Immune Tolerance Mechanisms in Human Breast Cancer

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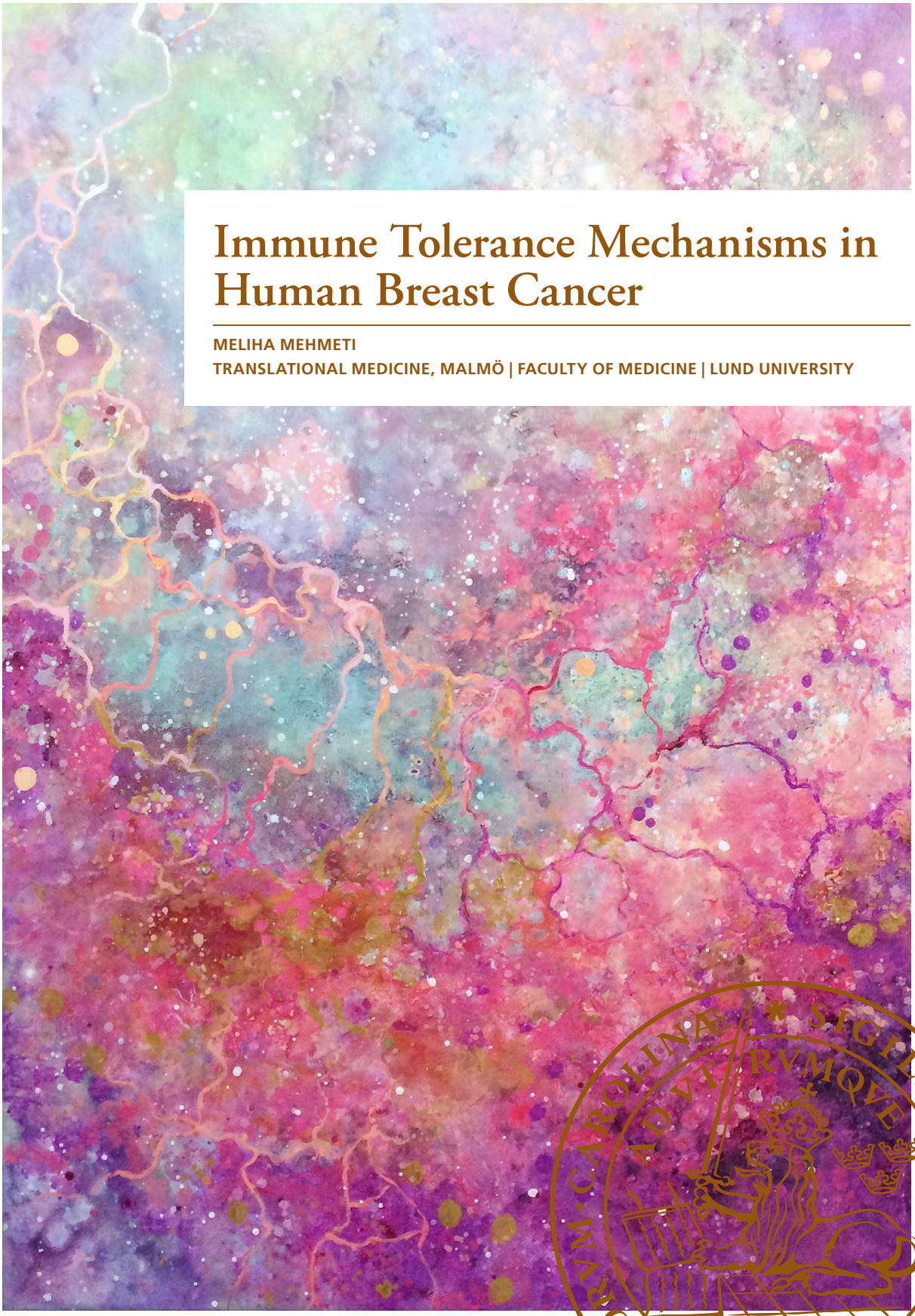

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LUND
UNIVERSITY

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Abstract

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In this thesis we explored various immunosuppressive tolerance mechanisms in the context of breast cancer. We report that the immune cell receptor TLR4 is expressed in human triple-negative breast cancer cells. TLR4 is there able to respond to both PAMPs and DAMPs, inducing inflammatory, tumor-promoting cytokines.

Furthermore, TLR4 expression is correlated with a shorter recurrence-free survival in $ER^{-}PgR^{-}$ breast cancer patients. We further report that S100A9, a TLR4 DAMP, is expressed both by malignant cells and anti-inflammatory $CD163^{+}$ immune cells in breast cancer. S100A9 binding to TLR4 elicits secretion of inflammatory, tumor-promoting cytokines and S100A9 expression is correlated with worse survival in $ER^{-}PgR^{-}$ breast cancer patients.

Further on in this thesis we show that Wnt5a, a factor implicated both in inflammation and cancer, is a novel endogenous TLR2/4 ligand. Through its binding to TLR2/4, Wnt5a is able to act as a DAMP but provokes an anti-inflammatory and a pro-inflammatory cytokine profile in human and mouse immune cells respectively. Moreover, Wnt5a promotes the differentiation of monocytes into an anti-inflammatory Mo-MDSCs cell population.

In the fourth project of this thesis, we studied the presence, origin and tumor-biological functions in breast cancer patients of another MDSC population, G-MDSCs. We report an enrichment of immunosuppressive G-MDSCs in metastatic breast cancer patients, while also showing that G-MDSCs comprise a morphologically heterogeneous population of alternatively-activated neutrophils promoting cell proliferation, angiogenesis as well as inhibiting myeloid immune cell infiltration.

Finally, we report a potential novel immune tolerance mechanism in breast cancer involving the expression of the transcriptional regulator AIRE, a factor with highly important role in central tolerance in the thymus. We show that is it expressed in malignant cells, CAFs and TAMs in breast cancer and that expression of AIRE in infiltrating TAMs is correlated with worse outcome in breast cancer patients.

Collectively, this thesis brings clarity on and introduces novel immune tolerance mechanisms exploited by breast cancer for the purpose of its progression

Key words: Breast cancer, immune tolerance, toll-like receptors, DAMP, Wnt5a, TAMP, myeloid-derived suppressor cells, autoimmune regulator

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List of Abbreviations

AIRE	autoimmune regulator
AP-1	activator protein 1
APC	antigen presenting cell
Arg1	arginase-1
BMM	bone marrow macrophage
BRCA	breast cancer gene
CAF	cancer associated fibroblast
CaMKII	calmodulin-dependent kinase II
CAR	chimeric antigen receptor
CARS	compensatory anti-inflammatory response syndrome
CD	cluster of differentiation
CIS	carcinoma *in situ*
CTL	cytotoxic T-lymphocyte
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
DAMP	damage-associated molecular pattern
DC	dendritic cell
DVL	dishevelled
ECM	extracellular matrix
ER	estrogen receptor
FZD	frizzled
G-MDSC	granulocytic-MDSCs
GSK3β	glycogen synthase kinase-3 beta
HER2	human epidermal growth factor receptor 2
HMGB1	high mobility group box 1
ICI	immune checkpoint inhibitor
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
IRF	interferon regulatory factor
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
LRP	lipoprotein receptor-related protein
M1	pro-inflammatory macrophage
M2	anti-inflammatory macrophage
MAPK	mitogen activated protein kinase
MBC	metastatic breast cancer
MCP-1	monocyte chemotactic protein-1
MDSC	myeloid-derived suppressor cell
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
Mo-MDSC	monocytic MDSC
mTEC	medullary thymic epithelial cell
MyD88	myeloid differentiation primary response 88
NET	neutrophil extracellular trap
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
PD-1	programmed death 1
PD-L1	programmed death-ligand 1
PgR	progesterone receptor
PMN	polymorphonuclear
PRR	pattern-recognition receptor
ROS	reactive oxygen species
SERM	selective estrogen receptor
SIRS	systemic inflammatory response syndrome

TAA	tumor associated antigen
TAMP	tolerance-associated molecular pattern
TAM	tumor associated macrophage
TCR	T cell receptor
TDLU	terminal duct lobular unit
TGF	transforming growth factor
Th	T helper cell
TNBC	triple negative breast cancer
TLR	toll-like receptor
TNF	tumor necrosis factor
Treg	regulatory T cell
TSA	tissue specific antigen
VEGF	vascular endothelial growth factor
Wnt	wingless-related MMTV integration site

Abstract

One of the most critical events in cancer progression involves the acquired ability of the tumor to avoid the immune system. One way to achieve a successful evasion is establishment of an immunosuppressive and tumor-promoting microenvironment.

In this thesis we explored various immunosuppressive tolerance mechanisms in the context of breast cancer. We report that the immune cell receptor TLR4 is expressed in human triple-negative breast cancer cells. TLR4 is there able to respond to both PAMPs and DAMPs, inducing inflammatory, tumor-promoting cytokines. Furthermore, TLR4 expression is correlated with a shorter recurrence-free survival in ER^-PgR^- breast cancer patients. We further report that S100A9, a TLR4 DAMP, is expressed both by malignant cells and anti-inflammatory $CD163^+$ immune cells in breast cancer. S100A9 binding to TLR4 elicits secretion of inflammatory, tumor-promoting cytokines and S100A9 expression is correlated with worse survival in ER^-PgR^- breast cancer patients. Further on in this thesis we show that Wnt5a, a factor implicated both in inflammation and cancer, is a novel endogenous TLR2/4 ligand. Through its binding to TLR2/4, Wnt5a is able to act as a DAMP but provokes an anti-inflammatory and a pro-inflammatory cytokine profile in human and mouse immune cells respectively. Moreover, Wnt5a promotes the differentiation of monocytes into an anti-inflammatory Mo-MDSCs cell population. In the fourth project of this thesis, we studied the presence, origin and tumor-biological functions in breast cancer patients of another MDSC population, G-MDSCs. We report an enrichment of immunosuppressive G-MDSCs in metastatic breast cancer patients, while also showing that G-MDSCs comprise a morphologically heterogeneous population of alternatively-activated neutrophils promoting cell proliferation, angiogenesis as well as inhibiting myeloid immune cell infiltration. Finally, we report a potential novel immune tolerance mechanism in breast cancer involving the expression of the transcriptional regulator AIRE, a factor with highly important role in central tolerance in the thymus. We show that is it expressed in malignant cells, CAFs and TAMs in breast cancer and that expression of AIRE in infiltrating TAMs is correlated with worse outcome in breast cancer patients.

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Populärvetenskaplig sammanfattning

Det är av största vikt att vårt immunförsvar kan skydda vår kropp från fara. För att kunna göra det använder immunsystemet olika receptorer som kan känna igen farosignaler från smittämnen som bakterier och virus (pathogen-associated molecular patterns, PAMPs). Dessa receptorer benämns som Toll-lika receptorer (TLRs) och fungerar som första försvarslinjen genom att känna igen olika faromolekyler. Vid bindning till dessa molekyler framkallar de ett inflammatoriskt svar, vilket i bästa fall eliminerar faran. Inflammatoriska svaret involverar bl.a. produktion av olika pro-inflammatoriska signalsubstanser. Det är samtidigt lika viktigt att immunsvaret är strikt kontrollerat eftersom en för stark inflammation kan leda till förödande skador på kroppen. För att dämpa inflammationen, aktiverar immunsystemet olika tolerans-mekanismer såsom anti-inflammatoriska celler (t.ex. myeloid-derived suppressor cells; MDSCs) som motvikt för att skapa balans.

Cancer är ett samlingsnamn för en grupp komplexa sjukdomar som uppstår som följd av skador i vårt DNA. Under normala förhållanden är celldelning en kontrollerad process som styrs av signaler från omgivningen, men på grund genetiska förändringar (mutationer) kan en cell emellertid uppnå förmågan av okontrollerad och oändlig celldelning och så småningom därmed utvecklas till en tumör. En cancercell skiljer sig från en normal cell; den beter sig annorlunda och ser annorlunda ut, något som immunsystemet reagerar mot. Efter upptäckandet av cancerceller initierar immunsystemet processen för avläsning av tumören där flera olika immunceller rekryteras till platsen för att delta i kampen. I de flesta fall avlägsnas tumören, men i vissa fall kommer tumören undan och med tiden lyckas den manipulera immunsystemet till sin fördel. En viktig del i cancerns framgång hänger på att undkomma immunsystemet, och det gör den bland annat genom att skapa en anti-inflammatorisk mikromiljö som främjar dess tillväxt.

Den här avhandlingen fokuserar på olika mekanismer i förvärvandet av immuntolerans i bröstcancer.

I avhandlingens första del var målet att studera uttrycket och funktionen av olika TLRs i bröstcancer. TLRs utgör en familj av tio olika mönster-igenkännande receptorer (PRRs) som känner igen olika PAMPs och som i vanliga fall utrycks av olika immunceller. På senare år har dock flera studier rapporterat att TLRs även uttrycks av olika cancerformer där de bidrar till cancerprogression. I *Artikel I* visar vi att flera TLRs (TLR2, 3, 4, 9) uttrycks av bröstcancerceller. Uttrycket var mest

uppenbart i cancerceller som tillhör den trippel-negativa bröstcancer-undergruppen, en mycket aggressiv typ av bröstcancer som är förknippad med dålig prognos. Vidare i arbetet upptäckte vi att TLR4 var fullt funktionell med förmågan att framkalla utsöndring av olika cancer-främjande signalsubstanser. Vi fann även att förekomsten av TLR4 är korrelerad med snabbare återfall hos $ER^{-}PgR^{-}$ bröstcancerpatienter.

Fara uppenbaras inte enbart i form av smittämnen. Förutom att aktiveras av PAMPs, kan TLRs även känna igen och aktiveras av kropps-egna molekyler (danger-associated molecular patterns; DAMPs). Vid trauma, stress eller radio/kemoterapi kan vävnader skadas och detta kan i sin tur leda till att celler börjar läcka ut sitt innehåll, bland dem DAMPs. Å andra sidan kan DAMPs aktivt utsöndras av cancerceller och därmed framkalla en steril inflammation. I avhandlingens andra delarbete fokuserade vi på S100A9, en DAMP som binder till TLR4. Tidigare studier har rapporterat att S100A9 är involverad i olika inflammatoriska processer och att den har förmågan att locka till sig anti-inflammatoriska celler (MDSCs). I *Artikel II* fann vi att S100A9 uttrycks av bröstcancer celler och genom att binda till TLR4 inducerar S100A9 utsöndring av olika cancer-främjande signalsubstanser. Dessutom kunde vi också visa att förekomsten av S100A9 är förknippad med sämre överlevnad hos $ER^{-}PgR^{-}$ bröstcancerpatienter. Sammanfattningsvis visar vi att bröstcancerceller har förvärvat en komplett uppsättning av verktyg för att kunna inducera inflammation som främjar dess tillväxt.

I *Artikel III* fokuserade vi på Wnt5a, ett ämne som har kopplats både med inflammation och cancer. Vi visar för första gången att Wnt5a binder till TLR2 och TLR4, och att genom denna bindning är den kapabel att framkalla ett pro-inflammatoriskt (DAMP) och ett anti-inflammatoriskt (tolerance-associated molecular pattern; TAMP) svar i mus respektive humana immunceller. Vi visar även att Wnt5a är förmögen att omvandla pro-inflammatoriska monocyter (vit blodkropp) till anti-inflammatoriska monocyt-MDSCs (Mo-MDSCs). Förekomsten av MDSCs kan vara livsviktigt i sjukdomar som kännetecknas av överdriven inflammation, såsom blodförgiftning, genom att dämpa inflammationen. Men i samband med cancer kan MDSCs förlama anti-cancerimmunsvaret och därigenom bidra till cancerprogression. MDSCs är en heterogen cell population och förutom Mo-MDSCs innefattar den även granulocyt-MDSCs (G-MDSCs). Båda cellerna är kraftigt immundämpande, men i motsats till Mo-MDSCs är G-MDSCs ursprung samt tumör-främjande funktioner i människa mindre studerade. I *Artikel IV* visar vi att G-MDSCs utgör en heterogen cellpopulation av alternativt-aktiverade neutrofiler (granulär vit blodkropp) som är berikad hos metastaserande bröstcancerpatienter. Genom att skapa en bröstcancer-musmodell upptäckte vi att G-MDSCs bidrar till ökad cancertillväxt och bildandet av nya blodkärl. Dessutom hindrar G-MDSCs andra immunceller att infiltrera tumören. Således bidrar G-MDSCs till cancertillväxt.

I sin sökning av fara är det absolut nödvändigt att immunförsvaret kan diskriminera mellan farosignaler och normala kropps-specifika molekylär (själv-antigen) och därmed visa tolerans mot sig själv. För att undvika förödande autoimmuna sjukdomar, måste T celler (antigen-specifik vit blodkropp) gå igenom en mognadsprocess (central tolerans) i thymus där självtoleranta T celler klarar sig och får lämna thymus och självreaktiva T celler avlägsnas. En viktig komponent i mognadsprocessen är Autoimmune regulator (AIRE), ett ämne som uttrycks i thymus. Mutationer i AIRE är kopplad till olika autoimmuna sjukdomar. Ibland kan det hända att självreaktiva T celler undkommer eliminering och tar sig ut i perifera organ. Som backup, har kroppen installerat en perifer tolerans där självreaktiva T celler upptäcks och oskadliggörs ute i kroppen. Man har bland annat upptäckt uttrycket av AIRE i periferin. I sista delarbetet, *Artikel V*, har vi upptäckt att AIRE uttrycks i cancerceller men även i en anti-inflammatorisk, tumör-främjande immuncellpopulation (tumor-associated macrophage, TAM) hos bröstcancerpatienter där uttrycket av $AIRE_{TAM}$ och bröstcancer är korrelerad med sämre prognos. Vi undersöker nu om AIRE i bröstcancer bidrar till immuntolerans genom att avvärja T celler som i normala fall skulle angripa cancern.

Introduction

Cancer

During thousands of years, cancer has proven enigmatic, challenging both physicians and patients. Already in 3000 BC, cancer was documented by Egyptians as an incurable illness. Considered as the second leading cause of death, cancer is estimated to deprive over 9 million lives worldwide [1].

Cancer is a collective term that includes a group of diseases arising as a result of DNA mutations which subsequently leads to an uncontrolled division of abnormal cells. During the course of cell division, errors can occur in the replicating DNA. Additionally, various environmental factors, to which our body is exposed to including smoking and UV-light, can damage the DNA. Under normal circumstances, the multiple DNA repairing mechanisms employed by the cell are perfectly able to repair the damage, however in some cases, the cell fails to repair the damage and normal cells acquire mutations and eventually become cancerous. Cell division without restraint is generated in various ways, including mutations in protooncogenes involved in cell division and mutations in tumor suppressor genes involved in e.g. apoptosis and DNA repair. Loss of function in the latter further potentiates accumulation of a plethora of mutations aiding tumor progression [2]. Additionally, mutations can also be inherited e.g. BRCA1 and BRCA2 in breast cancer.

Besides an aberrant cell division, cancer development and progression involve a multiple-step process. In 2000, Weinberg and Hanahan summarized the main processes in cancer progression into six different events referred to as hallmarks of cancer [3]. Since then, the list has expanded and four additional hallmarks have emerged including the tumor-promoting inflammation and the immune destruction avoidance (**Fig. 1**) [4]. The hallmarks illustrate the complexity and heterogeneity of cancer. For a long time, cancer was believed to solely consist of tumor cells, however, today we know that this is not the case. In fact, cancer constitutes a microenvironment consisting of multiple cell-types, both cancerous and non-malignant, all believed to be involved in a reciprocal communication aiding tumor progression [5]. As part of the tumor microenvironment, immune cells have been shown to play a crucial role in cancer progression and patient survival. In the last two decades, a major effort has been invested in studying the role the immune

system plays in cancer, and this thesis has focused on how the immunosuppressive cells are generated.

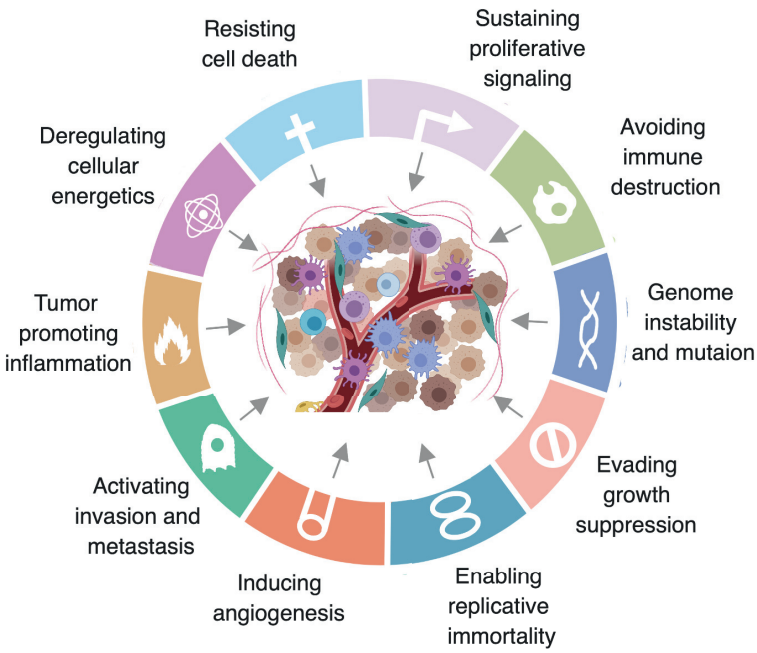

Figure 1. The hallmarks of cancer. Adapted from Hannahan and Weinberg, 2011 [4].

Breast Cancer

Epidemiology and Etiology

Breast cancer is the most prevalent malignant disease in women worldwide. In Sweden alone, over 8900 breast cancer incidences are reported annually, accounting for approximately 30 % of all cancer cases in women. Today, one of ten women are diagnosed with breast cancer and the incidences have constantly been increasing in the last 20 years, most probably due to an increasing aging population and the introduction of screening [6,7]. Nevertheless, the prognosis is generally good with a relative 5-and 10- year survival of approximately 90 % and 80 % respectively [8].

Risk factors associated with breast cancer development are plentiful. As with most cancers, breast cancer is a disease of aging, where around 70 % of all breast cancer incidences are reported in women above 55 years of age. The length of exposure to estrogen hormones has strongly been attributed to breast cancer development, comprising an early menarche, late menopause, late first birth as well as exposure to hormone replacement therapy. Other risk factors include diet, smoking, and mammographic density [9].

Among all risk factors associated with breast cancer development, after age, the genetic predisposition is the most important. It is estimated that around 5 %- 10 % of all breast cancer incidences arise as a result of somatic genetic mutations [10]. *BRCA1* and *BRCA2* are tumor suppressors which play important roles in the maintenance and repair of DNA double strand breaks and somatic mutations in these genes account for around 40 % of all familial breast cancers [11]. This implies that other genes ought to be responsible for the remaining hereditary breast cancers, and although rare, mutations in *PTEN* and *TP53* have also associated with hereditary breast cancer [12].

Breast Cancer Progression

A normal breast comprises two different compartments, a glandular tissue which represent the functional part of the breast and a surrounding stromal tissue. A. mature glandular tissue has a tree-like appearance and consists of a terminal duct

lobular unit (TDLU), the functional unit of the breast, responsible for milk production and transportation. Both the lobules (alveoli) and the ducts are constructed of a lumen lined by an inner luminal epithelial and an outer myoepithelial cell layer and a basement membrane. The surrounding stroma is fat-rich, where fibroblasts, immune cells and blood vessels are also present (**Fig. 2**) [13,14]. Majority of breast cancers originate from the TDLU and the development of breast cancer involves a series of pathological events which start with an appearance of lesions or flat epithelial atypia. At this stage, the cells in questions might display an abnormal response to both growth factors and apoptosis signals. This is followed by atypical hyperplasia and subsequently into the development of carcinoma *in situ* (CIS). The transition between atypical aplasia and CIS is characterised by increased genetic changes and genetic instability. Approximately one fifth of all detected breast cancers upon a mammography screening are accounted for by CIS cases [15,16]. The shift from a carcinoma *in situ* to an invasive cancer involves further recruitment of stromal cells and extracellular matrix (ECM) proteins which aid the invasive process by producing factors that breaks down the basement membrane and thus allowing the spread of malignant cells into the surrounding stroma (**Fig. 3**) [17].

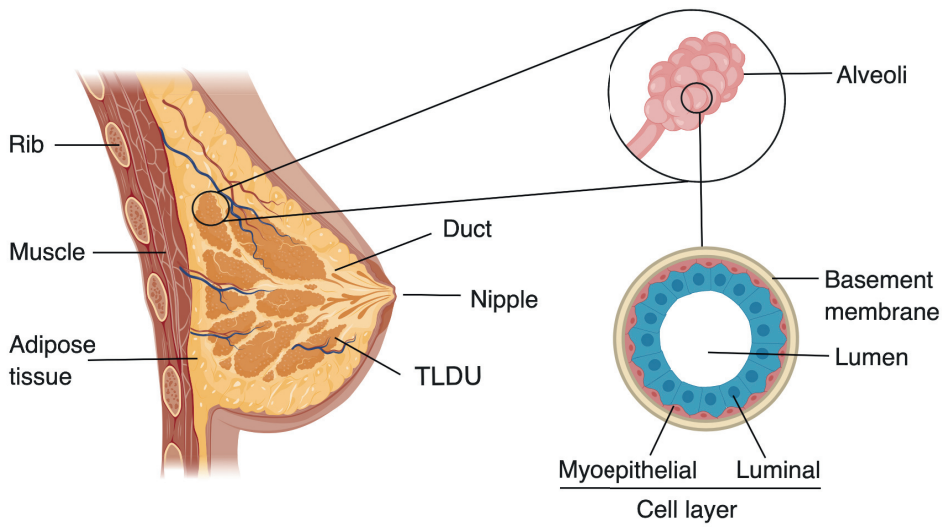

Figure 2. Schematic illustration of the normal breast.
The functional unit of the normal breast comprise the terminal duct lobular unit (TDLU), a branch-like complex extending from the nipple. The TDLU is composed by an inner lumen lined by lumen epithelial cells, myoepithelial cells and basement membrane (BM).

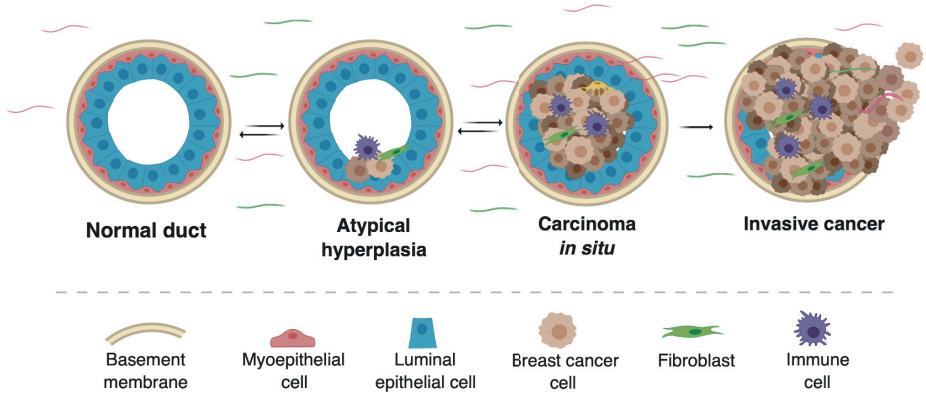

Figure 3. A simplified overview of the breast cancer progression.
The breast cancer progression summerized in four main steps represented by the normal intact duct, the appearance of lesions leading to atypical hyperplasia, followed by carcinoma *in situ* characterized by the gain of genetic instability and recruitment of various immune and stromal cells resulting in invasive cancer featured by the disruption of basement membrane.

Breast Cancer Classification

Breast cancer is a heterogeneous disease. The morphological and immunohistopathological characteristics divides the tumor into several subgroups, providing a useful tool when it comes to the choice of treatment and prognosis.

Histological classification

Based on the growth pattern, tumors can be classified into various histological subgroups, where the two major groups constitute the invasive breast carcinoma and non-invasive carcinoma *in situ.* Around 50-80 % of breast cancers reported are invasive breast carcinomas [18], representing the most common histological type. The invasive breast carcinomas are further sub-classified in smaller groups as reviewed in [19]. In addition, both the invasive and non-invasive carcinomas can origin from the lobular and the ductal unit of the breast [19].

The Nottingham histological grade and TNM staging

The Nottingham histological grading (NHG) is recognized as a valuable prognostic tool when assessing the aggressiveness of breast tumors. The degree of

differentiation of a tumor is evaluated based on morphological characteristics including the mitotic count, tubule formation and nuclear polymorphism upon which the tumor is assigned a grade. The classification system includes grades I-III, where grade I tumors are well differentiated, grade II tumors are moderately differentiated, and grade III tumors are poorly differentiated. The NHG provides predictive information regarding the overall survival and recurrence free survival [20].

Breast cancer progression can further be assessed by the use of TNM staging, which takes tumor size (T), lymph node involvement (N) and distant metastases (M) into account. By combining the values obtained from the three parameters, the clinical stage (I-IV) of the tumor is assessed [21].

Immunohistochemical markers

One of the most important steps in the process of determining the prognosis and the treatment of breast cancer is the assessment of estrogen receptor (ER) and progesterone receptor (PgR) expression as well as the HER2 amplification. ER and PgR expression, which is present among 80% of all breast cancer patients, is evaluated with immunohistochemistry. Patients exhibiting ER/PgR-positive tumors are candidates for endocrine therapy. Furthermore, the amplification status of HER2 gene (*ERBB2*) is assessed, and this helps to determine whether the patient is a candidate for HER2-targeted therapy. Approximately 15 % of all breast cancer patients display HER2 overamplification. Additionally, the expression of the proliferation marker Ki67 is also analyzed [21]. Tumors that lack all three receptors ($ER^-PgR^-HER2^-$) are referred to as triple negative breast cancer (TNBC).

Molecular classification

Gene expression profiling has proven groundbreaking for defining molecular subtypes of breast cancer. As the molecular background of breast cancer provides useful information when predicting clinical features such as the overall survival and relapse-free survival, assessing the differences in molecular characteristics of breast cancer is of high clinical relevance [22]. Today, six different breast cancer subtypes are identified including; Luminal A, Luminal B, HER2 enriched, Basal-like, Normal-like and Claudin-low (summarized in **Table 1**) [19,22,23].

Majority of Luminal A and Luminal B tumors are characterized by positive expression of ER and PgR and the absence of HER2 amplification [21]. While most Luminal A tumors exhibit a low histological grade, a low proliferation index and good outcome, Luminal B tumors on the other hand display a higher histological grade, a high proliferation index and intermediate/poor prognosis [18]. HER2-enriched tumors exhibit a varied ER and PgR expression and, as the name implies, they

display HER2 amplification. Additionally, HER2-enriched tumors display high histological grade and proliferation index and although HER2-enriched breast cancer patients benefit from targeted therapy, the outcome still remains poor [22]. Most Basal-like tumors and Claudin-low tumors lack the expression of ER and PgR and lack over-amplification of HER2 and are commonly referred to as TNBC. Moreover, they are also characterized by a low expression of cell adhesion molecules and elevated mesenchymal features [23]. The scarcity of treatment options due to the lack of drug targetable receptors as well as the tumors display of high histological grade and proliferation, results in Basal-like breast cancer patients having a very poor outcome [18,22,24].

Table 1. Molecular subgroups of breast cancer and their characteristics [18-20,22-24].

Subtype	Molecular profile	Frequency	Histological grade	Proliferation, Ki67	Outcome
Luminal A	$ER^+PgR^+HER2^-$	50-60%	Low	Low	Good
Luminal B	$ER^{+/-}PgR^{+/-}HER2^{-/+}$	10-20%	Intermediate or high	High	Intermediate or poor
HER2	$ER^-PgR^-HER2^+$	10-15%	High	High	Poor
Basal-like	$ER^-PgR^-HER2^-$	10-20%	High	High	Poor
Claudin-low	$ER^-PgR^-HER2^-$	12-14%	High	High	Poor
Normal-like	$ER^{-/+}HER2^-$	5-10%	Low	Low or intermediate	Intermediate

Breast Cancer Treatment

Upon the discovery of breast cancer, the most prominent and common treatment is surgery. Depending on the stage of the tumor, its spread to adjacent lymph nodes and existence of metastases, adjuvant radiation- and/or chemotherapy is introduced to the treatment [25]. Further treatment options include endocrine therapy such as selective estrogen receptor modulators (SERMs) with Tamoxifen and aromatase inhibitors as the most usually employed for ER^+ tumors, and targeted therapy as trastuzumab or lapatinib for HER2-amplified tumors [26,27]. As patients with TNBC do not benefit from the targeted or endocrine therapy mentioned above, the current treatment strategy remains surgery and/or chemotherapy [28]. Nevertheless, Lynparza, a PAPR (poly-ADP ribose polymerase) inhibitor, has been approved earlier this year by the FDA for treatment of breast cancers displaying BRCA1 and/or BRCA2 germline mutations [29]. As around 35 % of TNBCs display mutations in BRCA1 and around 8 % are positive for BRCA2 mutations [30], it is comforting to state that a good portion of TNBC patients might benefit from this treatment in the future. In a phase III clinical trial, metastatic TNBC patients assigned to a combination therapy of atezolizumab (programmed death ligand 1 (PD-L1) inhibitor) and nab-paclitaxel

showed encouraging results of significantly prolonged progression-free survival (PFS) and overall survival (OS) in comparison to patients receiving nab-paclitaxel only. Moreover, the PFS and OS was longer in patients with positive PD-L1 tumors in comparison to patients lacking PD-L1 expression [31].

The Tumor Microenvironment

A tumor aspires to satisfy the requirements needed to tick all the boxes on the list of hallmarks of cancer. However, a tumor cannot sustain a successful growth by itself and therefore relies on help from non-transformed cells; either already present in the tumor microenvironment or acquired to the tumor site. The tumor microenvironment is a dynamic organ comprised of a mixture of malignant and non-malignant cells including cancer-associated fibroblasts (CAFs), various immune cells such as tumor-associated macrophages (TAMs), and cells composing the vasculature, including endothelial cells, pericytes and the ECM (**Fig. 4**) [4,5,32].

Fibroblast are spindle-shaped cells of the connective tissue with important functions in the ECM homeostasis by producing ECM-regulating components such as collagen, fibronectin and matrix metalloproteinases (MMPs). Furthermore, they play an important role in wound repair. During a non-cancerous wound healing process, fibroblasts are transiently activated and are then referred to as myofibroblasts. However, in the context of cancer, the transient activation remains persistent. Consequently, CAFs encompass one of the most prominent non-malignant cells in the tumor microenvironment [32,33]. They are involved in several tumor-promoting processes including chemoattraction, proliferation and invasion by secreting various factors such as CXCL16, TGF-β and MMPs [5,33-35].

A vascular network within the tumor microenvironment is crucial for the prosperity of a tumor as it is a vital source of oxygen and nutrients [36]. This is highlighted by the fact that tumor cells residing too far away from a vessel, or a tumor growing at a faster speed than the vessel formation, often suffers from necrosis [37]. Endothelial cells comprise a fundamental component of the vascular architecture playing an important role in tumor angiogenesis. Tumors secrete various angiogenesis-promoting factors, including the prototypic vascular endothelial growth factor (VEGF). Upon release, VEGF activates endothelial cells to produce MMPs which subsequently breaks down the ECM, facilitating the endothelial cell migration and *de novo* vessel-formation [36].

Another cell type present in the tumor microenvironment, aiding tumor progression are the so called pericytes. In healthy tissues, pericytes can be found lining around the basement membrane of blood vessels, and foremost, exert important functions in vessel development and maintenance [38]. In tumors, the functional role of pericytes is rather unknown, however, they seem to exert a less firm grip and display an

abnormal coverage around blood vessels [39]. For instance, an increased pericyte coverage stabilizes the vessels, thus supporting tumor growth [40]. On the other hand, a low pericytes coverage results in increased vessel permeability and leakage favoring tumor invasion and metastasis. Additionally, a low pericyte coverage is correlated with worse outcome in breast cancer patients [41].

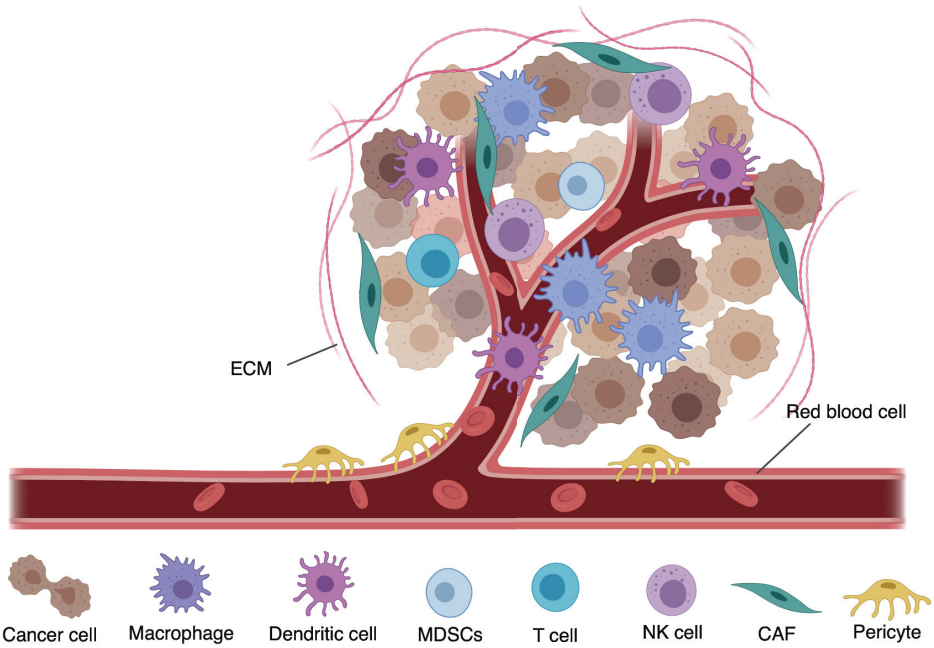

Figure 4. The tumor microenvironment.
The tumor microenvironment constitues multiple cell-types, all involvolved in a reciprocal communication aiding the tumor progression.

The ECM represents another important factor of the tumor microenvironment driving tumor progression. It comprises numerous non-cellular components including collagen, fibronectin, proteoglycans and laminin, which in healthy tissue does not only provide structural support but also plays an important role in cell adhesion, cell proliferation and in the communication between cells [42]. A tumor ECM differs largely from normal ECM, displaying remodeling and a stiffer structure. It has been demonstrated that tumor stiffness characterized by increased collagen crosslinking can stimulate integrin signaling, which furthermore, promoted tumor invasion in murine mouse model [43]. This is further supported by the notion that women with extensive mammographic breast density have an increased risk of developing breast cancer [44]. CAFs represent a major source of ECM components which also are subjected to degradation by different MMPs, an event which

subsequently triggers cell motility and invasion [33,45]. MMPs on the other hand are involved in several key regulatory processes important in tumor progression including angiogenesis, invasion and metastasis, and have been associated with worse prognosis in various cancer types [45-47].

The tumor microenvironment also consists of infiltrating immune cells. It has lately become apparent that the immune response acts in favor of the tumor and therefore is part of the tumors´ natural microenvironment. How this is all orchestrated, and which immune cells are responsible, will be described in detail below.

The Innate Immune System

The human body is under constant threat from pathogens such as bacteria, viruses and fungi that when infecting the host, interfere with its normal functions. Over hundreds of millions of years, the immune system has evolved to act as a shield against exogenic intruders while at the same time maintaining tolerance to self. The immune system consists of two branches, the innate and the adaptive immune system, whose complementary functions provide the host with full protection.

The innate immune system comprises a general first-line defense that involves recognition of foreign patterns as being non-self, thus enabling an immune response. To fulfil its tasks, the innate immune system employs several defense mechanisms including physical barriers such as the skin and the secreted mucosal layer lining the respiratory- and gastrointestinal tract [48]. In case the pathogen manages to breach the physical barriers, the innate immune system engages a variety of pattern recognition receptors (PRRs), including the most studied Toll-like receptors (TLRs). These sense the intruder and subsequently initiate a proper immune response by e.g. the upregulation of MHC and co-receptor molecules responsible for antigen presentation and the induction of different cytokines and chemokines which act as messengers playing important roles in the further activation and recruitment of different immune cells [49,50,51]. The cellular defense of the innate immune system includes natural killer (NK) cells, monocytes, neutrophils, macrophages, dendritic cells (DCs), eosinophils and basophils, which exert various functions such as direct killing of the pathogen through the release of antimicrobial compound, wound healing, pathogen phagocytosis and antigen presentation to lymphocytes [48,52]. Below, some important mediators of the innate immune response related to this thesis will be described.

Monocytes

Monocytes are bone-marrow derived blood cells from the myeloid lineage that comprise around 10 % of leukocytes in human blood. They are circulating cells playing important roles in the early inflammatory responses, host defense during infections, homeostasis and tissue repair as well as in the replenishment of both recruited and tissue resident macrophages [53,54]. Morphologically, monocytes are

characterized by their diverse shape and size, kidney-shaped nucleus and a high cytoplasm-to nucleus ratio. The challenging task in identifying monocytes based on their morphology has been facilitated by the use of flow cytometry where monocytes are identified by the expression of the lipopolysaccharide (LPS) receptor CD14 and their light-scatter characteristics [54]. Nonetheless, monocytes comprise a heterogenous cell population which is further divided into several subtypes based on the presence and the density of cell-surface markers CD14 and CD16 (Fcγ receptor III). They are thus grouped into the classical ($CD14^{++}CD16^{-}$), the intermediate ($CD14^{++}CD16^{+}$) and the non-classical ($CD14^{+}CD16^{++}$) subtypes [55,56,57].

Besides playing a crucial role in host defense, monocytes have also been associated with cancer progression. One example is that monocytes, after being recruited by CCL2, secreted from metastatic tumor cells or the tissue stroma in the target organ, have been shown to generate metastatic associated macrophages which promote breast cancer metastasis [58]. Monocytes isolated from renal cell carcinoma (RCC) patients displayed a protumor gene- and protein profile and promoted an invasive behavior and angiogenic activity of RCC cells when co-cultured *in vitro.* Additionally, the monocytes subsequently infiltrated the tumor giving rise to TAMs [59]. Furthermore, metastatic behavior was also observed *in vitro* in interactions between various monocytic- and breast cancer cell lines, where monocytes promote motility and invasiveness of breast cancer cells [60,61]. In addition, reprograming of monocytes towards a myeloid immunosuppressive cell population by the tumor microenvironment, has been shown to be more prevalent in advanced cancer [35,62]. The heterogeneity of monocytes as well as their ability to differentiate to different types of macrophages including M1, M2, TAMs and MDSCs (which will be reviewed later on in this thesis) demonstrates an incredible versatility and plastic nature of monocytes.

Macrophages

Over a century ago, macrophages were discovered by Ilya Metchnikoff [63], and ever since, we have learned about their versatile functions including their central role in the immune responses upon an infection, their tumoricidal ability and contribution to tissue remodeling and cancer progression. During an infection, macrophages can be recruited from circulating monocytes. Additionally, various tissues comprise a tissue-resident macrophage population, including microglia in the central nervous system and Kupffer cells in the liver and similarly to the monocyte-derived macrophages, they are involved in processes such as immune surveillance [64].

Macrophages comprise a versatile cell population, differing in both phenotype and function. Depending on the signals received from the microenvironment,

macrophages can either undergo a classical or alternative activation and thus differentiate towards the M1 or the M2 macrophages respectively. The polarization towards classically activated M1 macrophages is induced by TLR-ligands such as LPS and pro-inflammatory cytokines including IFN-γ and TNF-α [65,66]. M1 macrophages are characterized by their role in driving T_h1 immune responses, upregulation of pro-inflammatory mediators such as TNF-α and IL-12, elevated antigen presentation as well as antimicrobial and tumoricidal activities [67]. On the other side, the polarization towards alternatively activated M2 macrophages is induced by IL-4, IL-10 and IL-13. Functions employed by M2 macrophages include tissue-remodeling, wound healing and anti-inflammatory attributes with an IL-10^{high}IL-12^{low} cytokine profile as well as a high expression of scavenger receptors [65,66,68]. However, it should be taken into consideration that M1 and M2 polarization merely represents a simplification and that in-between these extremes, a wide spectrum of different polarization states exists.

Tumor associated macrophages (TAMs) have been described as one of the most prominent players in tumor progression. They represent a major leukocyte population within a tumor, sharing common attributes with M2 macrophages including the anti-inflammatory IL-10^{high}IL-12^{low} cytokine profile and tissue remodeling properties [68]. Additionally, TAMs display impaired phagocytosis [69]. The presence of TAMs has been described in various cancer types including breast cancer, classic Hodgkin's lymphoma and bladder cancer, where they have been correlated with higher tumor grade and worse overall survival [68,70-72]. Moreover, TAMs have also been described to promote proliferation, invasiveness and angiogenesis [68].

Neutrophils

Neutrophils represent the predominant circulating human leukocyte population with an essential role in the innate immune responses [73]. In the host defense, neutrophils are among the first cells at site of infection and employ a variety of defense mechanisms including phagocytosis of microbes, release of antimicrobial peptides and induction of neutrophil extracellular traps (NETs) [74,75]. Neutrophils are short-lived cells with a half-life of approximately 8 h in circulation in humans. Nonetheless, their lifespan can extend during inflammatory conditions enabling them to also participate in inflammation resolution [73]. Neutrophils were originally believed to comprise a rather homogenous population, however, in recent years, it has been shown that neutrophils indeed are phenotypically and functionally heterogeneous with several subtypes, including the anti-tumor N1 and the pro-tumor N2 neutrophils, being described [76,77].

In the context of cancer, neutrophils play a paradoxical role. Increased levels of neutrophils have been associated with poor outcome in various cancers [78-80], while in others, neutrophils employ tumoricidal effects and are correlated with a better outcome [81 82]. Indeed, controversy seems to surround neutrophils. Sharing similar phenotype and function it has been proposed that neutrophils might give rise to, or even are equal to an immunosuppressive subset of granulocytic-myeloid derived suppressor cells (G-MDSCs).(reviewed in [83,84]), .This hypothesis will be discussed in more detail further below in this thesis.

Toll-Like Receptors, PAMPs and DAMPs

To be able to recognize invading pathogens, TLRs act as first line of defense by recognizing molecular patterns conserved among microbial species, and thus alarm the body of the danger [85]. TLRs are members of the pattern recognition receptor (PRR) family and today there are ten (1-10) TLRs identified in humans. They are located both on the cellular membrane (TLR1, TLR2, TLR4, TLR5, TLR5, TLR6) and on intracellular endosomes (TLR3, TLR4, TLR7, TLR8 and TLR9) of various immune cells including monocytes, DCs, neutrophils, macrophages as well as epithelial cells [86-91]. Besides being expressed on immune cells, TLR expression has also been reported in various types of cancers, including melanoma, neuroblastoma, esophageal cancer, breast cancer and renal cell carcinoma [92,93,94,95,96,97]. An increasing amount of evidence reports that TLRs employ a variety of pro-tumor activities including proliferation, metastasis and are correlated with high relapse rate [94,95,98]. Consequently, TLRs have been described as an important link between inflammation and tumor progression. On the other hand, TLR expression has also been demonstrated to exhibit reduced proliferation, a protecting role against tumor development and better outcome [96,97,99]. TLRs are type I integral membrane glycoproteins belonging to the interleukin-1 receptor superfamily. They consist of an extracellular domain characterized by a leucine-rich repeats region and a conserved intracellular TIR (Toll/ IL-1R) domain, displaying homologies with the intracellular domain of the IL-1 receptor. TLRs are able to sense a broad range of molecular patterns conserved among microbial species. These recognizable molecules are referred to as pathogen-associated molecular patterns (PAMPs) and include e.g. LPS from Gram- bacteria, viral double stranded RNA, flagellin and CpG motifs recognized by TLR3, TLR4, TLR5 and TLR9 respectively [100]. Upon ligation, the intracellular TIR domains dimerize resulting in a TLR conformational change necessary for the recruitment of intracellular adapter proteins essential for the downstream signaling [101]. All TLRs, except TLR3, signal through the MyD88-dependent pathway, which consequently activates NFκB and MAPK (AP-1, p38 and JNK) signaling pathways. Following their activation, NFκB, AP-1, p38 and

JNK induce the transcription factors of various pro-inflammatory mediators. TLR3 signals through the MyD88-independent (TRIF-TRAM) pathway which ultimately results in the activation of the IRF3 pathway resulting in the transcription of type I IFNs [101,102]. In addition to the cell membrane location, TLR4 can also be internalized into the cytosolic endosomes and signal through TRIF-TRAM signaling pathway (**Fig. 5**)[103].

TLRs have been studied extensively as adjuvants in anti-cancer vaccine therapy. As vaccines based on antigens and peptides are poorly immunogenic, delivery of TLR agonists has been shown to enhance inflammatory responses [104,105]. The idea of boosting the immune system to eradicate tumors is not new. Already a hundred years ago, William Coley discovered that administration of dead bacteria (*S. pyogenes* and *Serratia marcescens)* in cancer patients induced a fever which in many cases caused tumor regression [106].

TLR-induced inflammation can also be achieved in the absence of PAMPs. In situations of trauma and tissue injury, endogenous molecules, referred to as damage-associated molecular patterns (DAMPs), are released from necrotic or stressed cells [107]. Additionally, in response to hypoxia as well as to radio- and chemotherapy-induced cell damage, tumors release DAMPs which consequently bind to TLR, thus provoking a sterile inflammation [108]. An increasing list of intracellular DAMPs have been recognized, including high-mobility group box protein-1 (HMGB1), heat shock proteins, RNA and uric acid, just to mention a few [107,109,110]. Generally, DAMPs act as alarmins to report danger and induce tissue repair [110], however they have also been implicated in different malignancies [111].

S100A9 is a TLR4-binding DAMP that has been been strongly associated with tumor progression [112,113]. It belongs to the Ca^{2+}-binding S100 protein family consisting of 21 members to date and exists as a S100A9 homodimer or a S100A8/S100A9 heterodimer [114]. Produced primarily by myeloid cells, in particular neutrophils and monocytes, the presence of S100A9 has been associated with various inflammatory processes. S100A9 proteins are for instance involved in the early macrophage differentiation, migration of phagocytes through modulation of the cytoskeleton and recruitment of inflammatory cells [115-118]. As mentioned above, S100A9 has been implicated with various malignancies. Expression of S100A9 was reported in numerous cancer types including hepatocellular carcinoma [119], breast [120], colorectal [121] and gastric cancer [122]. Increased expression of S100A9 has been correlated with worse overall survival in breast- and prostate cancer patients [120,123]. In addition, S100A9 expression was correlated with high grade prostate cancers, whilst in a prostate cancer mouse model, $S100A9^{-/-}$ mice display reduced tumor growth [112,122]. Additionally, S100A9 promotes anti-inflammatory responses by accumulating immunosuppressive MDSCs [124]. On the contrary, anti-tumor behavior employed by S100A9 has also been reported. In a study of acute myeloid leukemia

(AML), the S100A9 has been shown to arrest the proliferation and induce differentiation in AML cells as well as improve the survival in an AML mouse model [125]. In colorectal cancer, high density of S100A9 was detected in tumor infiltrating lymphocytes, suggesting that S100A9 might play a role in anti-tumor responses [121].

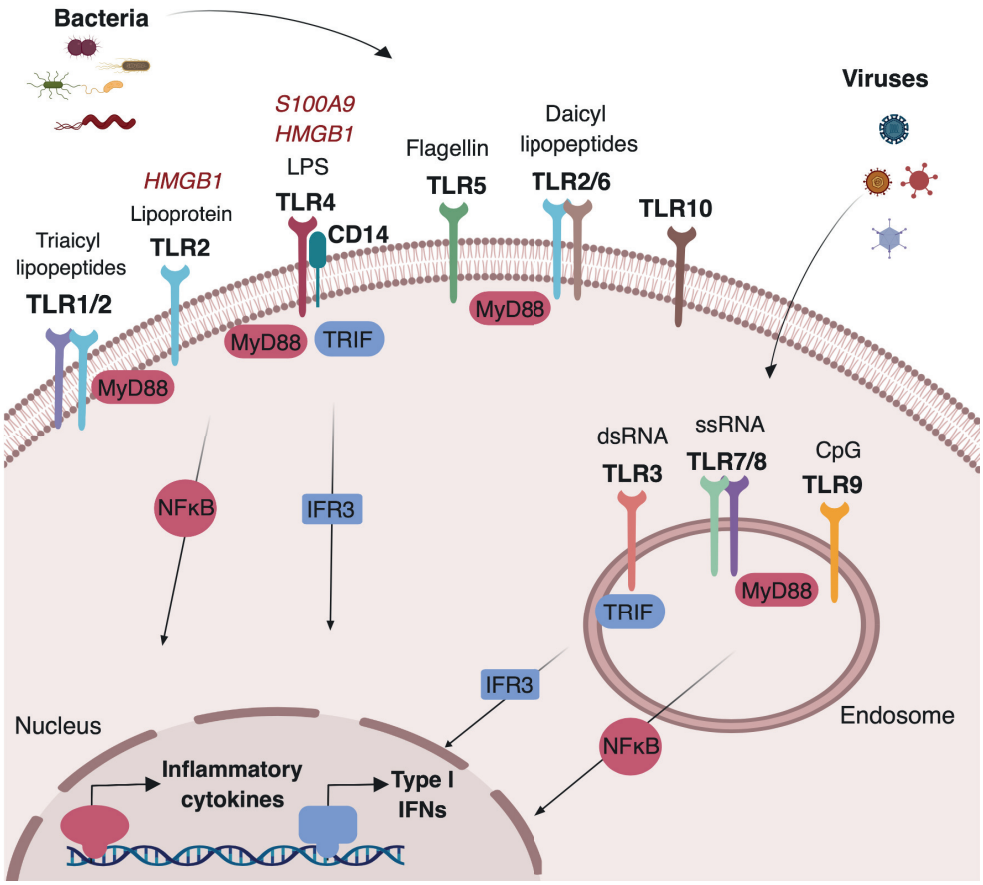

Figure 5. Overview of toll-like receptors and their ligands.
Ten different toll-like receptors are identified in humans, and they can be located both on the cellular membrane (TLR1, 2, 4, 5, 6 and 10) and in intracellular endosomes (TRL3, 7, 8 and 9). TLRs recognize a broad range of microbial pathogenic molecules (in black) and endogenous danger molecules from stressed and necrotic cells (in italic red). Upon binding to putative ligand, TLRs elicit inflammatory responses by activating varios intracellullar signaling pathways e.g. NFκB and IRF3.

The Adaptive Immune System

In case the innate immunity fails to quickly neutralize a pathogen or if an infection becomes too challenging for the innate immunity to eliminate, the adaptive immune system is activated. In contrast to the less specific innate responses, the adaptive immunity mediates a finely tuned antigen-specific response mediated by the antigen-specific T-cell receptors (TCRs) and B-cell receptors expressed by T cells and B cells respectively.

The T cells are divided into two major types: the $CD4^+$ T-helper (T_h) cells which can differentiate into one of several T-helper subtypes (T_H1, T_H2, T_H3, T_H9, T_H17, T_{regs}, T_{FH}) each and one of them characterized by the type of cytokines they secrete or in case of T_{FH} their localization, and the effector $CD8^+$ cytotoxic T cells (CTLs) [126]. The contact between T cells and an antigenic peptide is mediated through TCR binding to either MHC class I or MHC class II molecules. MHC class I molecules, which are expressed on virtually all nucleated cells in the body, present cytosolic antigens recognized by $CD8^+$ T cells, while MHC class II molecules, expressed by specialized antigen presenting cells (APCs), present foreign antigens recognized by $CD4^+$ T cells. Depending on the nature of the encountered antigen, the T_h cells orchestrate a proper immune response by secreting a specific range of cytokines, thus provoking various immune activities such as enhancing T- and B cells responses, boosting antibody production and isotype switching as well as activating CTLs killing of infected cells [126]. The killing mechanisms employed by the $CD8^+$ CTLs include contact-dependent release of granules containing perforin, granzyme and apoptosis-inducing proteases as well as the upregulation of CTL- membrane bound Fas ligand which upon interaction of Fas receptor on the target cell, induces apoptosis [126].

Together with T cells, the adaptive immune system also comprises the B cells which when activated differentiate into antibody producing plasma cells that aid the combat through e.g. antibody dependent cellular cytotoxicity (ADCC), antibody-mediated neutralization of toxins, facilitating phagocytosis of bacteria as well as inhibiting their entry into host cells [127,128]. Besides the highly antigen-specific responses, the other cornerstone of the adaptive immunity is its ability to create an immunological memory, meaning that upon a future encounter with same antigen, the adaptive immune responses are developed more promptly and effectively [48].

Besides the highly antigen-specific lymphocytes, the lymphoid cell family also comprises γδ T cells and Natural Killer T (NKT) cells. Owing to their invariant TCRs and an innate immune receptor profile, they are referred to as "innate lymphocytes". Contrary to the conventional T cells, γδ T- and NKT cells are not dependent on the classical MHC:antigen presentation to become activated. Instead they utilize receptors such as NKD2 and CD1d to recognize their target. They also employ similar cytotoxic mechanisms as CTLs including the production of IFNγ and the release of granzyme and perforin, thus playing important role in anti-tumor immunity, although a pro-tumor role has been reported as well [129-133].

Immune Tolerance

Immune tolerance is a crucial aspect of the well-being and integrity of the body. It is imperative that the immune system is able to discriminate between self and non-self and with respect to this, central tolerance has been developed. It is a sophisticated system that takes place in the thymus, whose prime function is clonal selection: meaning, survival of self-MHC reactive T-cell progenitors, and death of self-antigen reactive T-cell progenitors [134]. Central tolerance is an umbrella term comprising two main processes; the positive selection, taking place in the thymus cortex and the negative selection taking place in the thymus cortico-medullary junction and medulla. The T-cells that develop in the thymus derive from a small group of T cell progenitors presented as $CD4^-CD8^-$ (double negative, DN) cells which subsequently proliferate and give rise to a $CD4^+CD8^+$ (double positive, DP) TCR^+ cell population. During the positive selection, majority of DP T-cell progenitors die by neglect as they lack or present very low affinity to either MHC class I and- MHC class II molecules. Conversely, DP T progenitor cells showing affinity to MHC class I differentiate to single positive (SP) $CD8^+$ T cells, and DP T progenitor cells showing affinity to MHC class II on the other hand differentiate to single positive (SP) $CD4^+$ T cells [135]. In the thymic cortico-medullary junction and medulla, T cell progenitors displaying a too strong affinity to MHC:antigen molecules die by negative selection. The antigens are a broad range of tissue-specific antigens (TSAs) presented by MHC on the surface of medullary thymic epithelial cells (mTECs) and DCs, and T cell progenitors which have a too strong affinity to these, are hence deleted [136].

However, the central tolerance can be leaky and some T cells, despite being autoreactive, manage to escape the clonal deletion and enter the periphery where they potentially might give rise to autoinflammatory diseases [137]. To retaliate towards these cells, self-tolerance is also maintained in the periphery through a process referred to as peripheral tolerance, which employs a variety of regulatory processes including the pivotal immunosuppressive regulatory T cells (T_{reg}), peripheral deletion, an anti-inflammatory cytokine environment, as well as upregulation of inhibitory co-receptors regulators programmed death 1 (PD-1) and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) (**Fig. 6**)[134,138-142]. Immune tolerance is however not exclusively apparent during the process of self and- non-self-discrimination but can also become subject of manipulation by a cancer for its own advantage.

Autoimmune Regulator

Autoimmune regulator (AIRE) is a transcription factor primarily expressed in the thymus by mTECs. AIRE plays a critical role in the negative selection of T cells and mutations in the *AIRE* gene results in loss of self-tolerance and autoimmune diseases [143]. AIRE induces a "promiscuous" gene expression of a broad range of TSAs [144,145], which subsequently are presented by MHC class II on the cell-membrane where they come in contact with T cells undergoing negative selection. Only self-tolerant T cells leave the thymus and collateral damage is avoided [146]. Thymic DCs are also involved in the tolerization of T cells (**Fig. 6**)[147].

As some autoreactive T cells manage to escape the negative selection in the thymus, it is of highest importance to maintain a peripheral tolerance [137]. Although AIRE is also expressed in non-thymic tissues, its functional significance in the peripheral tolerance remains poorly understood. Pollani *et al*. performed extensive immunohistochemistry of various human tissues and reported that, besides in the thymus, AIRE expression was observed in peripheral lymphoid organs, including lymph nodes, tonsils and gut-associated lymphoid tissues in colon and appendix.

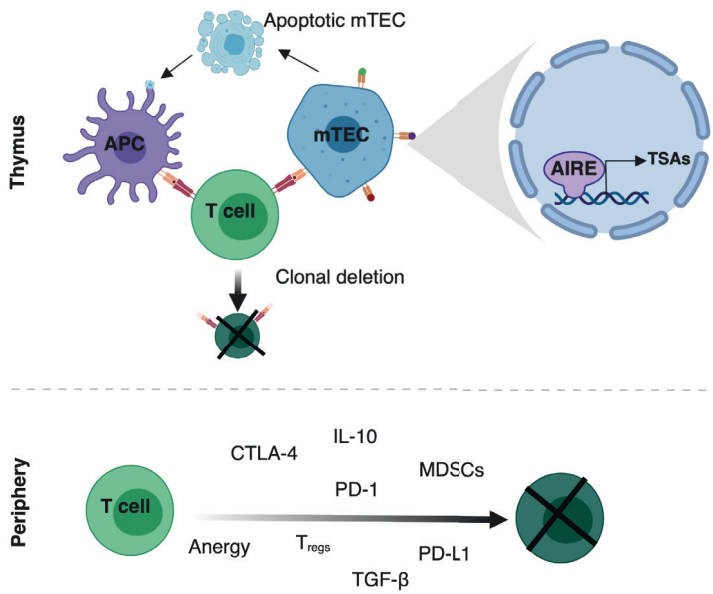

Figure 6. Simplified overview of the mechanisms of central and peripheral tolerance.
Autoimmune regulator (AIRE) induces transcription of numerous tissue-specific antigens (TSAs), which subsequently are presented by MHC molecules on the cell surface of meducally thymic epithelial cells (mTECs) and dendritic cells (DCs). T cells displaying a too strong affinity for TSAs are subjected to clonal deletion. In case some auto-reactive T cells escape clonal deletion in the thymys, multiple mechanisms are employed in order to mainatin a peripheral tolerance. Adapted from Mathis *et al.*, 2009 [143]

Additionally, they reported that AIRE was expressed by mature DCs, which displayed a tolerogenic phenotype by increased transcripts of various TSAs and the production of anti-inflammatory cytokine IL-10 as well as indoleamine 2,3-dioxygenase (IDO) [148]. AIRE expression in peripheral DCs has also been shown to reduce autoreactive $CD4^+$ T cell viability, decrease the expression of PD-1 and increase the expression of TSAs in a diabetes type I mouse model, all indicating a tolerogenic role of AIRE [149]. Furthermore, it has been shown that T_{regs} deviate into an autoreactive $CD4^+$ T convectional cell population in *Aire*$^{-/-}$ mice, demonstrating T_{reg} involvement in the peripheral tolerance [150]. On the contrary, another study showed that AIRE-induced genes in peripheral DCs did not include any TSAs but rather genes relevant in DCs maturation, suggesting functional divergence between peripheral and thymic AIRE-expressing cells [151]. In cancer, AIRE expression has been assessed in a small cohort consisting of 39 breast cancer patients. However, AIRE expression was only evaluated in ER^+ breast cancer patients and no conclusion regarding its localization or the functional role was drawn. However, when transfecting ER^+ breast cancer cell lines with an AIRE vector, they exhibited increased G1-phase and reduced S-phase as well as increased apoptosis [152].

Endotoxin Tolerance

During an infection, it is of prime importance that a proper immune response is induced in order to clear the pathogen. It is likewise important that the immune response is tightly regulated as an overt inflammation might result in tissue damage and harm the host. One clinically relevant example demonstrating this is the course of sepsis, which usually is simplified as a two-step process. Following a bacterial infection, the systemic inflammatory response syndrome (SIRS) is induced, a state characterized by the release of various pro-inflammatory cytokines such as TNF-α and IL-6 which, if not controlled, might result in multi-organ failure and death [153-155]. Subsequently, the second state is characterized by the compensatory anti-inflammatory response syndrome (CARS), as an effort to dampen the excessive inflammation. However, this state might pose the same danger as an overt inflammation, as immunocompromised patients are highly susceptible to secondary infections which might be life-threatening [155,156].

Functional parallels can be drawn between CARS and endotoxin tolerance, a mechanism described as a state of reduced responsive ability of monocytes and macrophages upon a repetitive LPS stimulation [157]. Endotoxin tolerance was described over 70 years ago by Paul Beeson which discovered that rabbits repeatedly injected with typhoid vaccine displayed reduced fever [158]. Today, multiple molecular mechanisms of endotoxin tolerance have been reported. As the principal receptor for recognizing LPS [159], it is not surprising that TLR4 and its

downstream signaling pathway is involved in endotoxin tolerance. Repetitive LPS stimulation decreases the expression of TLR4 in neutrophils [160] and macrophages [161] impairing the TLR4-MyD88 complex formation in monocytes [162]. Furthermore, endotoxin tolerant monocytes fail to generate activation of IRAK-1 [162]. Expression of an alternatively spliced form of MyD88 (MyD88s), lacking the intermediary domain crucial for IRAK-1 and IRAK-4 binding, diminishes the subsequent downstream activation of NFκB [163]. MyD88s expression in monocytes has been shown to increase upon LPS treatment [164]. Besides MyD88s, several other negative regulators of TLR4 downstream signaling pathway have been reported, including factors such as IRAK-M, MKP-1 and A20, which inhibit IRAK1/4, MAPK and TRAF6 respectively [165,166]. Endotoxin tolerance can also be induced by p50p50 homodimer formation, which not only induces IL-10 and inhibits TNF-α transcription [161], but also binds to various DNA promoters and by acting as a steric hinder, p50p50 inhibits the p65p50 activity [167]. Endotoxin tolerant cells are characterized by a diminished ability to present antigens [168-170] and to produce inflammatory cytokine such as IL-6, TNF-α and IL-1 [171], whilst producing anti-inflammatory cytokines such as IL-10 [170].

Myeloid-Derived Suppressor Cells

First reports describing cells with immunosuppressive properties emerged in the late 70's [172,173], and in the mid 90's, the first evidence of suppressor cells in tumors emerged [174,175]. Originally described with an ambiguous terminology, in 2007, the term myeloid-derived suppressor cells (MDSCs) was coined by Gabrilovich *et al*. to describe the origin as well as the distinctive immunosuppressive function of these cells [176].

MDSCs comprise a heterogenous immature myeloid cell population usually divided in two different subgroups; the polymorphonuclear or granulocytic MDSCs (G-MDSCs) and the monocytic MDSCs (Mo-MDSCs). The phenotype of human MDSCs still remains poorly defined, and characterizing them solely by their cell-surface marker expression is challenging due to overlap in marker expression with other myeloid cells [83]. Generally, G-MDSCs are described as immature $CD11b^{+}CD33^{+}HLA\text{-}DR^{-/low}Lin^{-}$, the latter as they lack lineage markers. However, the expression of CD15 and CD66b as well as a low density in Ficoll gradients has also been recorded. G-MDSCs have been described to resemble neutrophils both morphologically and phenotypically. Mo-MDSCs, which phenotypically and morphologically are similar to monocytes, are defined as $CD14^{+}CD11b^{+}HLA\text{-}DR^{-/low}CD15^{-}$ [177,178]. In contrast to human MDSCs, characterizing mouse MDSCs phenotype is less challenging. Mouse MDSCs are described as $CD11b^{+}Gr1^{+}$ cells,

where G-MDSCs and Mo-MDSCs are defined as $Ly6G^+/Ly6C^-$ and $Ly6G^-/Ly6C^+$ respectively [142].

MDSCs have been described in different pathological conditions such as sepsis [179-181], chronic infection[182-184] and autoimmune disease [185,186]. However, the most extensive research on MDSCs has been focusing on cancer [178,187], where MDSCs have been associated with tumor angiogenesis, metastasis and tumor burden both in cancer patients and in mouse models [188-190].

The expansion and activation of MDSCs has been described as a two-signal process which includes multiple factors produced by activated T cells, tumor stromal cells and tumor cells themselves [84]. The first signal involves expansion of MDSCs which is mediated by a broad range of factors including GM-CSF, G-CSF, M-CSF, IL-6, VEGF, and COX2, with STAT3 as the main signaling pathway through which they operate. The second signal involves activation of MDSCs, which on the other hand is mediated by NFκB through various factors such as TLR ligands and different pro-inflammatory proteins. [142]. On the contrary, it has also been reported that the expansion signal is instead mediated through NFκB, while STAT3 is responsible for the MDSCs activation [191]. Furthermore, S100A8 and S100A9 proteins have also been shown to play a role in the expansion of MDSCs [124]. However, the generation and activation models do not necessarily cover both G- and Mo-MDSC.

The most distinctive feature of MDSCs is their incredible immunosuppressive ability, which until today remains the golden standard for MDSCs characterization (**Fig. 7**). One potent mechanism through which MDSCs induce immunosuppression is by inhibition of T cell function. Production of arginase-1 (arg-1) and iNOS by MDSCs results in depletion of L-arginine, which is crucial for T cell proliferation. T cells present in an L-arginine- deficient environment remain in their G0-G1 cell-cycle phase [142]. Another way MDSCs can impair T cell function is through the production of reactive oxygen species (ROS) which exert several tumor-promoting activities such as inducing downregulation of T cell receptor ς chain and thus inhibiting T cell activity, enhancing the malignant cell resistance against CTL cytotoxicity and impairing the T cell infiltration into the tumor site [178,192]. MDSCs further induce immunosuppression by producing immunosuppressive cytokines including IL-10 and TGF-β, and are also able to promote angiogenesis through the production of e.g MMP9 [178,190]. MDSCs have also been reported to induce T_{regs}, which subsequently inactivate T cells, as well as to impair the cytotoxic function of natural killer cells. Generally, immunosuppressive function between Mo-MDSCs and G-MDSCs differ being characterized with following profiles; Mo-MDSCs: Arg/IL-10/IDO and G-MDSCs: ROS [84,178,193,194].

While the function of MDSCs has been extensively studied, the origin of MDSCs still remains an unexplored field. There are various theories explaining their potential origin as reviewed in [83,84]. In *Paper IV*, we have put effort in exploring the

origin of G-MDSCs where we show that G-MDSCs from metastatic breast cancer (MBC) patients cluster together with healthy donor neutrophils in a gene expression profile array, sharing genes relevant in angiogenesis and immunosuppression among others leading us to the conclusion that G-MDSCs might actually be a subset of neutrophils. This controversial theory has been contemplated by others owing to the phenotypical and morphological resemblance between G-MDSCs and neutrophils, the fact that activated neutrophils have been discovered in the low-density mononuclear cell fraction of Ficoll density gradient in cancer patients, and most importantly, the ability of neutrophils to induce ROS-dependent immunosuppression [83,195]. Indeed, MDSCs and neutrophils share many similarities and one resent study has shown that MDSCs are able to produce NETs, a mechanism traditionally performed by neutrophils [196].

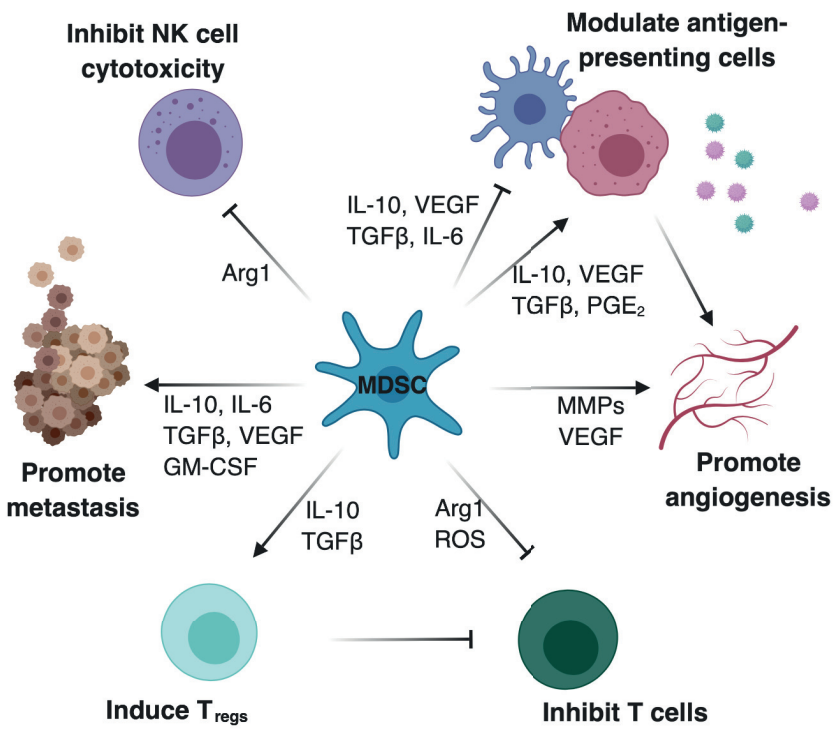

Figure 7. Myeloid-derived suppressor cells and their multiple immunosuppressive functions.
Illustration adapted from Millrud *et al.,* 2017 [84].

The Immune System and Cancer

Over a century ago, Paul Ehrlich predicted that our immune system plays a protective role against cancer. Fifty years later, Burnet and Thomas continued on that path and proposed that our immune system, involving T cells as principal effectors, is able to detect and eliminate cancerous cells. Consequently, the term "immunosurveillance" was coined. The concept of immunosurveillance was however reconsidered and the theory that our immune system not only protects us from cancer but is also involved in processes of sculpting the cancer emerged. The dynamic process involving the immune system and its host-protective, tumoricidal actions against tumors, together with its involvement in shaping the tumor characteristics, gave rise to a new term referred to as "cancer immunoediting" including three phases; elimination, equilibrium and escape (**Fig. 8**)[197-199].

During the elimination phase, the immune system is activated by tumor associated antigens (TAAs) and when primed, it is able to provoke anti-tumor responses [198]. Innate lymphocytes, including NK, NKT and γδ T cells are able to recognize and kill transformed cells, and lymphocyte tumor infiltration has been correlated with better prognosis in several cancer types [200-204]. Phagocytosis of TAAs by DCs consequently results in T cell activation through antigen presentation in the lymph nodes. Ultimately, tumor specific adaptive T cells are recruited to the tumor site and participate in the process of tumor elimination [198]. IFN-γ and IL-12 represent some of the principal cytokines in tumor rejection [139,198,205,206], and other tumoricidal molecules employed by the immune system include perforin and TRAIL [207-209]. The impact of the adaptive immune system in anti-tumor immunity has been demonstrated by the RAG2$^{-/-}$ mice characterized by their lack of mature T, B, γδ T and NKT cells. These mice display an increased frequency of carcinogen-induced and spontaneous tumor development [139,210]. The importance is further demonstrated in immunosuppressed patients which may develop various cancer forms following e.g. organ transplantation [211].

In situations where a tumor survives the immunosurveillance, it subsequently enters the equilibrium phase. During this phase, there is still an ongoing anti-tumor response, however, not potent enough. Instead of completely eradicating the tumor, the surviving tumor cells are subjected to a "survival of the fittest" selection which ultimately results in the generation of immune-resistant tumors [197].

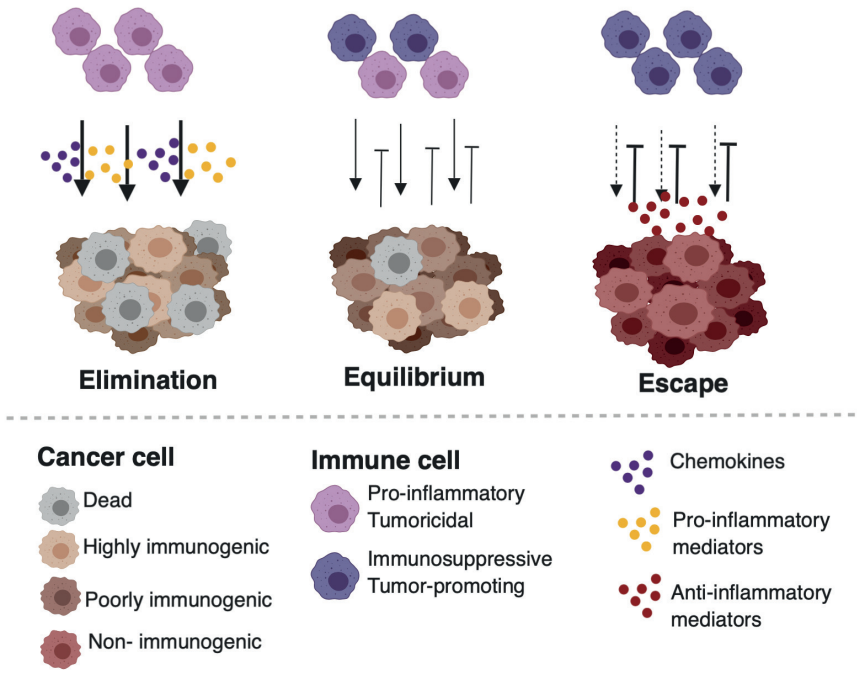

Figure 8. The three E's of of cancer immunoediting.
During *elimination* phase, the highly immunogenic cancer cells are detected by the immune system and are successfully eradicated. Over time, the tumor enters the *equilibrium* phase, characterized by a balance between tumor death and tumor growth. Ultimately, cancer cells become non-immunogenic and the tumor creates an immunosppressive environment in which it thrives and *escpaes* the immune destruction. Adapted from Dunn *et al.* 2002, 2004 [197,198].

During this phase, there seems to be a balance between anti-tumor and immunosuppressive cells and mediators [198].

During the escape phase, the tumor fully engages in mechanisms of avoiding the immune system. One such mechanism includes camouflaging by e.g. reducing the expression of MHC class I molecules and TAAs [198]. One major obstacle of successful anti-tumor responses is T-cell exhaustion. In the context of an infection, a persistent activation of T cells may result in the development of exhausted T cells as a regulatory mechanism of bringing a balance between inflammation and host-protection. Several mechanisms lie behind T cell exhaustion including a reduced ability to produce IL-2 and IFN-γ cytokines as well as upregulation of PD-1 [212,213]. This is also seen in tumors. To further avoid anti-tumor immunity, tumors promote accumulation of immunosuppressive cells, generating an immunosuppressive environment. The most prominent immunosuppressive cells in a tumor microenvironment are the TAMs and MDSCs. Both cell types suppress T cell activity through the secretion of immunosuppressive mediators as described above

[68,142,178]. Another potent immunosuppressing cell population include the $CD4^{+}CD25^{high}FOXP3^{+}T_{regs}$. The prevalence of T_{regs} is increased in various malignancies and they apply their immunosuppressive functions through e.g. expressing CTLA-4, affecting DC maturation, sequestering IL-2 and the release of anti-inflammatory IL-10 and TGF-β [214-219]. Essentially, the immunoediting process develops tumor cells resistant to an immuno-competent environment.

Cancer Immunotherapy

Cancer immunotherapy is a collective term for a broad range of therapeutic strategies which utilize the immune system to fight cancer. These therapeutics have expanded immensely in the last two decades and revolutionized the way various cancers are treated. It is therefore not surprising that James P. Allison and Tasuku Honjo were awarded with Nobel Prize in Physiology and Medicine, 2018 for their contribution to the field of cancer immunotherapy. Their discoveries are perhaps among the most notable success stories within the field which include the practice of inhibiting negative T cell regulators; CTLA-4 and PD-1, in order to boost the anti-tumor responses [220,221]. A complete T cell activation requires binding of a TCR to an antigen-presenting MHC-molecule together with the binding of costimulatory molecules CD28 and B7 present on T cells and APCs respectively. Also present on T cells are CTLA-4 and PD-1, which are CD28 homologues that impair T cell activation by distinct ways; CTLA-4 by inhibiting the CD28:B7 binding by binding to B7 itself in a competitive manner, and PD-1 by binding to its own ligand PD-L1, which often is present on tumor cells [222]. Currently, monoclonal antibody-mediated CTLA4 and PD-1 inhibition (also referred to as immune checkpoint inhibition (ICI)) is used to treat various malignancies including melanoma, kidney cancer, bladder cancer and non-small cell lung cancer. Several ongoing phase II and III clinical trials are exploring the effects of the ICI on multiple other malignancies [222,223]. Apart from ICIs, another therapeutic strategy explored include adoptive therapy where autologous T cells are modified *ex vivo*. One example are the chimeric antigen receptor (CAR) T cells which are not dependent on the classical TCR:MHC binding for their activity, but are already loaded with an antigen. Non-Hodgkin lymphoma and acute lymphoblastic leukemia patients are currently benefiting from this immunotherapy as CD19-directed CAR T cells have been approved by the FDA last year [224,225]. The use of monoclonal antibodies (targeted therapy) has successfully been used in some cancer types by means of targeting the TAAs. In breast cancer, the function of HER2/neu receptor is inhibited by Trastazumab [26].

Wnt

Wnt proteins constitute a family of secreted cysteine-rich glycoproteins highly conserved among species, holding important roles in tissue homeostasis, embryonic development, and cancer [226-228]. The significance of Wnt's in oncogenesis was first evident upon the discovery of *Integration 1* (*Int1)* in mice, a gene to which the oncogenic mouse mammary tumor virus (MMTV) integrates [229]. Later, it was found that *Int1* and Drosophila *wingless* (*wg*) were orthologs [230], and consequently by combining *wg* and *Int,* the term *Wnt1* was coined [231]. To date, 19 Wnt proteins in mammals have been identified and they operate through multiple receptors and co-receptors including the seven-transmembrane G-coupled Frizzled protein family (FZD), receptor tyrosine kinase (RTK), ROR1, ROR2 and the low-density lipoprotein receptor-related proteins 5 and 6 (LRP 5/6) [227,232]. To become fit for action, Wnt proteins are subjected to a series of post-translational modifications giving Wnt's their hydrophobic properties crucial for their secretion and function [233,234]. Wnt proteins are generally divided into two different subgroups; the transforming vs. the non-transforming subgroup based on their ability to transform C57MG mammary epithelial cells or the canonical and the non-canonical subgroups based on the signaling pathway they activate [235].

Canonical Wnt signaling

Wnt/β-catenin signaling pathway is one of the main signaling pathways activated by Wnt proteins. It involves the transcription factor β-catenin whose transcriptional activity relies on a canonical Wnt protein (e.g. Wnt1, Wnt3a and Wnt8) binding to a FZD receptor and the LRP5/6 co-receptor [236,237]. In the absence of a Wnt protein, β-catenin is bound and regulated by a destruction complex consisting of several intracellular proteins including Axin, casein kinase 1 (CKI), adenomatous polyposis coli (APC) and glycogen synthase kinase-3 beta (GSK3β). β-catenin is subsequently phosphorylated and ubiquitinated and ultimately subjected to proteasome degradation, keeping intracellular β-catenin levels low. The engagement of a Wnt protein with its putative FZD receptor along with the co-receptor LRP5/6 ultimately results in the dissemblance of the destruction complex and the release of β-catenin. The disruption of the destruction complex is mediated through the translocation of

Dishevelled (Dvl) to the cellular membrane and its recruitment of GSK3β and Axin complex [237]. Conclusively, the unbound and stable β-catenin is able to translocate to the nucleus and activate the transcription of various TCF/LEF inducible genes e.g. *c-myc* [236,238].

Non-canonical Wnt signaling

The best described non-canonical Wnt signaling pathways, which also are referred to as β-catenin independent, include the Wnt/Ca^{2+}, the Wnt/planar cell polarity (PCP) and the Wnt/ROR2 signaling pathways. These pathways are activated by non-canonical Wnt proteins (typified by Wnt5a and Wnt11) through their engagement with various FZD receptors alone or in combination with their co-receptor ROR2 [227,237,239].

Cell migration and cell polarity is regulated by the Wnt/Ca^{2+} signaling pathway. Activation of the signaling pathway is initiated upon the ligation of a non-canonical Wnt protein to its putative FZD receptor, resulting in the increase of intracellular calcium influx. The intracellular accumulation of calcium in turn activates various effectors such as protein kinase C (PKC), calcineurin and calmodulin- dependent protein kinase II (CaMKII), where the latter has been shown to play an inhibitory role of the β-catenin signaling pathway through its activation of e.g. nemo-like kinase (NLK) [240-242].

Wnt/PCP signaling pathway is another non-canonical pathway which controls the cytoskeletal reorganization. The cellular orientation and movement is achieved through the binding of a non-canonical Wnt protein binding to a FZD receptor which ultimately results in the activation of JNK and ROCK. The activation of the JNK and ROCK is mediated via Dvl which induces the activation of the small GTPases Cdc42, Rac and Rho [237,241]. Wnt/PCP signaling pathway is crucial during normal development and is activated during cancer [243].

Wnt5a

The most extensively investigated non-canonical Wnt protein is Wnt5a, a non-transforming secreted glycoprotein that activates the β-catenin-independent non-canonical Wnt signaling pathway. Since its discovery in 1990 [244], the biological role of Wnt5a has been studied in various fields including developmental biology, inflammatory diseases and cancer [245,246]. The importance of Wnt5a in development is illustrated by the knock-out of Wnt5a (*Wnt5a*$^{-/-}$) in mice displaying a phenotype of facial abnormalities, dwarfism and shortened limbs and tails, dysmorphic ribs,

heart defects, respiratory dysfunction and death shortly upon birth [227,246]. The role of Wnt5a in cancer has proven paradoxical as being recognized both as a tumor-suppressor and a tumor-promotor depending on the cancer type. Down-regulation of Wnt5a has been observed in several cancer types including breast cancer [247], acute lymphoblastic leukemias [248,249], and colon cancer [250]. Reduced Wnt5a levels have been correlated with worse overall clinical outcome in breast- and colon cancer patients [250,251]. A lower Wnt5a expression is also evident in the more aggressive ER^- MDA-MB-231 cell-line as compared with the ER^+ non-metastatic MCF-7 cell-line [247]. Furthermore, Wnt5a plays a tumor-suppressing role in thyroid carcinoma by inhibiting the proliferation and migration in a Wnt/Ca^{2+}/CaMKII signaling-manner leading to β-catenin degradation [252], and Wnt5a hemizygous mice display increased B-cell proliferation and develop myeloid leukemia [248]. In contrast, the tumor-promoting ability of Wnt5a has been reported in gastric cancer where Wnt5a promotes migration and invasion by activating the GTP- binding protein Rac and the focal adhesion kinase (FAK) protein. In addition, Wnt5a positivity in gastric cancer patients is correlated with higher tumor stage and worse overall survival [253]. Similarly, Wnt5a promotes invasiveness and motility by activating PKC in metastatic melanoma [254], induces increased proliferation and is associated with poor prognosis in non-small cell lung carcinoma patients [255] and induces EMT in pancreatic cancer [256]. Additionally, in several melanoma cell lines, Wnt5a has also been shown to induce exosome-mediated secretion of several tumor promoting mediators such as IL-8, VEGF, MMP2 and IL-6 [257].

Besides its crucial role in embryonic development and cancer, several studies have also addressed the involvement of Wnt5a in immune cell regulation. Bergenfelz *et al.* have shown that Wnt5a inhibits the monocyte differentiation towards M1 macrophages and instead induces an alternative monocyte activation producing a tolerogenic phenotype of macrophages [161]. Similarly, the Wnt5a-induced tolerogenic cell phenotype has also been observed in monocytes differentiated towards immature dendritic cells [258,259]. The tolerogenic DCs obtained displayed a reduced antigen-presenting ability as well as a reduced production of pro-inflammatory cytokines and increased producing anti-inflammatory cytokines [259]. Moreover, Wnt5a has been shown to reduce the generation of mature DCs [258] and to inhibit the B-cell proliferation [248] and T-cell development [260]. In contrast, Wnt5a has also been implicated in inflammatory responses. Increased expression of Wnt5a has been observed in several inflammatory diseases such as sepsis, atherosclerosis and rheumatoid arthritis [261-263]. Blumenthal *et al.* have shown that Wnt5a plays a role in Th1 responses by regulating the expression of IL-12 and IFN-γ in human macrophages and PBMCs [264]. Treatment of human macrophages with recombinant Wnt5a significantly induced the release of several pro-inflammatory cytokines including IL-1β, IL-6 and IL-8 [261]. Moreover, Wnt5a treatment enhanced the ability of human bone marrow stromal cells (BMSCs) to attract T-cells and monocytes

through several distinct pathways, including NFκB and MAPK [263]. It has been reported that recombinant Wnt5a induced pro-inflammatory cytokines in mouse macrophages in a TLR4-dependent pathway. The authors speculated that this might be an artifact caused by contaminated Wnt5a preparations [265]. Indeed, this is a logical explanation, however, in this context, quite far from the truth. In *Paper III,* we show for the first time that Wnt5a in fact is a novel TLR2 and TLR4 ligand eliciting pro-and anti-inflammatory cytokine profiles in human and mouse monocytes respectively.

The Present Investigation

Aims

The general aim of this thesis is to investigate various molecular mechanisms of immune tolerance in human breast cancer, including the generation of myeloid-derived suppressor cells (MDSCs) through a novel Toll-like receptor 4 (TLR4) ligand (Wnt5a), and the expression of Autoimmune regulator (AIRE).

The specific aims were:

I. To investigate the expression of TLRs in breast cancer, and to analyze the functional role of TLR4 and its clinical relevance in breast cancer patients.

II. To investigate the expression and the localization pattern of the DAMP S100A9, its functional role as well as its clinical relevance in breast cancer patients.

III. To investigate Wnt5a as a novel ligand for TLR2 and TLR4.

IV. To investigate the prevalence and generation of G-MDSCs in human breast cancer patients.

V. To investigate the expression of Autoimmune regulator (AIRE) and its functional characteristics in human breast cancer.

Paper I

Expression and function of toll-like receptor 4 correlates with worse overall survival in ER-/PR- breast cancer.

Background

Our immune system has a crucial task in protecting our body from infectious pathogens. In order to recognize intruders, the immune system has employed a group of pattern-recognition receptors known as TLRs. Being expressed on various immune cells, they are able to sense danger in form of exogenous PAMPs and endogenous DAMPs, thus inducing proper inflammatory responses [85,87,88,107]. An increasing amount of evidence reports that TLRs are expressed on various cancer cell types, contributing to cancer progression [94,95,98]. In *Paper I*, we aimed to investigate the expression of TLRs in multiple breast cancer cell lines with the focus on TLR4 expression and function. Additionally, we aimed to investigate the clinical relevance of TLR4 expression in breast cancer patients.

Results and Discussion

The expression of TLRs has previously been reported in several cancer types [92,94,95]. We started off by investigating the mRNA expression pattern of TLR2, TLR3, TLR4, and TLR9 as well as TLR4 co-receptors MD2 and CD14 in seven different breast cancer cell lines; four TN (MDA-MB-231, MDA-MB-468, SUM-149 and SUM-159) and in three ER^+PgR^+ (MCF-7, T47D and CAMA). The results showed that *TLR2* and *TLR3*, *TLR4* and the co-receptor *CD14* are favorably expressed in TNBC cell lines, whilst *TLR9* was generally expressed in all cell lines. Moreover, the co-receptor *MD2* was expressed in all TNBC cell lines, except in MDA-MB-468, meaning that three out of four TN breast cancer cell lines contain all necessary components for a functional TLR4 receptor. Furthermore, immunohistochemical analysis revealed a cytoplasmic TLR4 expression in MDA-MB-231, SUM-149 and SUM-159 cell lines, while being completely absent in MDA-MB-468 and in the ER^+PgR^+ cell lines. Although TLR4 is generally located on the cellular membrane, several studies have reported intracellular TLR4 [91,93,266,267]. To investigate the TLR4 functionality, we stimulated all seven cell lines with LPS. Our results show a marked increase of IL-6 and IL-8 and a low increase in TNF-α in the supernatants of MDA-MB-231, SUM-149 and SUM-159 cell lines. Moreover, NFκB activity was significantly increased when stimulating MDA-MB-231 cells with LPS. Constitutively active NFκB has been observed to be more prevalent in Basal-like compared to Luminal breast cancers. It has furthermore been correlated with

multiple tumor promoting activities such as proliferation, angiogenesis and metastasis [268,269]. The fact that TLRs studied in this project are preferentially expressed in TNBC tumors might be a causality to the constitutive activity of NFκB observed in ER^- breast cancers. In order to mimic a sterile inflammation, we treated TNBC cell lines with two DAMPs; HMGB1 and S100A9, and while HMGB1 had no effect, S100A9 induced a significant IL-6 and IL-8 increase in MDA-MB-231, SUM-149 and SUM-159 cell lines. These results are supported by previous findings showing similar S100A9 effects, where it induces IL-6 and IL-8 in a human monocytic cell line [270]. Furthermore, MDA-MB-231 cells transfected with siRNA against TLR4 showed a low, yet a significant decrease in both IL-6 and IL-8 release, suggesting other mechanisms involved in the IL-6/IL-8 induction. It has previously been shown that ER^- breast cancers display a rather inflammatory milieu characterized by cytokine production which is associated with tumor aggressiveness [271]. Moreover, the lack of ER expression has also been ascribed as one of the prognostic factors correlated with worse outcome in breast cancer patients [272]. In order to investigate the chemoattractant ability employed by different breast cancer cell lines, we co-cultured human primary $CD11b^+$ PBMCs together with conditioned media from two ER^+PgR^+ (MCF-7 and T47D) and two TNBC (MDA-MB-231 and MDA-MB-468) cell lines. Our results demonstrated that myeloid cells migrated towards MDA-MB-468 and MDA-MB-231, however, only statistically significant towards the latter. Additionally, MDA-MB-231 cells stimulated with LPS displayed a significantly higher invasive behavior in comparison to their untreated counterparts. LPS-induced TLR4 activation has previously been reported to promote metastasis [273]. A report by Volk-Draper *et al*. has also shown that TLR4 stimulation by paclitaxel induced breast cancer invasion and metastasis *in vivo* [274]. Lastly, in a TMA of 144 breast cancer patients, we investigated the expression of TLR4 and analyzed its expression with different histological and clinical parameters. From our analysis, we could draw the conclusion that TLR4 correlates significantly with the ER^-PgR^- breast cancer patient group and that high TLR4 expression is significantly correlated with worse recurrence-free survival. These results are in line with pervious published evidence shown the TLRs involvement in carcinogenesis [94,98].

In conclusion, functional TLR4 is expressed and is correlated with worse recurrence-free survival in ER^-PgR^- breast cancer patients. The ability of TLR4 to induce IL-6 and IL-8, two pro-tumorigenic cytokines, should be taken in serious consideration in terms of therapeutic targeting in order to reduce the tumor inflammation.

Paper II

S100A9 expressed in ER(-) PgR(-) breast cancers induces inflammatory cytokines and is associated with an impaired overall survival.

Background

S100A9 is a Ca^{2+}-binding protein that has been implicated in various inflammatory processes and cancer. S100A9 acts as a DAMP by binding to TLR4 and subsequently eliciting NFκB activity. Several immune cells, most notably neutrophils and myeloid cells, secrete S100A9, and although S100A9 protein expression in breast cancer has been investigated previously, the reports were rather inadequate [114-118]. I this paper, we wished to further elaborate on the expression pattern and function of S100A9 in breast cancer. We were also interested in investigating whether S100A9 expression correlated with any clinicopathological features that would be of relevance in breast cancer patients.

Results and Discussion

The expression of S100A9 has predominantly been attributed to immune cells and knowledge regarding its expression pattern in breast cancer is rather undefined [115,117]. Several attempts to explore the expression of S100A9 in breast cancer have been employed, both *in vitro* and in cohorts of patients. However, the cohorts used were either small or the assessments were incomprehensive [275-278]. To shed some light on this area, we started off by assessing the S100A9 expression in six different breast cancer cell lines: three ER^+PgR^+ (MCF-7, T47D and CAMA), two TNBC (MDA-MB-231 and MDA-MB-468) and one $HER2^+$ (SKBR3). Gene expression analysis revealed that *S100A9* was only expressed in the TNBC MDA-MB-468 and $HER2^+$ SKBR3 cell lines. These results were confirmed by the immunohistochemical (IHC) analysis on paraffin-embedded cell pellets from all mentioned cell lines, which demonstrated S100A9 protein expression in MDA-MB-468 and SKBR3 solely. We further investigated S100A9 protein expression pattern on a TMA consisting of 144 breast cancers. Our results demonstrated cytoplasmic, nuclear, membrane and stromal S100A9 expression in all compartments significantly correlated with ER^-PgR^- hormone receptor status. Reports regarding S100A9 expression in different breast cancers have indicated a tendency towards ER^- breast cancers [278]. However, one study showed that S100A9 is primarily expressed by non-invasive cell lines and primary breast cancers compared to the invasive counterparts [279]. In order to study the inflammatory responses S100A9 stimulation exerts on breast cancers cell lines, we performed a cytokine bead array.

Our results show that S100A9 induced a significant release of IL-8 and IL-1β in MDA-MB-231 cell line only. Also, S100A9 induced IL-6 release in T47D, CAMA and MDA-MB-231 cell lines, however, only significantly in the latter. In *Paper I*, we have demonstrated the ability of S100A9 to induce cytokine secretion, however, only in TNBC cell lines [280]. This experiment on the other hand includes a broader array of cell lines, providing a more comprehensive view of S100A9 effects in breast cancer. Similar effects exerted by S100A9 have been shown in myeloid cells [270]. Despite expressing the highest level of S100A9, MDA-MB-468 and SKBR3 cell lines did not respond to S100A9 treatment. This can be explained by our next experiment where we assessed the expression of TLR4. Neither MDA-MB-468 nor SKBR3 expressed *TLR4*, while on the other hand, *TLR4* was significantly expressed in the MDA-MB-231cell line. This is further confirmed by our results from *Paper I* where we report the expression and the lack of expression of TLR4 in MDA-MB-231 and MDA-MB-468 cell lines respectively [280]. As a ligand for TLR4, S100A9 elicits a NFκB activity [270]. To confirm this notion in breast cancer cells, we performed a Dual luciferase reporter assay with a NFκB reporter in MDA-MB-231, MDA-MB-468 and SKBR3. As expected, NFκB activity upon S100A9 stimulation was only observed in the MDA-MB-231 cell line. In order to investigate whether S100A9 expression was associated with any clinicopathological features or myeloid cell markers, we performed IHC on a TMA consisting of 144 breast cancers. Besides the correlation with ER^-PgR^- breast cancers as mentioned above, stromal S100A9 expression significantly correlated with HER2 and proliferation marker Ki67 positivity, larger tumor size and nodal stage. The stromal S100A9 expression in HER2 positive cancers is in line with our qPCR results, where the $HER2^+$ SKBR3 cell line was one of the cell lines that expressed S100A9. Similar observations of the correlation between S100A9 expression and HER2 positivity was previously reported by Arai *et al.* [275]. We further observed a significant correlation between stromal S100A9 expression and the anti-inflammatory $CD163^+$ myeloid cell, but not with $CD68^+$ macrophages. Interestingly, we have reported previously that $CD163^+$ myeloid cells are located in the stroma and not in the nest of breast cancer patients and in addition, S100A9 expression has been described in MDSCs [70,124]. The expression of S100A9, both by tumor and immune cells in different tumors has been correlated with worse outcome [123,275,281], therefore, it would be logical to speculate that the presence of S100A9 expression might have a negative impact on the outcome in the patients from our cohort. Indeed, the expression of S100A9 both in the stroma and the cytoplasm was significantly associated with a worse survival in ER^-PgR^- breast cancer patients.

Paper III

Wnt5a is a TLR2/4-ligand that induces tolerance in human myeloid cells

Background

In *Paper I* and *II*, we have shown that TLR4 is expressed in ER^- breast cancers is functional and more importantly, that it responds to DAMPs. In *Paper III*, we continued on this path and aimed to investigate Wnt5a as a novel endogenous TLR2/TLR4 ligand. Wnt5a is a non-canonical protein with important functions in development, cancer and inflammation [245]. Previous studies have reported elevated levels of Wnt5a in sepsis and as a result of LPS stimulation in myeloid immune cells.

Results and Discussion

Several independent studies have reported the ability of Wnt5a to induce various inflammatory mediators [161,263,265,282] and many of them in MAPK and NFκB-dependent manner, strikingly similar to those induced through TLR signaling pathways. A study from 2014 speculated that this might be a result of endotoxin-contaminated recombinant Wnt5a preparations [265], however, we though that it might be the other way around and hypothesized that Wnt5a is a ligand for TLR4. By means of several methodologies, including co-localization of transfected Wnt5a-HA and hTLR4-GFP in NIH3T3 cells, SRP binding Biacore analysis and *in vitro* binding assays, we confirm that Wnt5a is a novel ligand for TLR2 and TLR4. Confirming previous reports, we show that Wnt5a treatment of primary monocytes and a monocyte cell line; THP1, induces an anti-inflammatory cytokine profile characterized by the increased secretion of IL-10, IL-8 and IL-6 and the downregulation of TNF-α. On contrary, in primary mouse bone marrow macrophages (BMM) and the macrophage cell line RAW264.7, Wnt5a induced a pro-inflammatory cytokine profile characterized with an increased secretion of TNF-α and MCP-1 (CCL2). The contradictory Wnt5a-induced response in human and murine immune cells (anti-inflammatory vs pro-inflammatory respectively) was rather confusing in the beginning, however, when diving into the literature, we slowly realized that this same pattern has been observed by other research groups [161,259,283,284]. When performing NFκB reporter assays, Wnt5a did not activate NFκB in THP1-Blue cells. Hence, we propose that Wnt5a operates through alternative signaling pathways, including non-classical NFkB p50-homodimer formation as we previously showed [161], TLR4-PI3K pathway and cytokine secretion through

exocytosis [161,257,285]. When stimulating BMM from *MyD88*$^{-/-}$ and *TLR4*$^{-/-}$ mice with Wnt5a, we could see a completely abrogated TNF-α release in *TLR4*$^{-/-}$ BMM and partially in *MyD88*$^{-/-}$ BMM. These results further confirm hypothesis that Wnt5a is a novel TLR4 ligand. On the other hand, an increased MCP-1 secretion was observed in both *TLR4*$^{-/-}$ and *MyD88*$^{-/-}$ BMM. Furthermore, when treating human primary monocytes with Wnt5a in combination with TLR4 ligands (PAMP; LPS or DAMPs; HMGB1 and S100A9), we could observe increased prevalence of CD14^{+}HLA-DR$^{low/-}$ Mo-MDSCs cells *in vitro*. A Wnt5a-induced immunosuppressive phenotype has been observed previously. Valencia *et al.* reported that Wnt5a promoted an immunosuppressive phenotype of mDCs characterized by the reduction of HLA-DR and CD86 and upregulation of PD-L1 and a rendered LPS-induced activation [259]. Similar findings were reported in a study which demonstrates that Wnt5a blocks the M1 differentiation and instead induces a tolerogenic CD14^{+}HLA-DR$^{low/-}$co-receptor$^{low/-}$ cell population [161].

In conclusion, we report that Wnt5a is a novel TLR2/4 ligand and based on its opposite effects; anti-inflammatory in human vs pro-inflammatory in murine immune cells, we propose that it should be viewed as a tolerance-associated molecular pattern (TAMP) and a danger-associated molecular pattern (DAMP) in human and mouse myeloid immune cells respectively.

Paper IV

Human granulocytic myeloid-derived suppressor cells (G-MDSCs) in metastatic breast cancer patients is a heterogeneous population with tumor promoting capacity *in vivo*.

Background

Myeloid-derived suppressor cells (MDSCs) comprise a heterogeneous cell population with potent immunosuppressive properties that has been implicated in tumorigenesis. MDSCs are commonly divided into two subgroups; Mo-MDSCs and G-MDSCs. In *Paper III*, we described one potential mechanism involved in the generation of Mo-MDSCs. The origin of G-MDSCs, on the other hand, is less clear. One controversial hypothesis states that G-MDSCs are an immunosuppressive subset of neutrophils [83,195]. In *Paper IV*, we aimed to shed new light on the origin of G-MDSCs in humans and gain better understanding on the effects G-MDSCs might exert on tumor biological function *in vivo*.

Results and Discussion

The enrichment of G-MDSCs has been observed in various types of cancers [188,286-288]. In order to investigate the prevalence of G-MDSCs in metastatic breast cancer (MBC) patients, we performed flow cytometric analysis of cells from the Ficoll low-density mononuclear cell fraction by using antibodies specific for G-MDSCs cell surface markers. Our results showed that the levels of $CD33^{+}CD11b^{+}CD15^{+/low}CD14^{low}$ G-MDSCs was significantly enriched in patents with MBC as compared to healthy blood donors. We next assessed whether the enrichment of G-MDSCs was correlated with clinicopathological features, however, we did not observe any. This is in contrast to our previously published results where we report a correlation between enrichment of Mo-MDSCs and disease progression in MBC patients [62].

Further analysis revealed that G-MDSCs comprise a morphologically heterogeneous cell population, consisting of both blasts and polymorphonuclear (PMN) cells, where the PMN cells predominate in amount. The morphological heterogeneity has been reported previously [179,289], however, there is still no information regarding the functional differences or similarities between these cells. It is rather intriguing to speculate whether one type of the cells actually comprise the true G-MDSC cell population. We have tried to answer this question by performing single-cell RNA sequencing analysis on sorted G-MDSCs from MBC patients. However, we could unfortunately not harvest enough RNA to perform the analysis. Currently we are in the process of collecting samples in order to perform CyTOF mass spectrometry analysis, which hopefully will provide us with some enlightening answers.

We performed gene expression profiling analysis on G-MDSCs sorted from MBC and Gram+ sepsis patients as well as neutrophils and monocytes from healthy donors. Our results demonstrated that the gene expression from MBC G-MDSCs clustered with neutrophils from healthy donors, sharing similarities in gene expression relevant in angiogenesis, lymphangiogenesis and chemotaxis. Several overlaps between G-MDSCs and neutrophils have been reported. Alike G-MDSCs, neutrophils isolated on a density gradient have been found in the low-density mononuclear cell fraction [289,290]. Moreover, an immature ring-shaped and banded morphology of neutrophils has been described as well [289]. Concerning the function, immunosuppressive features in neutrophils have been also reported [195,291]. This supports the notion that G-MDSCs might be a subset of neutrophils. Surprisingly, gene expression profile from MBC G-MDSCs differed from G-MDSCs from Gram+ sepsis patients. One explanation to this discrepancy might be due to different generation mechanisms. While in the context of sepsis, G-MDSCs might be generated through stimulation by PAMPs, G-MDSCs generation in MBC patients might, on the other hand, be steered by DAMPs [84].

To broaden our knowledge about the effects that G-MDSCs exert on tumor-biological functions, we constructed a breast cancer xenograft model where we co-transplanted G-MDSCs sorted from MBC patients together with a TNBC cell line (MDA-MB-231) in immunocompromised NSG mice. Upon excising the tumors on day 21, we were struck by the apparent significant increase in tumor size in G-MDSCs/MDA-MB-231 xenografts compared to xenografts containing MDA-MB-231 cells only. As no difference in the Ki67 expression was observed, we concluded that the size difference is not a result of an increased proliferation. When staining for CD11b, CD163 and S100A9 (pan-myeloid cell, anti-inflammatory myeloid cell and MDSCs marker respectively [35]), we did not observe the presence of G-MDSCs, indicating that they did not survive until day 21. Despite their short lifespan, they seem to have an effect on the tumor growth early on in tumor development. This is in contrast to the transplantation survival of monocytes which survived in tumors until day 90 [35]. Immunohistochemical staining of the endothelial marker; CD31 and lymph vessel marker; Lyve-1, revealed an increase, although not significant, of both markers in G-MDSCs/MDA-MB-231 xenografts. These results are supported by the gene profile analysis where upregulation of genes relevant in angiogenesis and lymphangiogenesis was observed. As angiogenesis and lymphangiogenesis play essential role in tumor progression and act as source of nutrients [292], the observed increase in blood vessels in G-MDSCs/MDA-MB-231 xenografts might be an explanation to the increased tumor size. Interestingly, we also observed an infiltration of murine myeloid cells ($Ly6C^+$) in MDA-MB-231 xenografts while being completely absent in G-MDSCs/MDA-MB-231 xenografts, suggesting a novel G-MDSCs immunosuppressive mechanism. At this point we are however oblivious to the mechanism behind this process, and encourage further investigation in this particular field.

In conclusion, we report that G-MDSCs are enriched in MBC patients and comprise a heterogeneous cell population displaying both blast-like and PMN cell morphology. Furthermore, G-MDSCs contribute to tumorigenesis by inducing tumor growth and angiogenesis. We also report a novel G-MDSCs immunosuppression mechanism characterized by inhibition of immune cells infiltration within the tumor. Additionally, we hypothesize that G-MDSCs is an alternative immunosuppressive subset of neutrophils.

Paper V

Autoimmune regulator (AIRE) expressed in tumor associated macrophages is associated with worse prognosis in breast cancer patients.

Background

One highly critical aspect of the immune system is self-tolerance. Ideally, our immune system should recognize and eliminate pathogens and transformed cells while being tolerant to self. Autoimmune regulator (AIRE), is a transcription factor with an important role immune tolerance. Primarily, expressed by mTECs in thymus, AIRE induces transcription of a vast array of TSAs which subsequently are involved in the negative selection of T cells. The fate of T cells displaying a too high affinity for a TSA is death by apoptosis [143]. AIRE expression has also been reported in peripheral organs, most notably in DCs but also in cancer. However, the role of AIRE in peripheral tolerance is to date not clearly understood, and in *Paper V*, we aimed to elaborate on the expression and prognostic value of AIRE in breast cancer patients.

Results and Discussion

In order to evaluate the expression of AIRE, we stained a TMA of 144 breast cancer patients by means of immunohistochemistry. We observed a positive AIRE expression both in the cytoplasm ($AIRE_{cyt}$) and the nucleus ($AIRE_{nuc}$) of breast cancer cells as well as in CAFs ($AIRE_{CAF}$) and TAMs ($AIRE_{TAM}$). Nuclear AIRE expression in BC cells inversely correlated with nodal stage and tumor size, while $AIRE_{cyt}$ expression displayed an inverse correlation only with tumor size. However, we did observe a significantly positive correlation between $AIRE_{cyt}$ and $HER2^+$ BCs. It is worth mentioning that the $HER2^+$ BC population in present cohort was rather small (n=12), and that the AIRE expression in $HER2^+$ BCs deserves further investigation in a larger patient population. When assessing the $AIRE_{TAM}$ expression, we observed that $AIRE_{TAM}$ correlated with larger tumor size and NHG status. Furthermore, $AIRE_{TAM}$ expression displayed a significant correlation with infiltrating TAMs, both in the stroma and with overall presence. $AIRE_{TAM}$ also displayed an inverse correlation with $AIRE_{nuc}$ and $AIRE_{CAF,}$ shorter recurrence free survival and a borderline correlation with worse overall survival in breast cancer patients. These results indicate that AIRE expressing TAMs are most relevant regarding function in human BC. Additionally, $AIRE_{TAM}$ expression also correlated with TAM ($CD68^+CD163^+$) infiltration in the stroma but not in the tumor nest. The

correlation was particularly apparent in the presence of TAM rich tumors. When looking into other immune cells, including $CD3^+$ T cells, T_{regs}, T_h17^+ cells, mDCs and $S100A9^+$ myeloid cells, we could not observe any correlation between the above-mentioned parameters and $AIRE_{TAM}$. However, we noticed a borderline correlation between $FoxP3^+$ cells and $AIRE_{CAF}$, suggesting that CAFs might play a role in the T_{regs} accumulation in BC. Next, we performed gene expression analysis of AIRE in several BCs cell lines, *in vitro* differentiated monocyte-derived mDCs, M0, M1 and M2 macrophages and human breast cancer stromal cells. *AIRE* expression in peripheral DCs, has been reported previously [148,293] and this is further supported by our results. As expected, we observed *AIRE* expression in monocyte derived DCs (mDCs) on mRNA level. *AIRE* mRNA expression was also observed in breast cancer stromal cells and was particularly apparent in M1 and M2 macrophages. Interestingly, Kogawi *et al.* showed that $CD14^+$ monocytes as well as *in vitro* differentiated mDCs expressed AIRE both on mRNA and protein level [293]. These results suggest that *AIRE* expression in mDCs as well as in monocyte- derived M1 and M2 might be acquired early on in their development and preserved after their differentiation. Surprisingly, we did not observe *AIRE* mRNA expression in any of the seven BC cell-lines and as such this is in conflict with our IHC results where we observed a clear AIRE protein expression. The prime function of AIRE in the thymus is to regulate the maturation of T cells by inducing expression of TSAs [143]. Although the reports are quite few and inconstant, the expression of TSAs has also been observed in the periphery with the main hypothesis speculating that they are involved in the maintenance of peripheral tolerance [294]. Thus, it would be of interest to evaluate whether the AIRE expression in human BCs is functional by assessing the expression of TSAs.

In conclusion, when assessing the expression of AIRE in a cohort comprising 144 breast cancer patients, we report that AIRE is expressed in the cytoplasm and the nucleus of breast cancer cells ($AIRE_{cyt}$/ $AIRE_{nuc}$) as well as in CAFs ($AIRE_{CAF}$) and TAMs ($AIRE_{TAM}$). We also report a correlation between $AIRE_{TAM}$ and larger tumor size and NHG status, infiltrating TAMs and shorter recurrence free survival. Furthermore, we show *AIRE* mRNA expression in monocyte-derived DCs, M1 and M2 macrophages. In the future, we are planning various functional assays to investigate potential immunosuppressive function in BC.

Conclusions

I. TLR4 is predominantly expressed in TNBC cells and responds both to PAMPs and DAMPs by secreting inflammatory mediators. TLR4 expression is correlated with worse recurrence-free survival in $ER^{-}PgR^{-}$ breast cancer patients.

II. S100A9 is expressed both in malignant cells and in anti-inflammatory $CD163^{+}$ myeloid cells in human breast cancer. It induces secretion of pro-inflammatory mediators in a TLR4-dependent manner and its expression correlates with poor outcome in $ER^{-}PgR^{-}$ breast cancer patients.

III. Wnt5a is a novel TLR2/4 ligand that acts as a tolerance-associated molecular pattern (TAMP) in human myeloid cells and damage-associated molecular pattern (DAMP) in murine myeloid cells.

IV. G-MDSCs comprise a heterogeneous cell population enriched in metastatic breast cancer patients. G-MDSCs represent an alternative immunosuppressive subset of neutrophils that contribute to tumor progression.

V. AIRE is expressed in malignant cells, in CAFs and in TAMs in human breast cancer, and its expression in TAMs correlates with worse outcome.

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The Cover

”Akaleyi I” by Emma Lindström.