with disease progression and gene expression profiling strongly indicates that Mo-MDSCs derive from reprogrammed monocytes.

V. Distinct MDSC populations are enriched depending on microbial agent. Immunosuppressive G-MDSCs are preferentially enriched in patients with gram-positive sepsis and comprise a heterogeneous population expressing low levels of CD14.

Populärvetenskaplig Sammanfattning

Normalt sett skyddar immunförsvaret oss mot farliga och främmande ämnen. En cancercell uppfattas oftast som något defekt och farligt som måste elimineras. Men då och då lyckas cancerceller överleva och manipulerar då vårt immunförsvar till det sämre för oss.

Cancer är ett samlingsnamn på sjukdomar orsakade av en ansamling av genetiska förändringar. Dessa förändringar leder till att cellernas inbyggda kontrollsystem störs och cellerna börjar dela sig ohämmat och en tumör bildas. I flera årtionden har forskningen fokuserat på att hitta sätt att hindra cancercellers förmåga att bli självförsörjande på näringsämnen och att spridas till andra organ. Men en tumör består inte enbart av cancerceller. Friska celler från omgivningen där tumören växer, den så kallade mikromiljön, kan fastna eller till och med lockas till och inkorporeras i tumören. I många fall är antalet friska celler långt fler än antalet cancerceller i tumören. På senare tid har det visat sig att dessa friska celler från mikromiljön i hög grad kan påverka sjukdomsförloppet.

Bland de vanligaste cellerna i tumörers mikromiljö är olika typer av immunceller (t.ex. makrofager och dendritceller). Trots att tumörer uppstår från individens egna celler, så kan immuncellerna känna igen cancerceller som något farligt som måste avlägsnas. ”Farosignaler” på och omkring cancercellerna kan aktivera immunförsvaret och därför dödas troligen majoriteten av alla cancerceller innan de hunnit bilda en tumör. Men somliga cancerceller lär sig att manipulera immunförsvaret. Genom att släppa ut specifika ämnen kan tumörer omprogrammera immuncellerna och därmed stänga av försvaret mot cancercellerna. Som om det inte är illa nog så lurar tumören dessutom immuncellerna till att producera ämnen som främjar tumörtillväxten och prognosen blir nu sämre. Immunförsvaret förråder cancerpatienten!

Men hur kan detta ske? Normalt sett när en immuncell aktiveras, så startar en massiv reaktion för att avlägsna hotet mot individen (d.v.s. bakterier, virus eller cancerceller). Denna reaktion måste stoppas så snart hotet är borta för att undvika att friska celler skadas. Vid allvarliga infektioner som blodförgiftning (sepsis) är det av särskild vikt att kroppen reagerar kraftigt för att ta bort infektionen, men det är av lika stor vikt att den kraftfulla immunreaktionen stängs av för att undvika att viktiga organ påverkas eller till och med förstörs. Därför är immunceller skapade för att snabbt kunna

omprogrammeras (stängas av) samt att påbörja läkningsprocesser för att återställa den normala balansen igen. Baserat på detta lade vi fram hypotesen att det är dessa normala processer, som aktiveras i de omprogrammerade immuncellerna, som kan utnyttjas av cancercellerna. Genom att använda blodförgiftning som modell för avstängning av immunförsvaret studerade vi hur denna process fungerar i bröstcancer.

I den här avhandlingen studerar vi samspelet mellan bröstcancerceller och en specifik grupp av immunceller (myeloida celler). I Artikel I-II fokuserade vi på en typ av myeloida celler (monocyter) som när de lämnar blodet och går in i vävnader kan mogna ut till antingen makrofager eller dendritceller (DC). Förenklat kan dessa celler bli till celler som antingen dödar tumörceller (pro-inflammatoriska M1 makrofager och mogna DC) eller celler som främjar tumörtillväxten (anti-inflammatoriska M2 makrofager eller omogna DC), beroende på den lokala mikromiljön i vävnaden. Exakt vad som påverkar uppkomsten av tumör-främjande myeloida celler är fortfarande något oklart.

Wnt5a är ett ämne som kan produceras av tumörer och även av immunceller under allvarliga infektioner såsom blodförgiftning. I *Artikel I* fann vi att Wnt5a hindrar monocyter att mogna ut till tumördödande M1 makrofager i en miljö som borde gett M1 makrofager. Istället gynnade Wnt5a bildandet av tumörfrämjande monocyter (immunosuppressiva monocyter). Denna population var slående lik de omprogrammerade monocyterna som andra forskargrupper har identifierat i patienter med blodförgiftning. Utöver detta kunde Wnt5a även hindra monocyterna från att mogna till dendritceller (*Artikel II*). När vi injicerade monocyter tillsammans med olika typer av bröstcancerceller (av luminal eller basal typ) i möss som saknar egna immunceller, kunde vi se att monocyterna främjade bildandet av stödjevävnad i basala brösttumörer (*Artikel III*). Bröstcancer av denna typ kunde dessutom själva rekrytera monocyter, vilket tyder på att en patients egna monocyter kan luras till cancercellerna och där påverka bildandet av en tumör.

Eftersom vi i Artikel I kunde se att ämnen producerade av tumörer kan gynna uppkomsten av tumör-främjande monocyter, ville vi i *Artikel IV* se om dessa tumör-främjande monocyter även fanns i bröstcancerpatienter. Baserat på monocyternas egenskaper kunde vi se att de var påverkade tidigt under sjukdomsförloppet, men att den typiska omprogrammeringen vi såg i Artikel I uppkom något senare. Vi kunde även se en slående likhet mellan de tumörfrämjande monocyterna (även kallade myeloida suppressorceller) och de omprogrammerade monocyterna i blodförgiftningspatienter, vilket tyder på att uppkomsten av dessa celler är liknande. Att myeloida suppressorceller verkar finnas i sepsis är känt sedan tidigare. Men när vi undersökte detta närmare såg vi att andelen av de olika typerna av suppressorceller skilde sig beroende på vad som orsakade sjukdomen (gram-positiva eller gram-negativa bakterier; *Artikel V*).

Sammanfattningsvis har vi visat att myeloida celler är påverkade både i bröstcancer och i blodförgiftning (*Artikel I-V*). Wnt5a bidrar till uppkomsten av tumörfrämjande immunceller (*Artikel I*) och hindrar uppkomsten av immunceller som kan reagera mot tumörer (*Artikel II*). Monocyter kan påverka bildandet av stödjande vävnad i tumörer (*Artikel III*) och är omprogrammerade till en tumörfrämjande typ i bröstcancerpatienter (*Artikel IV*). Beroende på typ av stimulering så kan olika typer av suppressorceller anrikas även i patienter med blodförgiftning (*Artikel V*).

Acknowledgements

This work was carried out at Center for Molecular Pathology, Department of Laboratory Medicine, Malmö, Lund University, Skåne University Hospital, Malmö, Sweden. Financial support was provided by the Swedish Cancer Foundation, the Medical Research Council, Malmö Allmänna Sjukhus Cancer Research Foundation, Gunnar Nilssons Cancer Foundation, Ollie och Elof Ericssons Foundation, Kockska Foundations, Åke Wibergs Foundation, Alfred Österlunds Foundation and Gyllenstiernska Krapperups Foundation.

I would like to take the opportunity to express my deepest gratitude to the many people who have supported me throughout the years (and hopefully without forgetting someone..!):

Karin Leandersson, my supervisor! Vår resa började med orden ”Oj, det har jag glömt att säga till dig –men jag har ju tänkt att du ska börja hos mig”. Och vilken resa det har varit! Din entusiasm för forskning, för ”Mickey Mouse experiment” och din förmåga att hitta något positivt även i den fulaste blotten är helt fantastisk. Jag känner mig priviligerad att ha haft dig som mentor och jag har lärt mig så otroligt mycket av dig! Till och med jag uppskattar ”tvärtom-resultat” nu.

Sven Påhlman, my co-supervisor som alltid ställer de rätta frågorna. (Jag tycker fortfarande inte att de är dumma!) Tack för att du alltid ser saker från en annan synvinkel och peppar oss att sikta högt.

Past and present members of the Leandersson-group: **Caja**, för att du är en sån härlig person som alltid tänker ”outside the box”. **Elin**, för alla Wnt diskussioner, chokladtårta och för NLK-idén. Vi kommer långt med att tänka positivt! **Helena** för alla luncher, sepsis-diskussioner och för att du introducerade mig i infektionsläkarnas spännande värld. **Roni**, för alla sena kvällar i cellodlingen och alla diskussioner om choklad, träning och monocyter.

To all **past and present colleagues at CMP** for a nice working atmosphere, late nights in the lab, all Friday seminars, Breakfasts, Spring/Easter/Christmas get-togethers and MCC retreats –you are just the best!

Kris, oh how boring this journey would have been without you! Tack för att du alltid finns där som stöd, proof-reader och för att du försöker lära mig om bioinformatik. Du kan verkligen förgylla även den mörkaste labb-dagen! **Sofia**, finaste Sofini som funnits där hela vägen. För all pepp och sympati-depp när det gått dåligt, men framförallt för alla roliga retreats och AWs. High five till dig på alla sätt och vis! **Greta**, ma chérie, för alla fikor, AWs och härliga spontan-kramar i labbet. **Peppe**, för att jag alltid kan fråga dig om ”matte och prostata”... Jag kan fortfarande inte räkna till tio men -Chi dorme non piglia pesci!?! **Matilda**, för all handledning som exjobbare och för ditt härligt smittosamma skratt. **Marica**, för sällskap sena kvällar och för att du gillar att pyssla lika mycket som jag. **Sofie M**, min lilla hallonbåt, för diskussioner om allt från möss till Hunger Games. **Louise**, för sällskap vissa sena kvällar och för att du är en lika stor tidsoptimist som jag. **Susann**, for encouraging words and fun times in the lab and at a number of parties. **Tamae**, immune cells rock and so do you! Past and present office-mates **Jennifer**, **Reihaneh**, **Wasi**, **Christian**, **Karna**, **Susan**, **Nick**, **Aseem** and **Helén** (särskilt tack för all hjälp under slutspurten!) for all scientific and not-so-scientific discussions and wonderful time in the office and at AWs. And a special thanks to my “big-sister” **Gina**.

Elisabet J, **Christina**, **Siv**, **Filiz**, **Elise**, **Elisabeth O** och **Kristin** –labben står och faller med er! Tack för all hjälp under åren!

To my co-authors **Anders** (särskilt tack för bra samarbete!), **Ami** (vad hade jag gjort utan ditt kunnande om bröstcancer), **Lisa**, **Marlene**, **Karin J**, **Sabine**, **Björn**, **Helena**, **Kristina**, **Sara, Janne, Christer** and **Sofia**.

Till mina fina biomedicinar-tjejer **Jenny**, **Annelie**, **Iréne** och **Maria**, för allt roligt under utbildningen men framför allt för all fika, alla sockerkickar och diskussioner om forskning, böcker och annat. –MTFBWY!

Till mina underbara nära och kära!

Jennie och **Martin**, för att ni har förståelse för att jag är världens tidsoptimist och ändå förser oss med mat och vin efter långa arbetsdagar. Ni är guld värda!! **Ellie**, **Tobbe**, **Cicci, Kris** (igen) och **Niclas**, för alla härliga middagar, resor, wordfeudande och diskussioner om ditt och datt. **Cissi**, fina vän, oavsett när vi sågs senast känns det alltid som igår! Tack till **Fredrik** som underhåller och ”uppfostrar” Samme och alltid ställer upp för oss. **Jessica** och **Sara**, mina kära särbos! Tack för allt stöd, alla roliga/knasiga/udda utekvällar och mitt-i-veckan-mys i L-krona, HBG och Malmö. ”12 kexchoklad, tack”.

Clara och **Charlotte** –de finaste systrarna och bästaste vännerna någon kan ha. För att ni alltid finns där oavsett tid på dygnet, för att ni tog er tid att göra framsidan och för att ni helt enkelt är fantastiska. Den här avhandlingen är till er!

Mamma och **pappa**, jag hade inte varit här idag om det inte var för allt ert stöd! Tack för att ni tror på mig, uppmuntrar, peppar (även dagar då jag själv tycker att jag inte vill ha nån pepp!) och för att ni alltid frågar ”hur mår cellerna idag då”.

Och sist men inte minst **Samme**, min Tuss! Du hjälper mig upp när jag faller, får mig att skratta när jag behöver det som mest och tar mig tillbaka till verkligheten och nuet när jag snöat in på alltför nördiga saker. Tack för att du finns där varje dag! ♥

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