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TOLL-LIKE RECEPTOR 9 IN BREAST CANCER

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To my family

Education is a progressive discovery of our own ignorance.

-Will Durant

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Toll-like receptor 9 in breast cancer

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ABSTRACT

The innate immune system recognizes microbial features leading to the activation of the adaptive immune system. The role of Toll-like receptor 9 (TLR9) is to recognize microbial DNA. In addition to immune cells, TLR9 is widely expressed in breast cancer in addition to other cancers. Breast cancer is the most common cancer in women, affecting approximately one in eight in industrialized countries. In the clinical setting, breast cancer is divided into three clinical subtypes with type-specific treatments. These subtypes are estrogen receptor (ER)-positive, HER2-positive and triple-negative (TNBC) breast cancer. TNBC is the most aggressive subtype that can be further divided into several subtypes. TNBC tumors lack ER, progesterone receptor and HER2 receptor. Therefore, the current clinically used targeted therapies are not suitable for TNBC treatment as TNBC is a collection of diseases rather than one entity. Some TNBC patients are cured with standard chemotherapy, while others rapidly die due to the disease. There are no clinically used biomarkers which would help in predicting which patients respond to chemotherapy.

During this thesis project, we discovered a novel good-prognosis TNBC subtype. These tumors have high TLR9 expression levels. Our findings suggest that TLR9 screening in TNBC patient populations might help to identify the patients that are at the highest risk regarding a relapse. To gain better understanding on the role of TLR9 in TNBC, we developed an animal model which mimicks this disease. We discovered that suppression of TLR9 expression in TNBC cells increases their invasive properties in hypoxia. In line with the clinical findings, TNBC cells with low TLR9 expression also formed more aggressive tumors *in vivo*. TLR9 expression did not, however, affect TNBC tumor responses to doxorubicin. Our results suggest that tumor TLR9 expression may affect chemotherapy-related immune responses, however, this requires further investigation. Our other findings revealed that DNA released by chemotherapy-killed cells induces TLR9-mediated invasion in living cancer cells. Normally, extracellular self-DNA is degraded by enzymes, but during massive cell death, for example during chemotherapy, the degradation machinery may be exhausted and self-DNA is taken up into living cells activating TLR9. We also discovered that the malaria drug chloroquine, an inhibitor of autophagy and TLR9 signalling does not inhibit TNBC growth *in vivo*, independently of the TLR9 status. Finally, we found that ERα as well as the sex hormones estrogen and testosterone regulate TLR9 expression and activity in breast cancer cells *in vitro*. As a conclusion, we suggest that TLR9 is a potential biomarker in TNBC.

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Tollin kaltainen reseptori 9 ja rintasyöpä

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TIIVISTELMÄ

Sisäsyntyisen immuniteetin tehtävä on tunnistaa mikrobien molekyylirakenteita, mikä saa aikaan adaptiivisen immuunijärjestelmän aktivoitumisen. Tollin kaltainen reseptori 9 (TLR9) on dna:ta tunnistava sisäsyntyisen immuniteetin reseptori, jota ilmennetään myös useissa syövissä, kuten rintasyövässä. Rintasyöpä on naisten yleisin syöpä, johon joka kahdeksas nainen sairastuu elämänsä aikana. Kliinisesti rintasyöpä jaotellaan kolmeen alatyyppiin, joista kolmoisnegatiivinen rintasyöpä on aggressiivisin. Tämän tyypin syövät eivät ilmennä hormonireseptoreja (estrogeeni- ja progesteronireseptori) tai HER2-reseptoria. Tästä johtuen kolmoisnegatiivisten potilaiden hoitoon ei voida käyttää rintasyövän nykyisten hoitosuositusten mukaisia täsmähoitoja. Kolmoisnegatiivinen rintasyöpä ei kuitenkaan ole yksi sairaus, koska molekyylitasolla sen on osoitettu koostuvan lukuisista, biologialtaan erilaisista syöpämuodoista. Tällä hetkellä kliinisessä käytössä ei ole biomarkkeria, jonka avulla kolmoisnegatiivisen rintasyövän alatyypit voisi erottaa toisistaan.

Löysimme uuden kolmoisnegatiivisen syövän alatyypin, joka ilmentää vain vähän TLR9-proteiinia. Tällä alatyypillä on erittäin huono ennuste ja tulostemme perusteella TRL9-tason selvittäminen voisi seuloa huonoennusteiset syövät kolmoisnegatiivisten syöpien joukosta. Kehitimme eläinmallin, jolla voidaan tutkia matalan ja korkean TLR9-tason vaikutuksia kolmoisnegatiivisten kasvainten hoitovasteeseen. Toinen löytömme oli, että kemoterapialla tapettujen syöpäsolujen dna saa aikaan elävien syöpäsolujen TLR9-välitteistä invaasiota. Normaalisti entsyymit hajoittavat yksilön oman solunulkoisen dna:n. Erikoistilanteissa, kuten syöpähoitojen yhteydessä, jolloin solukuolema on massiivista, elimistön oma koneisto ei ehdi tuhoamaan solunulkoista dna:ta ja sitä voi kertyä eläviin soluihin, joissa se aktivoi TLR9:n. Kolmanneksi havaitsimme, että malarialääke klorokiini, joka estää TLR9:n toimintaa ja jolla on syövänvastaisia vaikutuksia soluviljelyolosuhteissa, ei estänyt TLR9-positiivisten tai TLR9-negatiivisten kasvainten kasvua käyttämässämme eläinmallissa. Neljänneksi soluviljelykokeittemme tulokset osoittivat, että sukupuolihormonit estrogeeni ja testosteroni sekä estrogeenireseptori osallistuvat TLR9:n ilmentymisen ja aktiivisuuden säätelyyn.

Tuloksemme osoittavat, että TLR9 potentiaalinen biomarkkeri kolmoisnegatiivisessa rintasyövässä.

TABLE OF CONTENTS

ABBREVIATIONS

AA	African-American
Ab	antibody
AMP	antimicrobial peptide
AP	adaptor protein
APC	antigen-presenting cell
BC	breast cancer
BCR	B cell receptor
BLBC	basal-like breast cancer
CEF	(cyclophosphamide, epirubicin and 5-fluorouracil (5FU)); first-line chemotherapy in all breast cancers in Finland
CpG-ODN	oligodeoxynucleotide containing a cytosine triphosphate deoxy-nucleotide followed by a guanine triphosphate deoxynucleotide
CNS	central nervous system
cPR	clinical partial response
CQ	chloroquine
CTC	circulating tumor cell
DAMP	danger/damage-associated molecular pattern
DC	dendritic cell
DFS	disease-free survival
DTC	disseminating tumor cell
EBV	Epstein-Barr virus
ECD	extracellular domain/ ectodomain
EMT	epithelial-to-mesenchymal transition
ER	estrogen receptor
ER	endoplasmic reticulum
HCQ	hydroxychloroquine
HER2	human epidermal growth factor receptor 2
HMGB1	high-mobility group box 1
HPV	human papillomavirus
HR	hormone receptor
HSV	Herpes simplex virus
IC	immune complex
IDC	invasive ductal carcinoma
IFN	interferon
IL	interleukin
ILC	invasive lobular carcinoma
IMO	immunomodulatory oligonucleotide
IRF	interferon regulatory factor
LCIS	lobular carcinoma *in situ*
LRR	leucine-rich repeat
MMP	matrix metalloproteinase
MyD88	myeloid differentiation factor 88
NET	neutrophil extracellular trap
NK cell	natural killer cell
NLR	NOD-like receptor
ODN	oligodeoxynucleotide
OS	overall survival
PAMP	pathogen-associated molecular pattern
PCa	prostate cancer
pCR	pathologic complete response
PFS	progression-free survival
PR	progesterone receptor
PRR	pattern recognition receptor
RFS	recurrence-free survival
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism
TAM	tumor-associated macrophage
TIL	tumor-infiltrating lymphocyte
TIR domain	Toll/IL-1R domain
TLR	Toll-like receptor
TN	triple-negative
TNBC	triple-negative breast cancer
TNFα	tumor necrosis factor α
Treg	regulatory T cell

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following publications, which are referred in the text by the Roman numerals I-IV. The original publications have been reproduced with the permission of the copyright holders.

I Sandholm J, Kauppila JH, Pressey C, Tuomela J, Jukkola-Vuorinen A, Vaarala M, Johnson MR, Harris KW, Selander KS. Estrogen receptor-α and sex steroid hormones regulate Toll-like receptor-9 expression and invasive function in human breast cancer cells. *Breast Cancer Res Treat*. 2012 Apr;132(2):411-9.

II Tuomela J*, Sandholm J*, Karihtala P*, Ilvesaro J, Vuopala KS, Kauppila JH, Kauppila S, Chen D, Pressey C, Härkönen P, Harris KW, Graves D, Auvinen PK, Soini Y, Jukkola-Vuorinen A, Selander KS. Low TLR9 expression defines an aggressive subtype of triple-negative breast cancer. *Breast Cancer Res Treat*. 2012 Sep;135(2):481-93.

III Tuomela J, Sandholm J, Kauppila JH, Lehenkari P, Harris KW, Selander KS. Chloroquine has tumor-inhibitory and tumor-promoting effects in triple-negative breast cancer. *Oncol Lett.* 2013 Dec;6(6):1665-1672.

IV Tuomela J, Sandholm J, Kaakinen M, Patel A, Kauppila JH, Ilvesaro J, Chen D, Harris KW, Graves D, Selander KS. DNA from dead cancer cells induces TLR9-mediated invasion and inflammation in living cancer cells. *Breast Cancer Res Treat.* 2013 Dec;142(3):277-87.

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1. INTRODUCTION

Toll-like receptors work as sentinels of the innate immunity system, as they recognize evolutionarily conserved microbial molecular patterns. Toll-like receptor 9 (TLR9) is an endosomal receptor for microbial DNA, which also recognizes self-DNA molecules in autoimmune settings when the clearance of dead cell material by DNAses is ineffective. TLR9 is expressed in several cell types of the immune system, such as plasmacytoid dendritic cells and natural killer cells. It is also expressed in several cancers, including breast cancer, where the role of TLR9 in breast cancer pathophysiology is currently unclear.

Breast cancer is the most common malignant disease in women of industrialized countries. According to WHO, there are 17 histological breast cancer subtypes. In addition, there is a great variation within the subtypes. Currently, some of the subtypes lack targeted therapies. Therefore, we urgently need new biomarkers for breast cancer treatment.

In breast cancer, the interplay between the tumor and immune system is of high importance. It is possible, if not probable that TLR9 has diverse roles in both immune and tumor cells. Therefore, it is crucial to study the role of TLR9 in the contexts of both the immune system and tumorigenesis.

2. REVIEW OF THE LITERATURE

2.1 The natural history of breast cancer

Breast cancer (BC) is the most common malignant disease in Western women, and the second leading cause of death after heart and coronary diseases. Globally, BC is responsible for 23% of female cancer diagnoses (1.38 million women) and 14% of cancer deaths (458 000 women) per year according to statistics in 2008 (Amaro *et al.*, 2013, Jemal *et al.*, 2011). As reported by the Nordic cancer registry, 4462 new BC diagnoses were made annually between 2009 and 2013 in Finland. Approximately one in eight Finnish women will be diagnosed with BC. Of all cancers in women in Finland, BC constitutes approximately 31% of them, and within the last 10 years, BC incidence has increased annually by 0.9% in women and 2.6% in men. In 1970, the age-standardized incidence was below 40 per 100 000. By 2010, the incidence had risen to approximately 90 per 100 000 (The NORDCAN project; http://www-dep.iarc.fr/nordcan.htm and www.cancer.fi; accessed September 12th 2015). Also, worldwide, BC incidence is increasing (Gluz *et al.*, 2009). In the end of 2013, there were approximately 62 000 women and 200 men alive who had or had had BC in Finland. Within one year of diagnosis, 97% of patients are alive and five years after diagnosis, 87% of patients are alive (The NORDCAN project; http://www-dep.iarc.fr/nordcan.htm; accessed March 8th 2015). This leads to the 5-year mortality rate of BC to be 13% of people diagnosed here in Finland.

Anatomically, the mammary gland resembles a tree, with ductal and lobular networks (Figure 1). The epithelium of normal breast tissue is built of a bilayer of inner luminal cells and a surrounding outer layer of basal (myoepithelial) cells. After pregnancy, the lobular secretory luminal cells produce milk, whereas the ductal and lobular myoepithelial cells are responsible for the milk release (Skibinski & Kuperwasser, 2015). Mammary gland development has several distinct stages (ectoderm - placode - rudimentary ductal structure present at birth - pubertal growth - pregnancy - lactation - involution after lactation - postmenopausal involution) (Macias & Hinck, 2012). The breast undergoes regeneration and regression cycles with successive menstruation cycles and pregnancies (Haricharan & Li, 2014). Hormones, for example estrogen, progesterone and prolactin are required to achieve the different developmental stages (Macias & Hinck, 2012). Haricharan and Li state that it is not surprising that BC is one of the most common malignancies among women, as the mammary gland is cyclically exposed to a large amount of growth factors and hormones which promote cellular expansion and regression (Haricharan & Li, 2014).

Most of the invasive BCs, and their *in situ* precursors, have their origin in acini, structures which form terminal ductal lobular units. An acinus consists of a hollow lumen which is surrounded by a single layer of polarized luminal epithelial cells (Sims *et al.*, 2007).

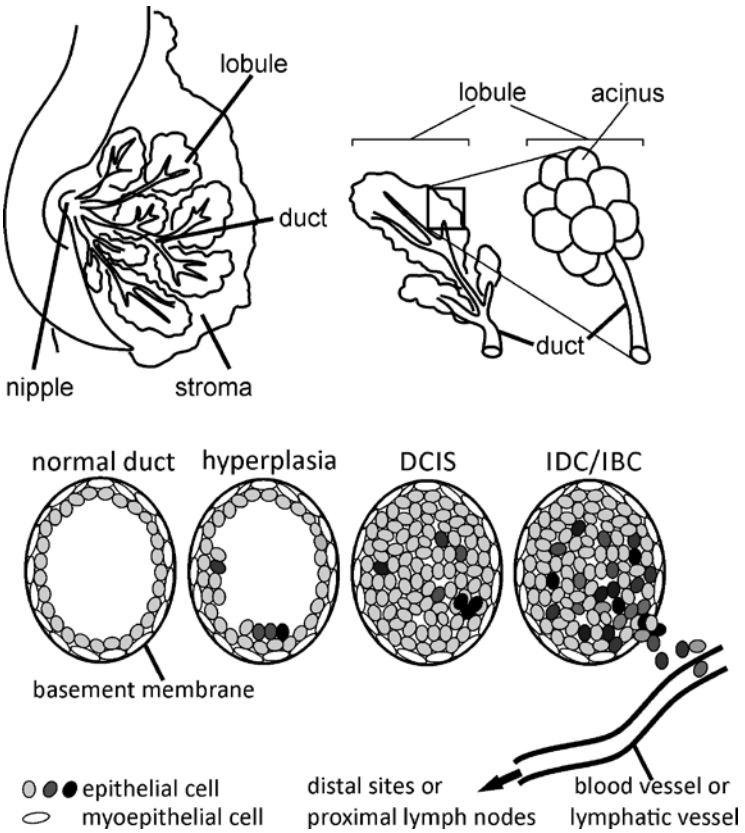

Figure 1. The structure of the breast and the evolution of invasive ductal carcinoma (IDC). The tumor originates from the ductal lobular units (acini) proceeding to an invasive adenocarcinoma either via a ductal or lobular carcinoma *in situ* phase. Only ductal pathway is shown. DCIS, ductal carcinoma *in situ*; IBC, invasive breast cancer. Adapted from Haricharan and Li, 2014.

The sex steroid hormones, estrogens are crucial for the normal development and maintenance of the breast, but their ability to promote proliferation also contributes to breast tumorigenesis (Stocco, 2012). Estrogen is the key promoter of BC, and most hormone receptor (HR)-positive tumors initially respond to anti-hormone therapy. Testosterone is protective in BC, except when aromatized to estrogen via aromatase enzyme, it promotes BC (Glaser & Dimitrakakis, 2015). Estrogen receptor (ER)α signalling promotes cell cycle progression leading to cell proliferation, whereas ERβ signals to the opposite direction promoting apoptosis and inhibiting proliferation.

Progesterone receptor (PR), which is also a gonadal hormone, is often expressed in BC, but its role is controversial (Risbridger *et al.*, 2010).

Tumorigenesis is a multistep phenomenon where cellular homeostasis is compromized. Survival-promoting mutations accumulate in cells resulting in a malignant phenotype. These mutations can have an inherited origin or they can be induced by external stimuli. Hanahan and Weinberg have compiled a list of ten hallmarks (capabilities), which tumors acquire during tumorigenesis. The hallmarks are: apoptosis resistance, genetic instability, angiogenesis induction, invasion and metastasis activation, replicative immortality, growth suppressor evasion, active proliferative signalling, cellular energetics deregulation and, highlighting the importance of immunity, protumoral inflammation and tumor cell immune evasion (Hanahan & Weinberg, 2011, Hanahan & Weinberg, 2000).

Wang and coworkers performed BC single cell sequencing to study tumor evolution, and they discovered that there were no genetically identical tumor cells. Instead, they claim that the large number of subclonal and *de novo* mutations in their data indicated that the tumors had evolved for a long time resulting in clonal diversity (Wang *et al.*, 2014). Autopsy studies have shown that genome alterations are more common than previously believed (occult cancer). 39% of women in their forties had histologic BC in a postmortem examination (Klinke, 2012, Bissell & Hines, 2011). All cancers carry somatic mutations, which often harbor areas of regional hypermutations that are common but variable in BC (Nik-Zainal *et al.*, 2012). More than 400 genes are implicated in the development of cancer in general, however the number of genetic alterations critical for carcinogenesis is not clear, yet it appears to be at least five to seven in solid cancers (Duffy, 2013).

Even though cancer cells are responsible for the disease, the surrounding tissue (the tumor microenvironment; the stroma) has a substantial significance on tumor growth, invasion and metastasis. In the stroma, fibroblasts, endothelial cells and immune cells interact with normal epithelium and BC cells. The gene expression profile of the stroma follows that of the histological tumor grade, and changes vastly during cancer progression, even prior to local invasion. As an example, BC-associated fibroblasts proliferate faster and secrete higher titers of growth and immunomodulatory factors, as well as extracellular matrix proteins (Sadlonova *et al.*, 2009, Dittmer & Leyh, 2015).

In order to grow into a clinically observable tumor, cancer cells have to escape immune recognition (Koebel *et al.*, 2007, Schreiber *et al.*, 2011). Both tumor cells and stroma, including infiltrated immune cells, participate in the protumoral immunoediting, where tumor cells acquire a non-immunogenic phenotype (Ma *et al.*, 2009, Bidwell *et al.*,

2012). In fast-proliferating tumors, low oxygen concentration (hypoxia) induces a metabolic switch (Warburg effect), where tumor cells shift from oxidative respiration to glycolysis (aerobic to anaerobic) as their primary source of energy (Bonuccelli *et al.*, 2010). The metabolic requirements and the distribution of nutrients and waste products in the BC often split the tumor into a proliferating outer shell, a senescent inner region, and a necrotic core (Klinke, 2012). Tumor treatment-associated cellular stress promotes epithelial-to-mesenchymal transition (EMT), a natural process for example in normal morphogenesis (Weinberg, 2008). Normally, epithelial cells can move only laterally in the plane of the epithelium, where they maintain contact with the basement membrane. Tumorigenic EMT induces gain of stem cell properties, accelerated invasion, metastasis, and resistance to therapy (Hanahan & Weinberg, 2011, Weinberg, 2008).

With solid tumors, most of the deaths are caused by metastases, which like primary tumors, also have the capability to evade immuno detection (Klein, 2009). After the tumor cells have entered the blood or lymphatic circulation, they colonize a distant site and grow. The seed and soil hypothesis introduced in 1889 by Stephen Paget is the prevailing view on metastasis formation (Paget, 1889). The theory states that the metastatic cell (the seed) has to find a suitable (congenial) microenvironment (soil) to prosper (Fidler & Poste, 2008). Tumor cells that have undergone EMT may use a reverse process (mesenchymal-to-epithelial transition), which may result in metastases with a histopathology of the primary tumor cells that never underwent EMT (Hanahan & Weinberg, 2011). Metastases can further disseminate secondary metastases in a process called shower of metastases (Klein, 2009, Weinberg, 2008).

In biological terms, tumors can be considered as Darwinian systems of cell populations, which obey evolutionary dynamics (Hanahan & Weinberg, 2011). It is possible that clonally homogenous tumors have a better treatment response as heterogenous tumors more probably have tumor cells which survive the treatment (Klinke, 2012). The selection for endocrine therapy-resistant tumor cell clones in an initially treatment-responsive tumor is one of the biggest challenges in BC therapy (Polyak, 2014). Whatever we do to the cancer, it introduces a new selective pressure on the tumor evolution.

2.2 Classification and clinical subtypes of breast cancer

WHO classifies at least 17 histological types of BC. The large majority (50-80%) are invasive ductal carcinomas (IDC) and the second most common type is invasive lobular carcinoma (ILC). Special histopathological types, which are not IDC (tubular, medullary, mucinous or papillary), ILC, or metaplastic, account for up to 25% of all invasive BCs. The scarcity of any special type, and their divergent histological features and clinical

implications result in difficulties to conduct advanced molecular studies (Weigelt & Reis-Filho, 2009).

As terms, ductal and lobular carcinomas do not reveal the tumor origin or histogenesis, but are based on tumor architecture, cytology and immunohistochemistry (Weigelt & Reis-Filho, 2009). The clinical phenotype of the tumor cannot necessarily be deducted from the histological type (Badve *et al.*, 2011).

BC classification is based on histopathology, molecular pathology, genetic analysis or gene expression profiling. The tumor grade is an evaluation of proliferation and the degree of differentiation (tubule formation and nuclear pleomorphism) (Weigelt *et al.*, 2005), and the histological tumor grade and tumor type are the most significant measures that can be identified by histopathological analysis. Immunohistochemical analysis of ER, PR and HER2 (human epidermal growth factor receptor-2/ERBB2/ Neu) classify the tumors as luminal ($ER^+/PR^+/HER2^-$), $HER2^+$ ($ER^-/PR^-/HER2^+$), or triple-negative (TN; $ER^-/PR^-/HER2^-$). A tumor is classified ER/PR-negative when <1% of the tumor cell nuclei immunostain for ER or PR. HER2-negativity is defined by immunohistochemistry when ≤2% of the cells are HER2-positive, when HER to CEP17 (centromeric probe for chromosome 17) FISH ratio is below 2.2, or if the average HER2 gene copy number per nucleus is below 6 (Pal *et al.*, 2011). Generally, 15-30% of all BCs have gene amplification or overexpression (or both) of HER2, a tyrosine kinase receptor, resulting in a more aggressive phenotype and a poor prognosis in sentinel lymph node-positive patients (Baselga *et al.*, 2012, Wolff *et al.*, 2007).

BC staging is based on the primary tumor size, sentinel lymph node positivity/negativity and the existence of metastases. The stage advances gradually from 0 to IV, where carcinoma *in situ* tumors are of stage 0, and a stage IV tumor has already metastasized to a distant location (Matsen & Neumayer, 2013).

In the seminal gene expression-based BC classification, tumor groups are defined by the molecular features of the mammary epithelium: ER^+/luminal-like, basal-like, $HER2^+$ and normal breast (Perou *et al.*, 2000). Currently, a 5-class grouping is used, although others have been proposed. The subtypes are luminal A, luminal B, normal breast-like, HER2, and basal-like. The clearest delineation is made between luminal and basal-like tumors as luminal tumors express ER and possibly PR. ER-negative basal BCs resemble the basal epithelial layer of normal breast, often have chromosome segment gains or losses, and a poor outcome (Sims et al., 2007, Badve et al., 2011, Kao *et al.*, 2009).

Even though we have vast theoretical knowledge of new putative predictive marker molecules, ER, PR, HER2 and Ki-67 (proliferation marker) stainings, and histological

gradus analyses are used in the everyday clinical practise, where they guide the initial treatment options.

Tumor tissue is defined well-differentiated when it resembles its tissue-of-origin, whereas loss-of-differentiation is often accompanied by increased aggressivity. BC subtypes reflect mammary epithelial cell development. Mammary stem cell-like claudin-low type BC is the most primitive (least differentiated) tumor type, and the luminal progenitor-like basal phenotype is more differentiated than the claudin-low type. HER2-positive tumors lose their basal phenotype and gain luminal features. Luminal A and B tumors are the most differentiated (Perou, 2010), therefore usually the least aggressive.

2.3 Triple-negative breast cancer (TNBC)

2.3.1 TNBC definition

TNBC is a diagnosis of exclusion ($ER^-/PR^-/HER2^-$). The frequency depends on the methods and thresholds used, and TNBC generally represents 10-24% of invasive cancers (Carey *et al.*, 2010). Most of TNBCs are larger in size, high-grade, IDCs of no special type, which are characterized by marked degrees of nuclear pleomorphism, the lack of tubule formation, and high proliferation. TNBCs are aggressive and often have local lymph node metastases at diagnosis (Turner & Reis-Filho, 2013). Less than 30% of patients with metastatic TNBC survive 5 years (Carey et al., 2010).

Most TNBCs are histologically basal-like BCs (BLBCs), but only half of TNBCs have the BLBC gene signature (Lehmann *et al.*, 2011). Also, up to 45% of BLBCs are not TN. TNBC and BLBC are overlapping and the terms are often used interchangeably. However, they are distinct, as TNBC diagnosis is based on three genes, and rigorous BLBC diagnosis depends on approximately 500 genes (Carey et al., 2010, Foulkes *et al.*, 2010b). In one study, Kreike and coworkers studied 97 TN tumors and concluded that TNBC and BLBC are synonymous (Kreike *et al.*, 2007). However, when outcomes of non-BL TNBC and BL TNBC were compared, BL TNBC patients had significantly decreased BC-specific overall survival (OS) showing that the subtypes are not identical (Gluz et al., 2009).

When ER^+ and TNBC cells were compared and sequenced at a single cell level, aneuploidy was discovered to occur early in tumor development in both types. Importantly, point mutation rates differed as ER^+ cells had approximately similar mutation rates as normal cells, but TNBC cells had an over 13-fold point mutation rate increase showing that TNBC is genetically very unstable (Wang et al., 2014). TNBCs frequently express poor prognosis-associated markers such as EGFR (epidermal growth factor receptor) and myoepithelial markers (for example caveolins 1 and 2, c-kit, and P-

cadherin). Epithelial markers, such as E-cadherin are less probably expressed in TNBC, whereas proliferation-associated genes, such as Ki-67 and TOP2A (DNA topoisomeraasi II alpha) are highly expressed in TNBC (Carey et al., 2010).

Deregulated metabolism/energetics is one of the aforementioned cancer hallmarks (Hanahan & Weinberg, 2011). In a metabolite analysis, TNBC tumors had higher levels of choline and glutamate, and lower levels of glutamine than $ER^+/PR^+/HER2^+$ tumors. This shows that the differential metabolic profile of TNBC could have implications in tumorigenesis (Cao *et al.*, 2014). For example, glutamate has been suggested to promote glioblastoma multiforme invasion (Oh *et al.*, 2012).

2.3.2 TNBC subclassification

Based on gene expression analysis, basal-like (50%-75%) and claudin-low (5%-10%) tumors are the main TNBC subgroups (Bayraktar & Glück, 2013, Perou, 2010), as most claudin-low tumors are TN. The novel claudin-low subtype tumors lack cell-cell tight junction components claudins 3, 4 and 7 as well as E-cadherin. However, different BC subtypes have variable expression of claudin family members. Claudin-low tumors generally have high amounts of infiltrated immune cells, show EMT and have poor prognosis (Kwon, 2013, Perou, 2010). The normal breast-like tumor group, or a part of it is possibly a subgroup of TNBC (Tan *et al.*, 2008). According to Perou, some tumors of HER2-enriched type, which are not HER-positive, should also be considered TNBCs. The HER2-enriched type is distinctive of the HER2-positive tumor type, and most of HER2-enriched tumors are ER-negative and clinically TN (Perou, 2010). Metaplastic carcinoma, a rare histological BC subtype, which contains neoplastic factors diverse from those expected to be present in adenocarcinomas, is characteristically a TNBC. Such carcinomas may behave aggressively and be less chemotherapy-sensitive than IDCs of TNBC type (Turner & Reis-Filho, 2013). Most TNBCs are ductal, but also other rarer, better prognosis tumors (for example medullary or adenoid cystic tumors) can be TN (Rody *et al.*, 2011, Carey et al., 2010).

Lehmann and coworkers presented 7 TNBC subtypes based on gene expression profiling. These subtypes are basal-like 1 and 2, immunomodulatory, mesenchymal, mesenchymal stem-like, luminal androgen receptor, and unstable.[1] The subtypes had no differences in tumor size and grade, but the luminal androgen receptor and mesenchymal subtypes had a significantly shorter recurrence-free survival (RFS) than the basal-like 1 subtype (Lehmann et al., 2011).

[1] Both TN cell lines (MDA-MB-231 and Hs578T) used in the Manuscripts I-IV are of the mesenchymal stem-like subtype.

2.4 BC risk factors

2.4.1 BC risk factors

Naturally, the female gender is the biggest risk factor for BC. In ER^+ cancers, nulliparity and delayed childbearing are risk factors. In ER^+/PR^+ tumors, late age at menarche has a clear protective effect. BC risk increases with increasing alcohol consumption in ER^+ cancers, as alcohol increases sex hormone levels in several ways (Chen & Colditz, 2007). In a prospective study by Liu and coworkers, alcohol usage before first pregnancy associated more with ER^+/PR^+ than ER^+/PR^- or HR-negative BC (Liu *et al.*, 2013). Ethanol itself is not a carcinogen (Pöschl & Seitz, 2004), but its metabolite, acetaldehyde contributes to the carcinogenicity of alcohol for example in head and neck and esophageal cancers, yet its role in BC remains unclear (Eriksson, 2015).

Epidemiologial studies show that elevated lifetime exposure to estrogen is a major risk factor for BC (Mense *et al.*, 2008). After menopause, the main estrogen source is the adipose tissue, where the aromatase enzyme converts androgenic precursors into estrogens (Chen & Colditz, 2007). Weight gain during adulthood and especially after menopause increases the risk of BC (Eliassen *et al.*, 2006). The dysfunctional adipose tissue of an obese person provides a pro-tumor microenvironment due to inflammation and angiogenesis (Pérez-Hernández *et al.*, 2014).

Infectious agents are thought to associate with approximately one fourth of human cancers. 80% of the agents are viruses (Bouvard *et al.*, 2009). Tumor viruses, such as the human form of the mouse mammary tumor virus, Epstein-Barr virus (EBV) or human papillomavirus (HPV) could be the underlying cause of BC. Approximately 5 % of breast milk samples contain viral sequences (Friedenson, 2013), and the mouse mammary tumor virus, EBV and HPV sequences are found in clinical breast tumors. The presence of these viruses correlates with a higher tumor grade and younger age at diagnosis (Glenn *et al.*, 2012). Anti-retroviral therapy in HIV treatment has been shown to decrease BC incidence (Salmons & Gunzburg, 2013). In the case of HPV, the data are conflicting, where much of the conflict stems from the lack of standardization of detection methods. Silva and da Silva studied the tumor DNA of 79 IDC patients and found no HPV (Silva & da Silva, 2011), whereas Herrera-Goepfert and coworkers studied 20 metaplastic BCs, and found high-risk HPV-16 and HPV-18 from 8 patients (7 HPV-16, 1 HPV-18) of this small Mexican cohort. They concluded that HPV does not play any role in breast carcinogenesis (Herrera-Goepfert *et al.*, 2013, Herrera-Goepfert *et al.*, 2011). However, most researchers believe that HPV-16 and HPV-18 have a role in breast cancer, even though there is no conclusive evidence. In a meta-analysis of 21 publications, there was a significant 3.63-fold increase in breast cancer risk after a HPV infection (Pereira Suarez *et al.*, 2013, Sigaroodi *et al.*, 2012, Li *et al.*, 2011). In the future,

the effect of current HPV vaccination strategies on BC incidence will shed light on the situation.

High breast density (mammographic density) is also a risk factor of BC (Huo *et al.*, 2014). In a study of 27347 postmenopausal women, hormone replacement therapy was shown to elevate BC risk (Manson *et al.*, 2013). Other causative agents include family history of BC, older age, older age at first childbirth or at menopause, height (taller), benign breast hyperplasia, Ashkenazi Jewish heritage, birth control pills, personal history of cancer, high androgen levels in blood, high prolactin levels, high estrogen levels in blood after menopause, radiation exposure during youth or from medical imaging, high bone mass density, and BRCA1 or BRCA2 mutations (http://ww5.komen.org/BreastCancer/ LowerYourRisk.html; accessed April 18th 2014).

2.4.2 TNBC risk factors

TNBC occurrence in African Americans (AAs) is 2-3 times higher (up to 47% of BCs) than in Caucasians (on average 17%). 8% of Japanese patients have a TN disease (Carey et al., 2010). The frequency of TNBC is even higher in native African women (55%; 27% BLBC and 28% unclassified) (Perou, 2010). However, there are probably no biological differences in TNBC tumors between AAs and Caucasians, as ethnicity does not influence the clinical presentation and outcome when patients receive similar therapy and follow-up (Pacheco *et al.*, 2013).

The shift towards anti-inflammatory Th2 immunity in AAs is thought to be an evolutionary response to helminths (parasitic worms) in sub-Saharan Africa, and consequently, the immune adaptations may differentially increase ER^- BC risk among AA women (Anthony *et al.*, 2007). Hong and coworkers showed that women with the highest ratios of proinflammatory Th1 cytokines to IL-5 (a Th2 cytokine) levels were least likely to have ER^- or TNBC compared to ER^+ or luminal A BC. Both ER^- and TNBC were associated with high levels of circulating IL-5, and the strongest trend was detected in premenopausal women. Hence, TNBC gains benefit from an environment where antitumoral Th1 immunity diminishes in relation to Th2 immunity. TNBC was also associated with increased parity. Pregnancy is characterized by a reduction of the Th1/Th2 ratio (Th2 bias). The restoration of normal Th1/Th2 immunity occurs postpartum (Hong *et al.*, 2013), when breastfeeding gives immunological benefits to mothers, resulting in Th1 activation and the reduction of ER^- and TNBC risk (Millikan *et al.*, 2008). Compared with white American women, AA women have higher circulating estrogen levels, higher fertility and higher body mass indices (Gong *et al.*, 2013). Both TNBC and BLBC are more frequent in abdominally obese women (Carey et al., 2010), affect younger (<50 years old) patients, are later often interval BCs (diagnosed between

mammograms), and show more varied histological features. Interval BCs associate with high expression of Ki-67, p53 and basal-like markers, and more dense breast tissue (Badve et al., 2011, Pal et al., 2011). BRCA1 mutation is a TNBC risk factor, as the majority of BCs with BRCA1 mutation are TN and resemble BLBC morphologically and immunohistochemically (Carey et al., 2010). It has been suggested that contralateral TNBC (primary tumor being ER-positive) may result from the use of the selective estrogen receptor modulator tamoxifen (Li *et al.*, 2009), and TNBC risk increases over four-fold in women under 40 years with ≥ 1 year oral contraceptive use (Dolle *et al.*, 2009). Also, Hispanic ethnicity (Liedtke *et al.*, 2008), lower socioeconomic status (Gluz et al., 2009) and metabolic syndrome (Maiti *et al.*, 2010) have been shown to be TNBC risk factors in the American population.

2.5 Breast cancer screening and therapy

2.5.1 Breast cancer screening with mammography

Mammography screening can find early stage tumors allowing curative therapy (Mook *et al.*, 2011, Jemal et al., 2011). Nevertheless, in 2000, Gøtzsche and Olsen declared that mammography is unjustified. They concluded that for every 1000 biennially screened women (for 12 years), one BC death is avoided, but total number of deaths increases by six (Gøtzsche & Olsen, 2000). The article restarted a vivid discussion about the issue known as mammography controversy. The discussion stems from overdiagnoses, false positives, costs, and the age of when to start screens. Collectively, it is about the trade-off between the benefits and harms of screening (Berry, 2013). Nevertheless, nine years later Gøtzsche and Nielsen concluded that screening reduces BC mortality. They estimated that for every 2000 screens for 10 years, a single patient's life is prolonged and 10 persons are unnecessarily treated. Also, over 200 women will psychologically suffer the consequences of over treatment due to false positive findings (Gøtzsche & Nielsen, 2009). According to Njor and coworkers, the European mammography screening programmes are a likely cause of the BC mortality reduction by 26% (Njor *et al.*, 2012). This is just an estimation, but population-based mammography screens have clearly resulted in a reduction of BC mortality within the EU, and the number of BC deaths avoided due to the screening exceeds the number of overdiagnoses (Paci & Group, 2012). Overall, the explanation for most of the mortality reduction is the joint effect of mammography and adjuvant therapy (Toriola & Colditz, 2013). In Finland, a national population-based mammographic screening program for women of ages 50–59 years was initiated in 1987. In Turku, Finland, screening was offered to all women of ages 40–74 years. Also in Turku, in a study of 527 invasive BCs, it was shown that screen-detection reduced local and distant recurrence both in patients aged 40-74 years and in a cohort of women aged 40-49. The screening interval

(annual versus triennial) had no significance (Parvinen *et al.*, 2011, Kauhava *et al.*, 2008, Immonen-Räihä *et al.*, 2005).

2.5.2 Neoadjuvant therapy

After the detection of a breast tumor, before the operation, neoadjuvant therapy can be applied to reduce the tumor size. Originally, neoadjuvant chemotherapy for BC was used only with a locally advanced and inflammatory disease[2] to make the tumor operable (Gampenrieder *et al.*, 2013, Mohamed *et al.*, 2014). Currently, it is also used to decrease the tumor stage, and consequently to increase the chance of breast conservation therapy. The other benefits of neoadjuvant therapy are faster evaluation of tumor sensitivity, testing for new treatment options, and the avoidance of acquired resistance (Gampenrieder et al., 2013). Neoadjuvant hormone therapy is at least as effective as neoadjuvant chemotherapy and is becoming a standard therapy in HR-positive BC patients with large inoperable tumors, and/or in older and weak patients unfit for chemotherapy (Saleh *et al.*, 2014). With neoadjuvant hormone therapy, no serious adverse effects have been reported. As previously mentioned, androgens are converted to estrogens by aromatase in the adipose tissue, but also in other tissues such as the brain and bones. In addition, a healthy breast expresses low levels of aromatase, but cancerous BC epithelial cells and surrounding fibroblasts produce a greater level of the enzyme (Stocco, 2012). In fact, aromatase inhibitors have been more effective than tamoxifen in neoadjuvant hormone therapy trials both in premenopausal and postmenopausal patients (Charehbili *et al.*, 2014, Saleh et al., 2014).

2.5.3 Adjuvant therapy

The usual procedure is that after neoadjuvant therapy, mastectomy (breast removal) or lumpectomy (breast-conserving surgery) is performed. Further treatment has to be considered as even though the visible tumor has been removed, the microscopic disease (circulating tumor cells (CTCs), disseminated tumor cells (DTCs) and micrometastases) has to be treated if the tumor characteristics warrant this. Treatment that aims at the clearance of residual microscopic disease is called adjuvant therapy. In BC, the choices for adjuvant therapy are chemotherapy and/or hormonal therapy and radiation therapy. The used chemotherapeutic agents are described in Table 1. (http://rintasyoparyhma.yhdistysavain.fi/ hoitosuositus; accessed June 7th, 2014).

[2] Inflammatory BC is rare and very aggressive BC subtype, which is typically $ER^-/HER2^+$, stage III or IV, and is more common among young women (Mohamed et al., 2014).

2.5.3.1 Pharmaceutical treatment and radiation therapy

Chemotherapeutic agents shown in Table 1, and combination treatments shown in Table 2 are currently in use for BC treatment. The use of multiple compounds in BC therapy aims at targeting the tumor with a variety of mechanisms. The treatment depends on the tumor type (HR and HER2 statuses) and tumor stage. The most used adjuvant chemotherapy treatment is CEF (Cyclophosphamide, Epirubicin and 5-fluorouracil (5FU)) with or without docetaxel (Table 1 and Table 2). In 2013, the Finnish Breast Cancer Group published the National Diagnostics and Treatment Recommendations for Breast Cancer (http://rintasyoparyhma.yhdistysavain.fi/hoitosuositus; accessed June 7th, 2014). Currently, there are several clinical trials studying optimal chemotherapeutic compositions alone or together with a specific target therapy, for example monoclonal antibodies targeted to oncogenic proteins critical to tumor progression (https://clinicaltrials.gov/; accessed October 12th, 2014).

Hormone therapy is the standard adjuvant treatment for HR-positive patients. In general, post-menopausal patients receive aromatase inhibitors, whereas pre-menopausal patients are treated with tamoxifen (Risbridger et al., 2010). In 1977, the U.S. Food and Drug Administration approved tamoxifen for metastatic BC treatment and since that, tamoxifen has been the most used therapy for the treatment of ER-positive BC worldwide (Higgins and Stearns, 2011). It has been shown that ER-positive patients with a high immunohistochemical androgen receptor expression have a better outcome and response to hormone therapy (Peters et al., 2009). In post-menopausal BC patients, breast tissue estradiol levels can be 10 times higher than serum estradiol levels, which stems from clearly increased aromatase levels in the tumor or peripheral tissues (Risbridger et al., 2010).

Table 1. Chemotherapeutic agent classes used in BC therapy.

Agent class	Agent examples	Way of function
Taxan	Docetaxel, Paclitaxel	Anti-mitotic, apoptosis-inducing
Anthracycline	Doxorubicin, Epirubicin	Inhibition of topoisomerase II, inhibition of DNA and RNA synthesis
Antimetabolite	Gemcitabine	Nucleoside analogue, apoptosis-inducing
Antimetabolite	Fluorouracil	Pyrimidine analogue
Antimetabolite	Methotrexate	Folic acid inhibitor
Antimetabolite	Capecitabine	Prodrug for 5-fluorouracil
Vinca-alcaloid	Vinorelbine	Anti-mitotic
Ketone analogue	Eribulin	Anti-mitotic, apoptosis-inducing
Alkylating agent	Cyclophosphamide	DNA damage
Platinum agent	Cisplatin, Carboplatin	DNA damage

Table 2. Examples of chemotherapy treatment strategies used in BC treatment. CEF is the first-line treatment for all Finnish BC patients (http://rintasyoparyhma.yhdistysavain.fi/hoitosuositus; accessed June 7th, 2014).

Treatment	Agents
CEF/FEC	Cyclophosphamide, Epirubicin and 5-fluorouracil (5FU)
CMF	Cyclophosphamide, Methotrexate and 5FU
VMF	Vinorelbine, Methotrexate and Fluorouracil
TC	Docetaxel (Taxotere) and Cyclophosphamide
AC	Doxorubicin (Adriamycin) and Cyclophosphamide
FinXX*	Capecitabine, Docetaxel, Cyclophosphamide and Epirubicin

*In the FinXX trial, patients with TNBC and patients with three or more positive axillary lymph nodes had longer RFS when capecitabine was added into the chemotherapy regimen. Also, HER2-positive patients in the capecitabine group, which were treated with trastuzumab had longer RFS than patients without capecitabine (Joensuu *et al.*, 2014, Joensuu *et al.*, 2012, Joensuu *et al.*, 2009).

Approximately 30% of BCs overexpress HER2, which correlates with an age older than 50 and an aggressive disease (Brufsky, 2014, Wilks, 2015). Trastuzumab (herceptin), a recombinant antibody targeting the extracellular region of HER2, was the first therapy approved by the U.S. Food and Drug Administration for the treatment of metastatic $HER2^+$ BC (Gluz et al., 2009). Unfortunately, almost 70% of $HER2^+$ BC patients have intrinsic trastuzumab resistance, and nearly all become unresponsive during the treatments (Brufsky, 2014, Valabrega *et al.*, 2005).

Most ER-positive BCs express PRs, which have served as a marker for functional ERα (Lanari *et al.*, 2012). Activation of PR has been shown to induce cancer cell growth and metastasis (Ogba *et al.*, 2014, Izzo *et al.*, 2014). Antiprogestin therapy targeting the PR-A receptor is not in routine use, but could be a valid treatment strategy together with tamoxifen for patients with high PR-A expression (Lanari et al., 2012).

For stage IV metastatic BC, the treatments vary. These second-line therapies are different from primary therapy since the primary therapy did not prevent the tumor spread (http://rintasyoparyhma.yhdistysavain.fi/hoitosuositus; accessed June 7th, 2014). Most clinical trials are carried out with this patient group making it possible for them to benefit from new therapies.

Radiation therapy aims at decreasing the risk for local recurrence. In an invasive disease, radiation therapy is recommended after the lumpectomy or mastectomy with sentinel lymph node metastases. Radiation therapy dosing is recommended to be designed by the support of a computer tomography 3D model of the tumor. Also, radiation therapy is used as a palliative treatment in cases of skeletal or soft tissue

metastases (http://rintasyoparyhma.yhdistysavain.fi/ hoitosuositus; accessed June 7th, 2014).

2.5.4 TNBC treatment

TNBC causes an unbalanced amount of BC deaths. The tumor grade, nodal involvement, tumor size and treatment do not affect the prognosis of TNBC. In comparison to non-BL TNBC, BL TNBC patients have a significantly decreased BC-specific OS due to the chemoresistant disease. BL TNBC patients represent over 50% of TNBC patients (Gluz et al., 2009).

There are no targeted therapies for TNBC, and the challenge is to discover new biomarkers, which would categorize the disease into subtypes that can be systemically targeted more efficiently. Currently, the only systemic adjuvant treatment for TNBC is chemotherapy, reducing the risk of death by 30%. The majority of TNBCs are oligo- or multiclonal at diagnosis, which presents a major challenge to precision medicine. Also, the vast majority of TNBCs remain TN upon recurrence (Turner & Reis-Filho, 2013, Badve et al., 2011). However, Wang and coworkers challenge the definition of a clone due to the fact that there were no two genetically identical cells present in their single-cell sequencing study (Wang et al., 2014).

In the neoadjuvant setting, the response of TNBC patients to neoadjuvant chemotherapy is better than that of the non-TNBC patients, and anthracycline + taxane therapy seems to be the best option for TNBC, as anthracycline-only patients had a pCR of 20%, whereas anthracycline+taxane group had a pCR of 28% (O'Toole et al., 2013). TNBCs with BRCA1 or BRCA2 mutations are highly sensitive to both platinum compounds as well as PARP inhibitors (Turner & Reis-Filho, 2013). In neoadjuvant settings, when either carboplatin or bevacizumab was added to the standard therapy of TNBC patients, pCR breast increased from 48% to 60% and 59%, respectively (Sikov *et al.*, 2015).

A TNBC meta-analysis showed that in comparison to CMF, anthracycline-based chemotherapy demonstrated a 23% increase in disease-free survival (DFS). However, a retrospective analysis with patients receiving either adjuvant CMF or FEC showed an increased DFS for CMF. Node-positive early BLBC had a 35% increase in DFS with FEC-D (FEC followed by docetaxel), compared with FEC. A similar retrospective analysis comparing taxotere, doxorubicin and cyclophosphamide (TAC) with cyclophosphamide, doxorubicin and fluorouracil (CAF) showed a 50% increase in DFS in the TAC arm (O'Toole *et al.*, 2013). TNBC and BLBC respond well to anthracyclines and taxanes, proliferate fast and have defects in DNA repair. Patients with TNBC, BLBC or HER2-enriched BC show a higher pathologic complete response (pCR), but also a worse OS

caused by a higher relapse rate, when compared to patients with luminal BC. Anthracyclines are not only directly cytotoxic, but they also activate the adaptive antitumor immune response when they induce immunogenic tumor cell death. Anthracyclines also induce the release of HMGB1 (high-mobility group box 1), a chromatin-binding protein, which facilitates tumor antigen cross-presentation to T cells (Carey et al., 2010, Loi *et al.*, 2013, Zitvogel *et al.*, 2008).

2.5.5 Male BC treatment

Male BC is rare. According to the Nordic Cancer Registry, there are approximately 19 new cases annually in Finland (http://www-dep.iarc.fr/nordcan/fi; accessed October 12th, 2014). Due to the rareness, the treatment of male BC is not based on clinical trials, but female therapy principles and indications are used. Male BCs resemble postmenopausal womens' BC (Zygogianni *et al.*, 2012), but unlike in female BC, where the expression of ERβ is reduced, ERβ protein is highly expressed in male BC (Murphy *et al.*, 2006). Most male BCs are ER-positive, and 10-15% of cases are HER2 positive (http://rintasyoparyhma.yhdistysavain.fi/hoitosuositus; accessed June 7th, 2014).

2.6 Breast cancer metastasis

> "If detected in the pre-cancerous stage or when cancer has only first made a definite beginning, removal of the affected organ will rarely be followed by recurrence... ...but if the disease be allowed to proceed unchecked till it assumes considerable proportions, how can it be hoped that some outlying portion of cancerous deposit of minute size will not be left behind even after an operation of great magnitude?" (Jennings, 1893).

In BC, like in most solid tumors, metastases cause most of the deaths. To successfully metastasize, the cell has to leave its site-of-origin, home to a distant tissue, resist apoptosis and antitumoral immunity, and finally colonize the new area. BC metastasizes to various organs. Locally first to the axillary lymph nodes, and distally most commonly to the bone, lung and liver. Almost 30% of patients with early-stage disease will have metastatic recurrence, even several years later (Redig & McAllister, 2013). BC patients with an aggressive disease develop metastases within 3 years from the diagnosis. Current clinical strategies cannot cure the metastatic disease or identify patients with a high metastasis risk (Weigelt et al., 2005, Redig & McAllister, 2013). The probability to get a metastasis is estimated to be a function of time and tumor size (Foulkes *et al.*, 2010a). The size of the tumor and the amount of positive lymph nodes correlate, as in most BC types, the presence of positive lymph nodes can be predicted from the tumor size. Importantly, nodal status is the strongest prognostic factor for BC progression and the overall outcome. In TNBC, nodal status correlates with survival, yet

nodal status and tumor size correlate variably (Foulkes et al., 2010a). Currently, there are three gene expression profile-based commercial molecular test kits for the estimation of BC recurrence risk: Oncotype DX (21 genes), MammaPrint (70 genes), and PAM50 (50 genes) (Kittaneh *et al.*, 2013).

In addition to invading microbes, the immune system should recognize and destroy abnormal cells. Both blood or lymph-resident CTCs and tissue-invaded DTCs have devised mechanisms to avoid immunologic destruction. When analyzing 7 European studies of DTCs, Braun and Naume show that the prevalence of DTCs in BC patients varies between 13% and 42% (Braun & Naume, 2005). Bone-infiltrating DTCs secrete a multitude of growth factors and proteolytic enzymes that impair bone structure and integrity. The composition of these molecules dictates whether the lesions eat the bone (osteolytic/osteoclastic disease) or induce the accumulation of sclerotic bone mass (osteosclerotic/osteoblastic disease). In both cases, the skeleton gets weaker (Hill *et al.*, 2006). BC lesions are for the most part osteolytic, but in 15-20% of patients, the lesions are predominantly osteosclerotic (Coleman & Seaman, 2001). As an example, prostate cancer (PCa) lesions are mostly osteosclerotic (Charhon *et al.*, 1983) and only multiple myeloma lesions are completely lytic (Oades *et al.*, 2002).

The bone marrow epithelium is a dynamic area with a high blood flow, making it a tempting site for metastases. The CTC migrates to the bone either straight from the primary tumor, or by first inhabiting a lymph node and subsequently the bone. In the bone marrow cavity, CTCs are generally recruited to the hard bone endosteal zone. The endosteal zone is the bone marrow area distal to the central vein. DTCs secrete molecules that help them attach to the stroma and bone matrix, and also express adhesion molecules, which support the attachment. Then, DTCs produce more pro-angiogenic and bone-resorbing molecules that improve the metastasis growth (Roodman, 2004, Hill et al., 2006). A vicious cycle exists between the osteolytic metastatic cells and the bone marrow stroma. Tumor cells secrete molecules which stimulate osteoclast (osteolytic metastasis) or osteoblast (osteosclerotic metastasis) activation, and the stroma promotes metastasis growth by producing growth factors, which stimulate tumor cells (Wong & Pavlakis, 2011, Onishi *et al.*, 2010, Guise *et al.*, 1996, Mundy, 2002).

Bone metastases are prevalent in up to 70% of BC patients with an advanced disease. Bone metastases induce pain, fractures, spinal cord compression and hypercalcemia (Coleman & Rubens, 1987, Mackiewicz-Wysocka *et al.*, 2012). In the USA, 350 000 patients bearing bone metastases originating from different cancers die annually (Roodman, 2004). Still, bone metastases are not usually the reason for mortality. In a study of 113 patients with BC as the primary cause of death, visceral metastases to the

liver, pleura, lung and brain were the most frequent causes of death (Ursaru *et al.*, 2015).

Metastatic inefficiency explains the time lag between the initial diagnosis and the diagnosis of metastases. The metastized cells have to acquire new genetic or epigenetic changes in order to be able to grow in the strange environment. A micrometastasis will eventually grow into a tumor by recruiting the necessary stroma and vasculature (Redig & McAllister, 2013, Urrutia *et al.*, 2015). Only under 1% of DTCs can grow into metastases. A meta analysis of 6800 patients revealed that in early stage BC, DTCs and CTCs are prognostic markers of DFS. Furthermore, in the case of metastatic BC, DTCs and CTCs are prognostic markers of progression-free survival (PFS). Often the metastatic lesion and the primary BC have a mismatch on the HR or HER2 statuses. Consequently, the histopathology of the metastasis should be the primary guide for the treatment of metastatic disease (Redig & McAllister, 2013). For example, HER2-positive CTCs have been detected in patients with a HER2-negative primary tumor suggesting that these patients could benefit from HER2-targeted therapies (Hoon *et al.*, 2011). Dormant cells are difficult to detect and target and can only be studied in accessible tissues, like the blood and bone marrow.

In the metastatic organ, cancer cells can exist in three distinct states: actively growing (angiogenic metastases-forming), micrometastases (no net size change in tumor volume as proliferation is balanced by apoptosis), or solitary, dormant cells. BC CTCs have been detected 22 years after diagnosis in clinically disease-free patients. It is currently poorly known how and why the cells switch from dormancy to growth (Goss & Chambers, 2010).

During the first 10 years after diagnosis, ER-positive patients have lower relapse rates than ER-negative patients, but after 10 years ER-positive patients have greater relapse rates. The recurrence risk of TNBC patients peaks during the first 3 years, and 50% of the patients die within 5 years (median time to death 4.2 years) after the initial treatment (Dent *et al.*, 2007, Gluz et al., 2009, Goss & Chambers, 2010, Foulkes et al., 2010b). Most TNBC patients who have only a partial response following neoadjuvant chemotherapy have a high relapse risk with the peak of metastases occurring at 1 year. Residual tumors are regarded as primarily treatment-resistant and, therefore, response to further systemic therapy and long-term favorable outcomes are rare (Bayraktar & Glück, 2013).

The reason for the use of multiple chemotherapeutic agents, especially in the metastatic setting, is that there is almost a universal resistance to a single agent (Szakács *et al.*, 2006). The major reason for an unsuccessful treatment of a metastatic

disease is either a primary or an acquired multidrug resistance to standard therapies (Bayraktar & Glück, 2013). Multidrug resistance can arise via the decreased transporter-dependent drug uptake, changes in tumor cell metabolism, or by increased drug efflux (Szakács et al., 2006).

In lymph node metastasis-negative patients, vessel invasion is a predictor for relapse. One third of lymph node-negative BC patients will have metastases, and one third of patients with lymph node-positive BC are metastasis-free 10 years after local therapy (Weigelt et al., 2005). ER^+ tumors most often form bone metastases. ILCs are more likely ER^+ than IDCs, and metastasize with increased bias in the gastrointestinal tract and ovaries (Arpino *et al.*, 2004). Already when the tumor has a diameter of 2 mm, it has blood supply and therefore, it is probable that CTCs have left the primary tumor before diagnosis (Weigelt et al., 2005). The first distant recurrence site differs between TNBC and non-TNBC patients (Figure 2). TNBC patients have an increased visceral versus bone metastasis rate, an increased rate of lung and brain metastases as the first site of recurrence, and an increased risk of central nervous system metastases (Gluz et al., 2009, Pal et al., 2011, Foulkes et al., 2010b).

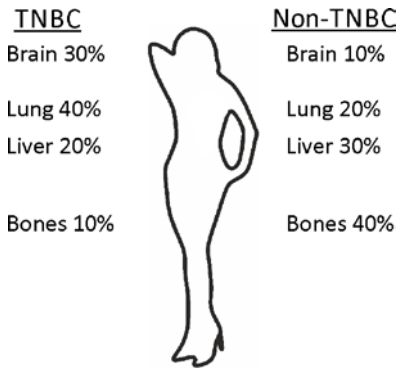

Figure 2. The first distant recurrence sites of metastatic TNBC versus non-TNBC patients. Adapted from Foulkes et al., 2010b.

2.7 Innate immunity

Pathogen-induced evolutionary pressure has modified the vertebrate immune system to its current shape, consisting of innate and adaptive/acquired immunity. Of those, innate immunity has existed prior to adaptive immunity (Medzhitov & Janeway, 1997). Innate immunity is the first line of defence, which discriminates non-self molecules from self molecules (Akira *et al.*, 2001) by recognizing invariant, evolutionally conserved microbial features. Pattern-recognition receptors (PRRs) initiate the innate immune response by recognizing conserved pathogen-associated molecular patterns

(PAMPs) (Medzhitov & Janeway, 1997). Sometimes, PRRs recognize also endogenous molecules (danger/damage-associated molecular patterns; DAMPs) (Lee *et al.*, 2012). Phagocyting cells (for example macrophages, neutrophils and DCs) monitor the extracellular environment and act as the major players in PAMP/DAMP detection. Pathogen recognition initiates production of several cytokines and the upregulation of co-stimulatory molecules in phagocytes. The antigen-presenting phagocyte (antigen presenting cell; APC) presents the processed PAMP/DAMP to a naïve T cell (cross-presentation), which induces antigen receptor production and T cell proliferation leading to protective adaptive immunity (Figure 3) (Medzhitov & Janeway, 1997). Cytotoxic $CD8^+$ T cell activation, which among others is responsible for tumor cell killing, requires antigen cross-presentation by APCs (Akira & Hemmi, 2003, Iwasaki & Medzhitov, 2010, Aderem & Ulevitch, 2000).

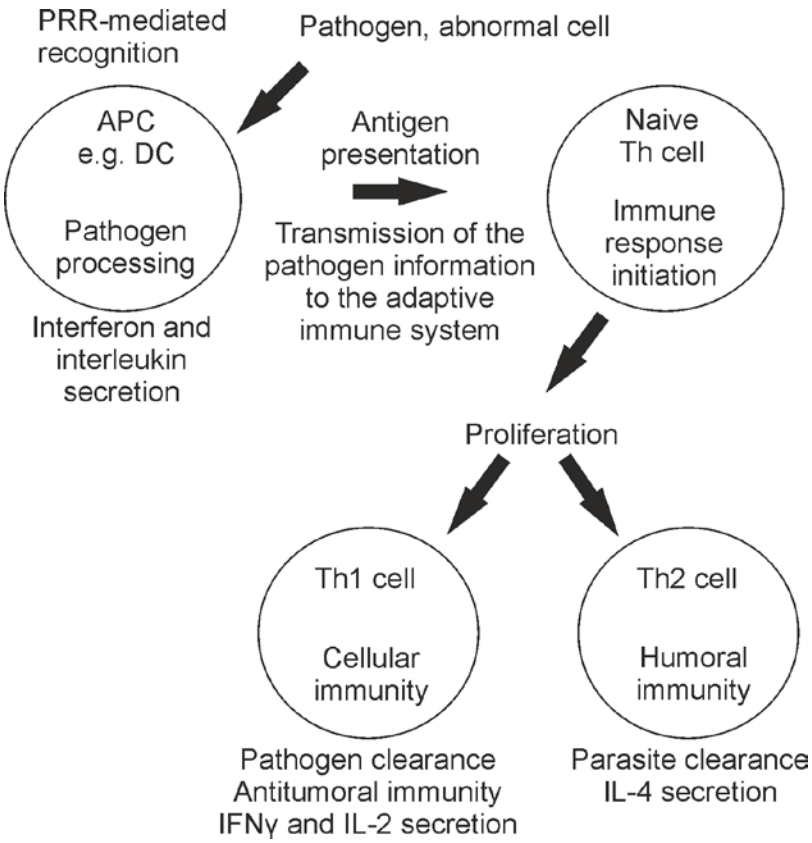

Figure 3. The concept of immune system activation. Innate immune cell processes the antigen and presents it to the adaptive immunity cell. For clarity, only the helper T cell side and two T helper subtypes are shown. APC, antigen presenting cell; DC, dendritic cell; PRR, pattern recognition receptor; Th, helper T cell.

2.7.1 Pattern recognition receptors

In humans, there are four PRR families: NOD-like receptors (NLRs), Toll-like receptors (TLRs), RIG-I-like receptors and C-type lectin receptors (CLRs) (Goutagny *et al.*, 2012, Oh & Lee, 2014). Viruses induce RIG-I-mediated type I IFN production. NLRs mainly have antibacterial functions via the activation of NF-κB or inflammasomes, protein complexes which activate inflammatory processes (Lee & Kim, 2007). The majority of TLRs and NLRs sense bacteria, whereas Rig-I-like receptors are primarily virus-sensing. C-type lectins and TLRs 2, 4, 8 and 9 recognize mycobacteria, and for example TLR9 and TLR2 cooperate to regulate the Th1 response against mycobacteria (Kumar *et al.*, 2013). Inflammatory responses are optimized by several TLR-TLR or TLR-NOD cross-talk combinations. For example, TLRs and NALP3 interact in IL-1β production showing that PRR crosstalk effectively directs immune responses (Lee & Kim, 2007).

NLR-activated inflammasomes regulate innate immune responses to various PAMPs. Specifically, inflammasomes stimulate innate immune cells by activating pro-caspase-1, which in turn induces the maturation of pro-IL-1β and pro-IL-18. The tumor cell AIM2 inflammasome is essential for caspase-1-dependent inflammation-induced programmed cell death, pyroptosis (Jinushi, 2012, Oh & Lee, 2014). DAI, AIM2 and NALP inflammasomes recognize DNA, and at least DAI detects both self and non-self DNA (Muruve *et al.*, 2008). The cytoplasmic DNA-sensing AIM2 functions as a NLR-independent inflammasome regulator (Jinushi, 2012). The activation of TLR3, 4 or 9 signalling in murine macrophages increases *Aim2* expression, showing the cooperation between these two PRR classes (Choubey, 2012). Complement is a part of the innate immune system, and consists of small proteins, which attack the pathogen cell membrane. TLR-complement crosstalk shows that independent systems triggered by different PAMPs can converge to boost the activation of innate immunity (Brencicova & Diebold, 2013). Microbe detection is highly redundant. Normally microbes present multiple PRR ligands, therefore, if a microbe avoids recognition by one PRR, it will most likely be recognized by another one (Blasius & Beutler, 2010).

2.7.2 Toll-like receptors (TLRs)

The *Toll* gene was characterized in 1985 by Christiane Nüsslein-Volhard, who shared a Nobel prize in Physiology or Medicine in 1995 for its discovery. The finding was that the *Toll* gene is crucial in the dorsoventral development of the *Drosophila* embryo (Anderson *et al.*, 1985b, Anderson *et al.*, 1985a, Vacchelli *et al.*, 2012). Toll is a type I transmembrane receptor, which in *Drosophila*, controls the transcription of drosomycin, an antifungal peptide essential for the survival of flies confronting fungal infections (Lemaitre *et al.*, 1996). Medzhitov and coworkers characterized the human homologue of *Drosophila* Toll showing that it signals through NF-κB and activates both

innate and adaptive immune responses (Medzhitov *et al.*, 1997). For finding the link between TLRs and the immune response, Jules Hoffman and Bruce Beutler were awarded the Nobel prize in Physiology or Medicine in 2011.

Toll receptors are evolutionarily ancient, and homologues exist in insects, plants and mammals. The cytoplasmic domain is homologous with the interleukin (IL)-1R cytoplasmic domain, and was consequently named as Toll/IL-1R (TIR) domain. The extracellular domain/ectodomain (ECD) either faces the exterior of the cell (cell membrane TLRs) or the lumen of an intracellular compartment (endosomal TLRs) where they encounter PAMPs. Nucleic acid-sensing TLRs have an endosomal location, whereas all other TLRs reside in the cell membrane. Due to their orientation, TLRs do not have cytoplasmic ligands. The ECD contains leucine-rich repeat (LRRs) motifs (Akira & Hemmi, 2003), of which human TLRs have 20-27 very variable LRRs (Matsushima *et al.*, 2007). The large size of the ECD probably explains the broad PAMP specificity of some TLRs (Bell *et al.*, 2003). TLR ligands are presented in Table 3.

TLRs have cell type, ligand and adaptor protein specificity, and they also differ in their way of inducing immune responses (Chaturvedi & Pierce, 2009). TLRs are crucial in phagocytosis (Blander & Medzhitov, 2004) and recruiting leukocytes to sites of infection (Ioannou & Voulgarelis, 2010). TLRs regulate tissue repair and regeneration to support tissue homeostasis, and inhibit apoptosis (Ioannou & Voulgarelis, 2010, Basith *et al.*, 2012, Blander & Medzhitov, 2004).

Table 3. Human TLR ligands. Modified from Goutagny et al., 2012.

Receptor	Ligand	Origin
TLR1/2	Lipoarabinomannan, peptidoglycan, lipotechoid acid, triacyl lipopeptide, zymosan	Bacteria
TLR2/6	Lipotechoid acid, diacyl lipopeptide	Bacteria
TLR3	dsRNA	Viruses
TLR4	LPS, viral proteins	Bacteria, viruses
TLR5	Flagellin	Bacteria
TLR7	ssRNA	Bacteria, viruses
TLR8	ssRNA	Bacteria, viruses
TLR9	dsDNA/ssDNA	Bacteria, viruses
TLR10	Unknown	Unknown

They also play a role in antitumoral immunity by recognizing tumor therapy-induced DAMPs and upregulating proinflammatory cytokines, such as type I IFNs (IFNα/β), which have antitumor effects. DAMPs may sensitize APCs to tumor antigen cross-presentation, and consequently T cell response initiation (Jinushi, 2012).

Humans and mice have 10 and 13 TLRs, respectively. TLRs 1-9 are conserved in mice and men. Based on their amino acid sequences and genomic structures, TLRs 1, 2, 6 and 10 constitute the TLR2 subfamily (Kawai & Akira, 2010). Based on phylogenetic analysis, TLRs 7-9 constitute the TLR9 subfamily (Du *et al.*, 2000). Cell surface TLRs (TLRs1, 2, 4, 5 and 6) are monomers, and form homodimers or heterodimers when exposed to PAMPs/DAMPs (Gay *et al.*, 2014). TLRs 1 and 6 form heterodimers with TLR2. (Kawai & Akira, 2010). TLR4 and TLR5 form homodimers, but TLR4 is also active as a heterodimer with TLR6 (Gay et al., 2014, Latz *et al.*, 2007). TLRs 3, 7, 8 and 9 are endosomal and the inactive TLR3 is a monomer. The TLR9 family members are synthetized as stable preformed dimers (Latz et al., 2007, Reuven *et al.*, 2014, Gay et al., 2014). The mouse TLR10 gene contains a retroviral insertion and is non-functional, and TLRs 11-13 have disappeared from the human genome. Mouse TLR13 is an endosomal receptor for bacterial DNA (Hidmark *et al.*, 2012). The human TLR10 is an orphan receptor with the capability to downregulate inflammatory reactions (Oosting *et al.*, 2014). For a more detailed view of the history of the TLRs, O'Neill and coworkers have written a review concerning it (O'Neill *et al.*, 2013).

2.7.2.1 TLR signalling

In the canonical pathway (Figure 4), the conformation and/or association of the TLR-ECD changes upon ligand binding, letting the cytoplasmic TLR TIR domain and MyD88 TIR domains to bind and initiate signalling.

Five TIR domain-containing adaptor molecules (MyD88, TRIF/TICAM1, TRAM, MAL and SARM) participate in the initiation of TLR signalling (Goutagny et al., 2012). All TLRs, with the exception of TLR3, which signals via TRIF, require MyD88 for their full signalling activity. TLR7 and TLR9 signalling is thought to be completely dependent on MyD88 (Blasius & Beutler, 2010), but it has been shown that in breast cancer cells, the invasion-inducing TLR9 signalling uses a MyD88-independent pathway (Ilvesaro *et al.*, 2008).

After MyD88 recruitment, MyD88 and IRAK4 death domains interact leading to IRAK4 further activating IRAK1 and IRAK2, which activate TRAF6, eventually leading to MAPK cascade activation and JNK, p38 and CREB phosphorylation, and NF-κB nuclear translocation. NF-κB induces the production of cytokines. Also, transcription factors IRF3 and IRF7 may get activated, and induce interferon production (Blasius & Beutler, 2010). When DAMPs activate TLRs, it results in a sterile inflammation, which happens

usually in a necrotic context (Chen & Nuñez, 2010). Sterile inflammation is associated with tumorigenesis as TLR expression in tumor cells/cell lines is often protumoral. TLR ligand-induced chronic inflammation creates a microenvironment, which enhances tumor cell survival and migration (Basith et al., 2012).

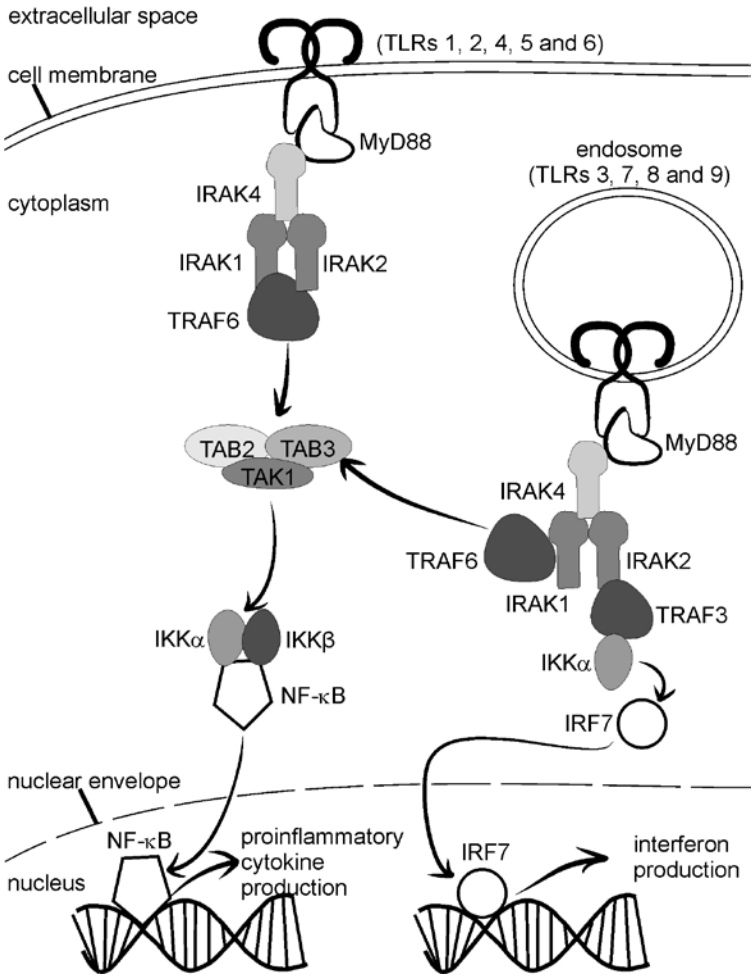

Figure 4. The canonical TLR signalling pathways. After ligand recognition, the adaptor protein MyD88 binds to the TLR TIR domain and initiates a signalling cascade, which results in the production of cytokines and interferons. Adapted from Blasius and Beutler, 2010 and O'Neill et al., 2013.

Also, TLR-activated tumor cells may release cytokines and chemokines, which induce cytokine and chemokine production in immune cells. This process can result in tumor growth and tumor immune tolerance (Basith et al., 2012, Pikarsky *et al.*, 2004, Sheyhidin *et al.*, 2011).

2.8 Toll-like receptor 9 (TLR9)

2.8.1 The origin of TLR9

Human *Tlr9* was first cloned in 2000, and was found to reside in the chromosomal location 3p21.3 in linkage disequilibrium with the *MyD88* gene (Du et al., 2000). A phylogenetic analysis of an amino acid sequence comparison showed that human TLRs 7, 8 and 9 constitute the TLR9 subfamily, which have a higher molecular weight than other TLRs. TLR9 was shown to be predominantly expressed in tissues, which contain high amounts of immune cells. These include the spleen, lymph nodes and bone marrow (Chuang & Ulevitch, 2000, Du et al., 2000). In the immune system, DCs are the most potent APCs. Human blood contains plasmacytoid DCs (pDCs) and two subsets of myeloid DCs (mDCs) (Hémont *et al.*, 2013). pDCs have a distinct TLR gene expression pattern with high expression of TLR1, TLR7 and TLR9, and minute amounts of other TLRs (Hémont et al., 2013, Krug *et al.*, 2001), whereas mDCs do not express TLR9 (Frenzel *et al.*, 2006, Hémont et al., 2013). TLR9 has been shown to locate in the ER and in endosomes (Sasai *et al.*, 2010). Nevertheless, splenic DCs have been shown to have cell surface TLR9 expression (Onji *et al.*, 2013).

In addition to classic TLR9, cDNAs for a non-splicing form and two alternatively spliced forms of human TLR9 were first isolated from human embryonic kidney 293 cells (Chuang & Ulevitch, 2000). Later, the human TLR9 gene was shown to have five N-terminal splice variants (isoforms), which have a differential expression in immune cell-rich tissues. The benefit of multiple isoforms is suggested to be a wider complexity of ligand recognition (McKelvey *et al.*, 2011).

2.8.2 TLR9 ligands

Before any knowledge of TLR9, it was known that bacterial DNA and synthetic oligodeoxynucleotides (ODNs) can trigger cytokine (IL-6, IL-12) and IFNγ production. A non-methylated 6 bp motif containing a CpG (cytosine-phosphate-guanine) dinucleotide was found to be responsible for the activation (Klinman *et al.*, 1996). Four years later, it was shown that *MyD88*$^{-/-}$ mice are non-responsive to CpG-DNA and that *Tlr9*$^{-/-}$ mice are resistant to the lethal effect of CpG-DNA. Subsequently, TLR9 was identified as the receptor that recognizes CpG-DNA (Hemmi *et al.*, 2000).

In vertebrates, DNA is usually methylated having less CpG sequences (Akira & Hemmi, 2003), except for mitochondria, which evolved from bacteria (Zhang *et al.*, 2010) and mammalian promoter cDNA, which contains hypomethylated CpG motifs (Leadbetter *et al.*, 2002). When released due to injury, mitochondrial DNA activates the adaptive immunity via TLR9 (Zhang et al., 2010). Both self-DNA and bacterial genomic DNA require endolysosomal DNase II-mediated degradation for TLR9 activation (Chan *et al.*,

2015, Pawaria *et al.*, 2015). Interestingly, structural studies on TLR9 ligand binding have suggested that single-stranded DNA is a more potent ligand, as double-stranded DNA has a much lower affinity to TLR9 (Ohto *et al.*, 2015).

CpG-ODNs are potent TLR9 agonists (Sommariva et al., 2011). A-type ODNs have one CpG motif and a 30 base poly-G tail on a mixed phosphorothioate-phosphodiester backbone, which induces CpG-A aggregation leading to a longer presence in the endosome. CpG-Bs have one or more CpG motifs on a synthetic phosphorothioate backbone (Blasius & Beutler, 2010). CpG-B maintains a linear, single-stranded form and induces a Th1 response by sensitizing B cells to antigens, thus inducing the birth of plasma cells. CpG-A forms higher-order structures and mainly induces IFNα secretion in pDC cells, NK cell activation, but also Treg generation showing that CpG-A has both immunogenic and immuno suppressive effects (Jinushi, 2012, Tian *et al.*, 2007). In pDC endosomal vesicles, TLR9-bound CpG-A (but not CpG-B) extends the duration of activation to induce IFNs (Honda *et al.*, 2005). CpG-C-ODNs share the properties of both A- and B-type of CpG-ODNs, contain a CpG within the stimulatory sequence at the 5' end and a palindromic sequence (Yang *et al.*, 2013).

Both CpG-ODN and non-CpG-ODN activate TLR9 in the pDC endosome. This shows why self-DNA, which has low levels of unmethylated CpG motifs, can induce TLR9 signalling in an autoimmune setting (Haas *et al.*, 2008). CpG-ODN classes have differences in their biological outcome depending on the target cell type and intracellular endosomal environment. Clinical trials on CpG-ODNs have mainly focused on B-type (B cell-targeting) CpGs as they are good vaccine adjuvants due to their activation of humoral immunity (Lahoud *et al.*, 2012). *In vivo*, *Tlr9*$^{-/-}$ mice weakly induce IFN after a CpG-ODN challenge indicating that there is redundancy in CpG-ODN recognition (Honda et al., 2005, Zhao *et al.*, 2004).

Foreign or self-generated DNA enters the cell by clathrin-mediated endocytosis (Tian et al., 2007) via DEC-205, which is the DNA receptor on the cell surface. Both pDCs and B cells have surface expression of DEC-205, but it is more critical for B cell signalling (Lahoud et al., 2012). This shows that the CpG-DNA endocytosis is not totally dependent on DEC-205. TLR9 and CpG-DNA interact in acidic (pH 5.0-6.5) endosomal, endolysosomal and lysosomal compartments, where CpG-DNA initiates signalling (Freed-Pastor *et al.*, 2012, Sasai et al., 2010). The binding affinity of CpG to TLR9 decreases when the pH increases (Kd is 20 nM in pH 6.0 and 2500 nM in pH 8) (Ohto et al., 2015), indicating that acidic compartments are optimal for TLR9 ligand recognition.

Stimulatory, control and inhibitory DNAs have similar affinities to TLR9, but only stimulatory DNA can induce changes in the TLR9 homodimer ECD conformation

showing that receptor activity does not always follow from binding to the receptor (Latz et al., 2007).

Several viruses, for example EBV, cytomegalovirus and hepatitis B virus create RNA-DNA hybrids, which bind TLR9 and induce downstream cytokine production via TLR9. Interestingly, the affinity of the RNA-DNA hybrid to TLR9 was higher than affinities of ssDNA or dsDNA (Rigby *et al.*, 2014).

2.8.3 Regulation of TLR9 expression

In pDCs, macrophages and B cells, CpG-DNA downregulates TLR9 mRNA expression (Janeway & Medzhitov, 2002, Hornung *et al.*, 2002). Further, it was shown that in B cells, transcription factors C/EBPα, CREB1, Elf1, Ets2 and Elk1 induce high TLR9 transcription. Also, CpG-DNA was shown to suppress the DNA binding activity of these transcription factors. Furthermore, transcription factors c-Jun and NF-κB p65 were shown to suppress TLR9 expression by inhibiting the binding of other transcription factors, showing that NF-κB, which mediates TLR9 activation-induced cytokine/chemokine production, provides a negative feedback loop in TLR9 regulation (Takeshita *et al.*, 2004).

In humans, many innate immunity pathway members have circadian expression patterns, typically peaking at night. The severity of many pathological inflammatory states, such as sepsis and rheumatoid arthritis, obey the circadian rhythm (Silver *et al.*, 2012). CpG-ODN-treated, circadian-deficient macrophages from mice with a defective circadian molecular clock, had significantly less TNFα and IL-12 production than normal macrophages. Splenic, macrophage and B cell TLR9 mRNA expression peaked at different times as TLR9 transcription is regulated by the core circadian transcription factors BMAL1, CLOCK and REV-ERBα. At the splenic peak time, CpG-ODN-treated mice had higher splenic TNFα expression at 2 h after challenge than mice that were challenged 12 hours earlier. The daily variation in TLR9 responses has long-term effects in adaptive immune responses, and it may be critical to the timely administration of adjuvant TLR9 (and also other TLR) agonists to maximize therapeutic efficacy (Silver et al., 2012, Curtis *et al.*, 2014). The effect of radiation and chemotherapy also depends on the timing of administration and, notably, altered expression of circadian-controlled genes are frequently observed in human cancers (Greene, 2012).

Several viruses like EBV, HPV and Merkel cell polyomavirus inhibit TLR9 expression, which is more specifically described in Chapter 2.10.1 (Shahzad *et al.*, 2013, Fathallah *et al.*, 2010, Hasan *et al.*, 2007).

2.8.4 TLR9 processing and cofactors

Protein synthesis of transmembrane proteins begins in the rough endoplasmic reticulum (ER). The polypeptide is first translated and then transported through the ER membrane. The ready-for-export protein then travels via the Golgi apparatus, where it will be modified (Chockalingam *et al.*, 2009). Earlier, it was thought that TLR9 does not pass the Golgi (Latz *et al.*, 2004, Leifer *et al.*, 2004). However, it has been shown that TLR9 contains Golgi-dependent glycan modifications and it has been in contact with a Golgi protease Furin, which cleaves RXRR sequences (R=arginine, X=amino acid) (Chockalingam et al., 2009). Activated TLR9 is found in endosomes, wheras the bulk of TLR9 resides in ER membranes (Latz et al., 2007). There is a constant supply of TLR9 to the endosomes/endolysosomes from the ER (Sasai et al., 2010, Chockalingam et al., 2009). Especially, the pool of TLR9 which signals from endosomes probably first travels through the Golgi apparatus. The presence of CpG-DNA further triggers more TLR9 export through the Golgi apparatus, which is essential for optimized TLR9 signalling (Chockalingam et al., 2009). The life cycle of TLR9 is described in Figure 5.

The carrier protein UNC93B1 is required for endosomal TLR signalling in both mice and humans (Blasius & Beutler, 2010). UNC93B1 mediates the packing of the endosomal TLRs into ER-budding COPII vesicles, which tansport cargo from the ER to the Golgi apparatus (Lee *et al.*, 2013). UNC93B1 regulates and is required for the ER exit of the TLR9, and it escorts TLR9 to the endosome both before and after TLR9 stimulation (Kim *et al.*, 2008b, Brinkmann *et al.*, 2007, Ewald *et al.*, 2008, Blasius & Beutler, 2010). TLR9 and TLR7 compete in UNC93B1 binding, yet UNC93B1 activity is biased toward TLR9 over TLR7 in nucleic acid detection, hypothetically to prevent pathogenic RNA-induced autoimmune effects (Fukui *et al.*, 2009, Lee et al., 2013). Mouse TLR9 transmembrane α-helix and human TLR9 cytoplasmic domain contain the endosomal localization signals for TLR9 (Chaturvedi & Pierce, 2009).

PRAT4A (Protein associated with Toll-like receptor 4) is an ER-localized protein, which interacts with several TLRs. In PRAT4A-negative B cells, TLR9 does not progress to the endolysosome from the ER (Liu *et al.*, 2010). The chaperone GRB94 (Glucose-regulated protein of 94 kDa/gp96/endoplasmin/HSP90b1) mediates protein folding, and is necessary for the function of TLRs 1, 2, 4, 5, 7 and 9 (Lee et al., 2012). Neither GRP94 or PRAT4A alone can fold TLRs to their full maturation status (Liu et al., 2010). The interaction between GRP94 and TLR9 is impaired when PRAT4A is absent, and the the interaction between PRAT4A and TLR9 is impaired when GRP94 is absent. *In vivo*, *Prat4A*$^{-/-}$ macrophages, bone marrow-derived DCs, and B cells produce less cytokines compared to PRAT4A-positive cells upon TLR9 activation (Lee et al., 2012).

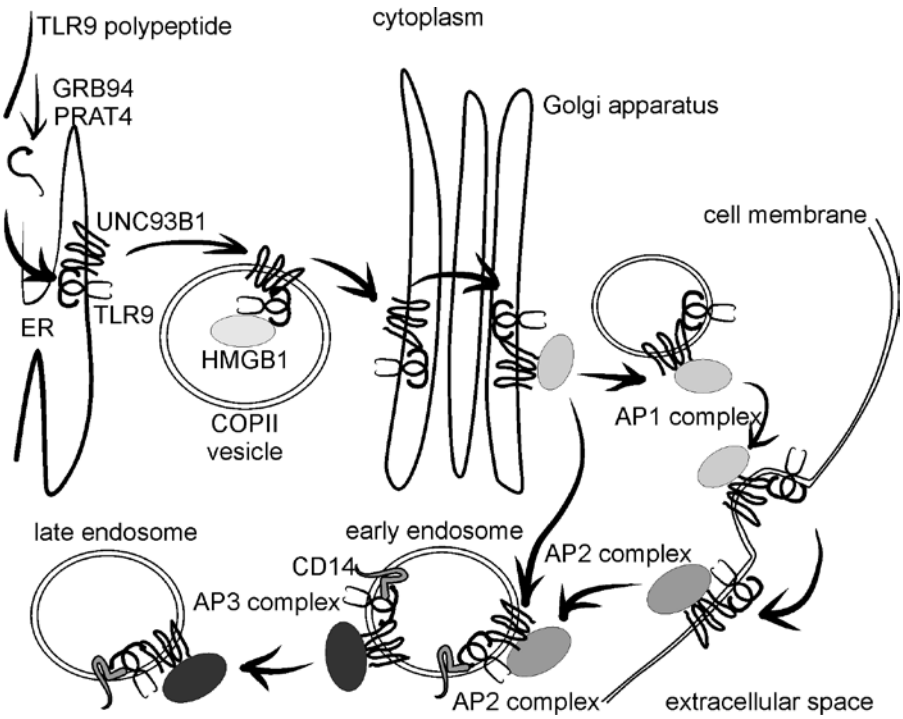

Figure 5. The life cycle of TLR9. GRB94 and PRAT4 support TLR9 polypeptide folding at the ER. UNC93B1 escorts TLR9 to the Golgi apparatus. Then, the TLR9-UNC93B1 complex either travels first to the plasma membrane (Gay et al., 2014), or straight to the endosome (Sasai et al., 2010).

Adaptor proteins (APs) are membrane proteins which select cargo to vesicles. TLR9 requires AP2 (an UNC93B1-mediated recruitment) to proceed to the endolysosome. The HRS/ESCRT (hepatocyte growth factor-regulated tyrosine kinase substrate/endosomal sorting complex required for transport) pathway participates in the post-Golgi delivery of TLR9 to the endosome, and AP2 complexes direct clathrin-mediated endocytosis from the cell surface. On the one hand, it has been proposed that the UNC93B1-TLR9 complex goes first to the cell surface and there AP2 internalizes the complex to the endosome (Lee et al., 2013). Also, there is evidence that TLR9 travels from the Golgi apparatus to the endosome without visiting the cell surface (Sasai et al., 2010).

HMGB1 is the archaeotypical DAMP. It functions in chromatin structure organization and transcription regulation, promotes protein-DNA interaction, and assists in V(D)J recombination, a process, which produces variability in the structure of T cell receptors and immunoglobulins. HMGB1 interacts with TLR9 and accelerates its CpG-ODN-induced movement from the ER to the endosome (Ivanov *et al.*, 2007).

CD14 is a coreceptor of TLR9, but it also binds TLR3 and TLR8 ligands poly(I:C) and imiquimod, respectively. CD14-depleted macrophages take up less CpG-DNA compared to wild type cells (Lee et al., 2012). The anti-inflammatory molecule lactoferrin inhibits TLR9 activity upon EBV infection by binding CD14 and preventing its interaction with TLR9 (Zheng *et al.*, 2014). The secreted cofactor Granulin upregulates innate immunity responses (Park *et al.*, 2011). In both pDCs and macrophages, Granulin depletion impairs the response of TLR9 to CpG-DNA, and addition of Granulin boosts the CpG-DNA-induced TNFα production. Granulin participates in CpG-DNA delivery to TLR9-containing vesicles, interacts with TLR9 and has the capacity to bind CpG-A, -B, -C, and inhibitory ODNs (Park et al., 2011).

DCs are specialized in macropinocytosis (fluid-phase endocytosis), which makes it possible to take up multiple PAMPs/DAMPs in a bolus of liquid. PAMP-containing endocytosed vesicles fuse with early endosomes, which are mildly acidic vesicles where the endocytosed cargo is sorted. The more acidic late endosomes (multivesicular bodies) are born by vesicle fusion, and harbor the further sorting of the cargo. Finally, the cargo-containing late endosome fuses with a lysosome, which degrades the remains of the material (Blasius & Beutler, 2010).

A mature endosome/endolysosome is required for TLR9 cleavage (Sasai et al., 2010), which has two steps. First, a part of the LRR-rich ECD is excised either by cysteine protease asparagine endopeptidase, or by cathepsins. Maximal signalling requires the subsequent second step, cathepsin-mediated cleavage (Ewald *et al.*, 2011). Asparagine endopeptidase-deficient pDCs secrete less cytokines upon TLR9 stimulation both *in vitro* and *in vivo* (Sepulveda *et al.*, 2009). Cathepsin inhibitors significantly suppress TLR9 ligand-induced NF-κB activation, and cathepsins are required for TLR9-assisted B cell proliferation (Matsumoto *et al.*, 2008). After the cleavage, TLR9 is fully functional (Latz et al., 2007). It has been proposed that the N-terminal cleavage product actively binds the TLR9 protein and participates in ligand recognition (Onji et al., 2013), but it is also suggested that the binding negatively regulates TLR9 (Lee *et al.*, 2014a).

In epithelial surfaces but also elsewhere, antimicrobial peptides (AMPs) fight against infectious agents. There are several classes of AMPs, including defensins and cathelicidins. The AMP constitution varies according to the tissue type, but mostly there is a synergy between AMPs in pathogen defense (Lai & Gallo, 2009). Human neutrophils express only one cathelicidin class AMP, hCAP18. LL-37, the C-terminal part of hCAP18, attacks bacteria and acts in synergy with the defensin class of AMPs. LL-37 is chemotactic for monocytes, neutrophils and T cells (Sørensen et al., 2001). In addition to neutrophils, also NK cells and mast cells produce LL-37, and skin, lung, gut and mammary gland epithelia secrete it (Lai & Gallo, 2009). LL-37 greatly potentiates CpG-

DNA-induced B cell and pDC stimulation. Without it, 20 times more CpG-B (B cells) or 30 times more CpG-A (pDCs) is needed to reach the same stimulation level as with LL-37, which can encounter and complex with bacterial DNA for example at neutrophil extracellular traps (NETs), which are induced by bacterial infection when neutrophils die and burst a web-like structure capturing microbes (Hurtado and Peh, 2010).

2.8.5 TLR9 signalling downstream of activation

Simply, TLR9 activation has two endpoints: the activation of NF-κB or IRF7 (Figure 6), which both result in an innate immune response. The canonical proinflammatory cytokine-inducing NF-κB pathway is described in chapter 2.8.2.1. NF-κB signalling is initiated earlier than IRF7 signalling in the endosomal life cycle (Sasai et al., 2010). CpG-DNA requires TLR9 signalling for IRF7 mRNA upregulation (Hemmi *et al.*, 2003). The type I IFN-inducing IRF7 activation requires APs. In the endosomal membranes, the AP3 complex recruits its cargo and delivers it to the late endosome. The AP3 complex is essential for TLR9-mediated IFN activation, and TLR9 cannot enter the late endosome in *Ap3*$^{-/-}$ cells. After stimulation, TLR9 is found in the early endosome. There TLR9 and the AP3 complex interact, and AP3 facilitates TLR9 trafficking to the late endosomal/lysosomal compartment. Only there TLR9 initiates IRF7 signalling. Also, UNC93B1 needs AP3 to access the late endosome (Sasai et al., 2010).

Quickly after CpG-ODN stimulation, pDCs and macrophages secrete HMGB1, which also interacts with CpG-ODN and enhances the immune response via TLR9 activation. The CpG-ODN-induced immune response is suboptimal without HMGB1 (Ivanov et al., 2007). RAGE is a multiligand receptor, which binds HMGB1 and the HMGB1-CpG-A complex. MyD88 and RAGE are essential for the HMGB1-mediated IFNα induction. After HMGB1-CpG-A stimulation, RAGE, MyD88 and TLR9 interact physically (Tian et al., 2007).

In pDCs, MyD88 and IRAK4 interact with TRAF6, TRAF3, IRAK1, IKKα and IRF7. IRAK1 and IKKα phosphorylate and activate IRF7. Also, TRAF6-induced ubiquination is required for IRF7 activation and type I IFN production (Blasius & Beutler, 2010, Kawai *et al.*, 2004). Normal breast tissue and primary BC very often express IRF7. Interestingly, its expression is downregulated in bone metastases both *in vivo* and in human BC. IRF7 has no effect on primary tumor growth *in vivo*, but it suppresses metastasis formation. In a cohort of 855 patients, the IRF7 signature (208 genes predicted to be regulated by IRF7) associated significantly with bone metastasis-free survival, and a low-IRF7 signature associated with a higher amount of bone metastases (Bidwell et al., 2012).

TLR9 also has a role in B cell activation. B cells function as APCs and antibody-producing cells, and have a specific receptor, the B cell receptor (BCR) on their plasma membrane. Upon antigen binding, the BCR initiates signalling from the cell surface, and continues

signalling while trafficking to the autophagosome. The BCR recruits TLR9 from the endosome to the autophagosome. This recruitment is necessary for the hyperactivation of MAPK signalling. The recruitment is, however, independent of TLR9 signalling as it occurs also in *MyD88*$^{-/-}$ mice (Chaturvedi *et al.*, 2008).

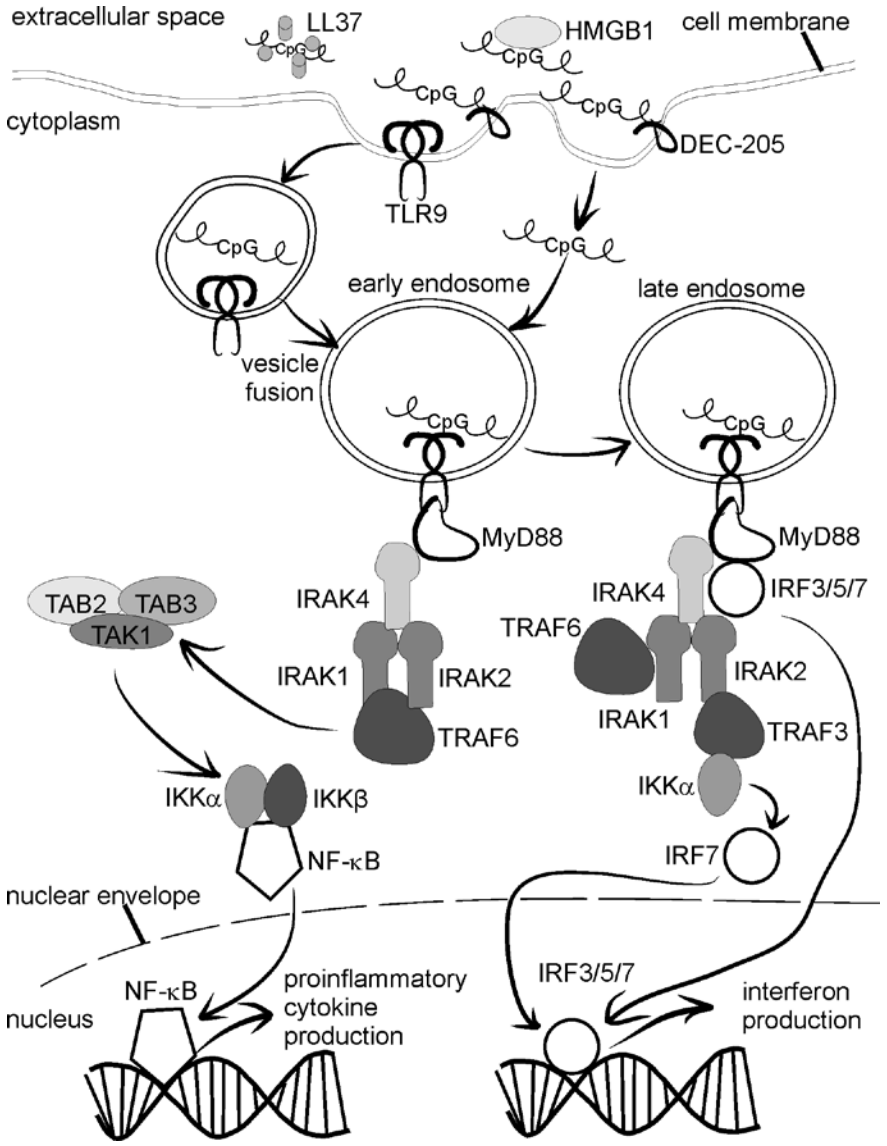

Figure 6. TLR9 signalling. After the Golgi apparatus exit, TLR9 is transported to the early endosome or the cell surface from where it is endocytosed. In the endosome, TLR9 is cleaved into a functional receptor. Ligand binding induces NF-κB activation and proinflammatory cytokine production. When signalling is attenuated and TLR9 signals from a late endosome, TLR9 activates the transcription factor IRF7 resulting in the production of interferons. Adapted from Sasai et al., 2010, Blasius and Beutler, 2010, and O'Neill et al., 2013.

Naive B cells cannot endocytose bacterial DNA and therefore DNA alone cannot induce the polyclonal activation of B cells (*i.e.* production of multiple antibodies by multiple B cells against the same antigen). It is probable that TLR9 encounters DNA in B cells only when the DNA-bound antigen is engulfed by the BCR (Roberts *et al.*, 2011). TLR9 ligands induce caspase-independent cell death in progenitor B cells, thus modulating B cell development and homeostasis. TLR9 signalling controls the balance of B cell lineages by favouring the expansion of the pre-B cells. This ensures the permanent renovation of the pro-B cell pool. Lalanne and coworkers suggest this to be a protective function, which helps to maintain the pre-B cell population that has undergone a successful V(D)J recombination (Lalanne *et al.*, 2010).

2.9 The many roles of TLR9 in health and disease

2.9.1 TLR9 in pathogen recognition

It has been estimated that 26% of human cancers associate with infectious agents, of which 80% are viruses (Bouvard et al., 2009). The HIV-1, hepatitis B virus, hepatitis C virus and human papillomavirus (HPV) are implicated in cancer development through persistent infections (Hirsch *et al.*, 2010). TLR9 has an important role in protection against viruses, and as an example TLR9 can recognize the Herpes simplex virus, Epstein-Barr virus (EBV) and HPV, which all are human pathogens (Fiola *et al.*, 2010, Hasan et al., 2007, Lund *et al.*, 2003).

Viruses have devised several strategies to inhibit receptors, which recognize them, and some oncogenic dsDNA viruses, like the Merkel cell polyomavirus, downregulate TLR9 expression. The Merkel cell polyomavirus, which is a cause for most Merkel cell carcinomas, inhibits TLR9 promoter activity by the downregulation of C/EBPα and C/EBPβ mRNA levels in human skin carcinoma and myeloma cell lines. Most of the inhibition results from the inhibition of C/EBPβ by the virus' large T antigen (Shahzad et al., 2013). When comparing normal cervical tissue and HPV-16-positive cervical cancer tissue, it was shown that HPV-16 oncoproteins E6 and E7 downregulate TLR9 expression utilizing both ERα and NF-κB p65 in the process (Hasan *et al.*, 2013). It has also been shown that HPV-16 genomic DNA can activate TLR9 via canonical signaling, and thereby induce NF-κB p65 and histone deacetylase 3 to bind the TLR9 promoter NF-κB *cis* element to inhibit TLR9 transcription (Zannetti *et al.*, 2014).

In vitro, EBV infects B cells, subsequently transforming them into indefinitely proliferating lymphoblastoid cells (Fathallah et al., 2010), and EBV infection strongly associates with Burkitt's lymphoma (Kutok & Wang, 2006). Addition of EBV particles to the TLR9-expressing human embryonic kidney cells (293T) activates NF-κB, and the EBV lytic phase protein BGLF5 reduces TLR9 expression through mRNA degradation (van

Gent *et al.*, 2011). Also, EBV inhibits TLR9 promoter activity in B cells via the oncoprotein LMP1 (Fathallah et al., 2010).

TLR9 also participates in protection against bacteria. TLR9 and MyD88-induced IFNγ is required for the protection of mice against *Listeria* infection (Ishii *et al.*, 2005). The *Staphylococcus aureus* cell wall contains TLR2 ligands, which are accessible to macrophage surface receptors. Wolf and coworkers found that both TLR2 and TLR9 are involved in recognizing *S. aureus*. The signalling is initiated at the cell surface when TLR2 is activated by *S. aureus*. By endocytosis and TLR9 activation, macrophages extend and maximize the immune response when both TLR2 and TLR9 signal (Wolf *et al.*, 2011). Other bacteria recognized by TLR9 include *Brucella abortus*, *Streptococcus pneumoniae*, *Mycobacterium tuberculosis* and *Helicobacter pylori* (Goutagny et al., 2012).

2.9.2 TLR9 in autoimmunity

TLR9 is linked to the pathophysiology of various autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus (SLE) and psoriasis (Lande *et al.*, 2011, Morizane *et al.*, 2012, Hennessy *et al.*, 2010). When an endosomal TLR is functional, it cannot tell the difference between microbial and self-nucleic acids, with the exception of TLR3, as long dsRNA stretches are absent from uninfected human cells. Therefore, as the molecular patterns of dsDNA, apart from their methylation level, are identical in microbes and men, TLR9 has the potency to recognize self-DNA (Brencicova & Diebold, 2013). Since the 1950´s, TLR9 antagonists, such as chloroquine (CQ), hydroxychloroquine (HCQ) and quinacrine have been used in RA and SLE treatment (Gambuzza *et al.*, 2011). The finding that CQ and quinacrine prevent endosomal acidification, thus inhibiting the activation of TLR9 and subsequent TLR9-CpG-DNA binding, led to the thought that TLR9 inhibition probably contributes to their therapeutic potential in autoimmune diseases (Rutz *et al.*, 2004).

The SLE patients' capacity to remove apoptotic cell debris, for example nuclear antigens, is often poor (Di Domizio *et al.*, 2012). Although apoptosis is thought to be non-immunogenic, the defective clearance of released self nucleic acids may activate the adaptive immunity leading to type I IFN production and a lupus-like autoimmune syndrome (Marshak-Rothstein & Rifkin, 2007, Barber, 2011). In SLE, self nucleic acids and their interacting proteins induce inflammation and damage to the tissue. This happens because the immune system has lost self-tolerance and self-antigens induce the production of pathogenic autoantibodies (Liu & Davidson, 2012). Neutrophil apoptosis is increased in SLE patients and the NET-originated immune complexes (ICs) effectively activate pDCs via TLR9. LL-37 has the property of being able to protect DNA from degradation. SLE patient sera contains LL-37-self-DNA ICs. When both LL-37 and

human neutrophil peptide-containing ICs are depleted, pDCs stop producing IFNα (Lande et al., 2011).

Psoriasis is an autoimmune disease manifested in the skin and joints. In the psoriatic skin, or after an insult in healthy skin, keratinocytes produce large amounts of AMPs, for example β-defensins and LL-37, a key factor that mediates pDC activation (Morizane et al., 2012). Healthy keratinocytes do not express LL-37. In psoriasis, LL-37 production together with a skin injury can induce pDC accumulation and their prolonged activation (Lande *et al.*, 2007). There are vast amounts of keratinocyte DNA in psoriatic lesions as the keratinocyte turnover rate is high (Baumgarth & Bevins, 2007), and TLR9 is significantly upregulated in psoriatic lesions in comparison to psoriatic non-lesional skin (Morizane et al., 2012).

Rheumatoid arthritis is a disease, where the functionality of the joints is compromized due to chronic synovitis (Meier *et al.*, 2013). TLR9 antagonism is considered as a potential treatment option in rheumatoid arthritis (Thwaites *et al.*, 2014, Hennessy et al., 2010), and TLR9 polymorphisms have been found to be associated with rheumatoid arthritis (Lee *et al.*, 2014b). The DAMP HMGB1 plays an important role in several diseases, including rheumatoid arthritis, where patients have chronic inflammation as well as cartilage and bone erosion. In rheumatoid arthritis, HMGB1 is a highly proinflammatory agent (Chen *et al.*, 2013).

For SLE and rheumatoid arthritis patients, HCQ treatment is effective, safe and cheap. In addition to effects on the autoimmune diseases, HCQ induces lower fasting glucose levels and lower insulin resistance (Penn *et al.*, 2010). Immunomodulatory ODN (IMO)-3100 (Idera Pharmaceuticals) which antagonizes TLR7 and TLR9 has proven to be clinically very effective against psoriasis in a phase 2 trial (http://ir.iderapharma.com/phoenix.zhtml?c=208904&p=irol-newsArticle& ID=1768483; accessed March 7th, 2015).

2.9.3 TLR9 agonists in cancer therapy

In 1995, five years before the role of TLR9 was found, CpG-DNA was shown to be able to activate immune responses (Krieg *et al.*, 1995). As a vaccine adjuvant, TLR agonists amplify both T and B cell responses (Huang *et al.*, 2008). Through DC activation, CpG-ODNs preferentially activate cytotoxic $CD8^+$ T cell responses (Xia *et al.*, 2014). Also, DCs are a potent target for immunotherapy as they are very effective APCs, and can induce cytotoxic $CD8^+$ T cell response and B cell maturation. Plasmacytoid dendritic cell (pDC) activation by an intratumoral TLR9 ligand injection induces pDC maturation into an APC, which potently activates cytotoxic $CD8^+$ T cells (Palma et al., 2012). CpG-ODNs are considered to be an effective immunotherapy in melanoma, basal cell carcinoma, renal

cell carcinoma and lymphoma to support immune responses and to prevent tumor immune evasion (Holtick *et al.*, 2011). For example, TLR9 activation renders both human and murine DCs resistant to Treg immunosuppression *in vitro*. Also, the reduction of Tregs by chemotherapeutic drugs such as paclitaxel enhances TLR9 agonist PF-3512676-induced antitumoral efficiency *in vivo* in a murine orthotopic renal cell carcinoma model (Vicari *et al.*, 2009). However, it has been shown *in vitro* that TLR9 ligands can induce either protumoral or antitumoral immunity by inducing or restraining immunosuppressive Tregs, respectively (Holtick et al., 2011). In a murine model, DAMPs inhibit antitumoral immunity by inducing Tregs and IL-10 production (den Haan *et al.*, 2007). In diffuse large B-cell lymphoma therapy, it has been suggested that adjuvant TLR ligands may cause side effects as therapy-induced DAMPs may induce tumor survival and progression (Huang *et al.*, 2012).

TLR9 ligands are widely studied as immune response-inducing adjuvants (den Haan et al., 2007). Second generation synthetic TLR9 agonists, IMOs restored colorectal and pancreatic cancer cell sensitivity to the anti-EGFR antibody cetuximab. The addition of IMO to cetuximab induced a strong, sustained co-operative antitumor effect with a significant tumor growth inhibition in pancreatic and colon carcinoma xenograft models (Rosa *et al.*, 2011). IMO dose-dependently inhibited non-small cell lung cancer cell survival and increased apoptosis *in vitro*. *In vivo*, IMOs inhibited lung tumor growth and sensitized tumors to chemotherapeutics (Wang *et al.*, 2006a). However, TLR9 stimulation in lung tumor therapy could cause adverse effects due to tumor TLR9 expression (Sautès-Fridman *et al.*, 2011) as TLR9 agonists can also mediate human lung cancer metastasis *in vitro* (Xu *et al.*, 2009).

Microglia cells have the capacity to activate both the innate and adaptive immunity. An immunosuppressive glioma microenvironment contains high numbers of microglia, and the glioma renders microglia less cytotoxic (Yang *et al.*, 2010, Carty & Bowie, 2011). In numerous mouse models, a direct intracranial CpG injection has had antitumoral effects. CpG-ODNs effectively induced cytokine secretion and NK and T cell activation, and stimulated a protective immune memory against tumor recurrence (Alizadeh *et al.*, 2010). In a Phase II study, an intracranially administered experimental TLR9 ligand CpG-28 showed a little response in recurrent glioblastoma. Of 34 patients, one had a radiological response (Carpentier *et al.*, 2010). Glioma patients have diverse TLR9 expression levels making it perhaps possible to find responders in a patient cohort (Carty & Bowie, 2011). *In vivo*, locally applied CpG-ODN most effectively eliminated intracerebral gliomas. CpG-ODN-induced TLR9 activation initiates an antitumoral immune response, which also requires TLR9-expressing non-tumor cells. In addition, CpG-ODN increased the amount of antitumoral tumor-infiltrating lymphocytes, namely

$CD4^+$ and $CD8^+$ effector T cells which secrete IFNγ (Grauer *et al.*, 2008). Importantly, it has also been shown that CpG-ODN induces glioma cell invasion but has no effect in glioma proliferation. Therefore, direct injection of TLR9 ligands into the tumor should be considered very carefully (Wang *et al.*, 2010). These data and the data in the previous chapter concerning lung cancer suggest that CpG-ODN treatment can have a double-edged sword-like nature depending on the cell type. Most publications report beneficial immunostimulatory effects following TLR9 stimulation. Importantly, as TLR9 activation has also been shown to induce tumor cell invasion, TLR agonist administration in relation to the tumor status requires further research.

2.9.4 The role of TLR9 in cancer pathophysiology

A local chronic inflammatory state is characteristic of several cancer types: ulcerative colitis and Crohn's disease for colon cancer, *Helicobacter pylori* infection for gastric cancer, bronchitis for lung cancer, pancreatitis for pancreatic cancer, cholecystitis for gall bladder cancer and HPV infection for cervical cancer (Basith et al., 2012, Friedman *et al.*, 2014). It is also probable that chronic prostatic inflammation (prostatitis) has significance in PCa development (Kundu *et al.*, 2008), and it has been speculated that TLR9-mediated infection and invasion leads to prostate cancer promotion (Ilvesaro *et al.*, 2007). Whether BC has viral etiology or not is currently under dispute (Pereira Suarez et al., 2013, Herrera-Goepfert et al., 2013).

Several human cancers express TLR9. TLR9 and also TLR5 protein levels gradually increase during the progression of cervical squamous cell carcinoma (Kim *et al.*, 2008a, Lee *et al.*, 2007). In two studies, TLR9 protein expression was shown to increase along with the histopathological grade of cervical cancer (Hao *et al.*, 2014, Fehri *et al.*, 2014). Human lung cancer tissues and various lung cancer cell lines express TLR9. Gastric cancer cells and *H. pylori* gastritis patients' metaplastic and dysplastic cells express TLRs 4, 5 and 9. When TLR1, 7 and 9 mRNA-expressing human myeloma cells are stimulated using bacterial ligands, tumor growth increases and cells become resistant to conventional therapies (Basith et al., 2012). Like benign B cells, also diffuse large B cell lymphoma cells often express TLR9 protein (Huang et al., 2012).

TLR9 has also indirect effects in tumors via infiltrating immune cells. In CpG-ODN-activated BC-resident pDCs, tumor-derived transforming growth factor (TGF)β and TNFα synergize to inhibit IRF7 expression and nuclear translocation, causing a decrease in type I IFN and TNFα production (Sisirak *et al.*, 2013). In mice, IL-12- or IFNα-dependent myeloid-derived suppressor cell differentiation is also achieved with CpG-ODN (van den Boorn & Hartmann, 2013). Gao and coworkers showed that TLR9 induces tumor regrowth after radiation therapy via IL-6 and STAT3 activation in myeloid cells, leading to inflammation and neovascularization (Gao *et al.*, 2013). Interestingly, it has

been shown that pDCs in the breast, ovary, and head and neck carcinoma microenvironment weakly respond to TLR9 stimulation (Hirsch et al., 2010).

TLR4 and TLR9 ligands induce enhanced proliferation of immortalized, but also primary prostate epithelial cells (Kundu et al., 2008). Both human PCa cells and clinical PCa samples have variations in TLR9 protein expression levels. CpG-ODN and bacterial DNA increase TLR9-positive PCa cell invasion. The invasion is associated with the activation of matrix metalloproteinase (MMP)-13, as its inhibition prevented invasion (Ilvesaro et al., 2007). A higher TLR9 protein expression was shown to associate with worse prognosis and PCa progression. TLR9 protein expression level increases along with the increase of PCa stage, with high Gleason score patients having the strongest signal. Also in PCa stroma, TLR9 is upregulated. Interestingly, a TLR9 increase also correlated with an increase in ERα expression in stroma (González-Reyes *et al.*, 2011, Väisänen *et al.*, 2010). Later, TLR9 protein expression was shown to be an independent poor prognosis marker in PCa (Väisänen *et al.*, 2013).

A significant increase in TLR3, 4, 7 and 9 mRNA levels was detected in esophageal squamous cell carcinoma patients. In tumor cells, TLR9 expression gradually increased with an increasing grade. High TLR9 expression only in tumor-residing fibroblast-like cells associated with low invasion and metastasis probability, suggesting that the surrounding connective tissue of the tumor is important for preventing tumor spread (Sheyhidin et al., 2011). Normally, TLR9 is expressed in the basal parts of the esophageal epithelium, with linearly decreasing intensity towards superficial layers. In high grade dysplasia, the whole epithelium expressed high TLR9 levels. In 76 squamous cell carcinoma samples, TLR9 expression correlated with an advanced stage, local metastases, previously undetected distant metastases, and poor OS (Kauppila *et al.*, 2011). Of 152 renal cell carcinoma patients, 81% had positive TLR9 immunostaining, which is a positive independent prognostic factor. The lack of TLR9 associated with poorer prognosis in patients with renal cell carcinoma (Ronkainen *et al.*, 2011).

In the whole brain mRNA analysis, 3 month old mice highly express TLRs 3, 7 and 9. At 10 months, TLR9 expression decreases by 50% (Letiembre *et al.*, 2007). In glioma cell lines and tumors, TLR9 expression correlates with malignancy, and TLR9 is an independent prognostic factor for PFS of glioblastoma (Wang et al., 2010). In glioblastoma patients, high TLR9 level correlates with shorter DFS and OS. In TLR9-negative glioblastoma, there was a PFS risk reduction of 43%. TLR9 and MMP-9 expression significantly correlated in glioblastoma tissue, and TLR9 status was a significant independent prognostic factor for OS (Leng *et al.*, 2012). Neuroblastoma is the most common extracranial childhood solid tumor. Brignole and coworkers tested 15 neuroblastoma cell lines and showed that they all express functional TLR9 (Brignole

et al., 2010). Furthermore, CpG-ODN inhibited neuroblastoma proliferation and induced apoptosis, and the *in vivo* administration of neuroblastoma-targeted CpG-containing liposomes induced a survival benefit. Also, non-targeted CpG prolonged survival, but in a lesser extent. In primary neuroblastomas, TLR9 expression inversely correlated with the disease stage, but high TLR9 expression associated with low age at diagnosis (Brignole et al., 2010).

Single nucleotide polymorphisms in TLR9 have been shown to predispose individuals to various diseases, including cancer. In primary biliary cirrhosis patients, the 2848 A/A TLR9 genotype associated with a significantly higher TLR9 expression and higher B cell count after CpG-ODN treatment (Kikuchi *et al.*, 2005). In Chinese Han women, the 2848 G/A polymorphism associated with a higher cervical cancer risk in the presence of HPV-16 infection (Lai *et al.*, 2013). In a Polish cervical cancer cohort, TLR9 polymorphisms 1486 T/C and 2848 C/T associated with a higher cervical cancer risk (Roszak *et al.*, 2012). In a study of 233 polymorphisms in TLR and NF-κB pathways, one TLR9 polymorphism was significant: the synonymous polymorphism rs352140(P545P) and BC risk were weakly associated (Resler *et al.*, 2013). In a meta analysis of 34 studies, the 2848 G/A polymorphism significantly associated with lower BC risk compared with 2848 G/G. In the study, no difference was found between Asian and Caucasian patients (Wan *et al.*, 2014).

2.10 The current understanding of the role of TLR9 in breast cancer

Many BC mutations, such as mutations in *Nos2*, *Ets1*, *Aim2*, *Gata3* or *Hdac7* genes cause defects in antiviral defence mechanisms increasing viral infection risk in the breast. Consistent with this concept, immune suppression before kidney transplantation increases the risk for BC (Friedenson, 2013). BC patients have a general immune system dysfunction, with impaired production of IFNγ and other cytokines. For example, IFNγ production was more blunted in HR-positive patients than in HR-negative patients (Campbell *et al.*, 2005).

In human BC cell lines, treatment with IMO decreased cell survival and proliferation and increased apoptosis and herceptin efficacy. *In vivo*, IMO had anticancer activity against murine syngeneic BC tumors (Wang *et al.*, 2006b). Similarly, in a BC model, IMO and herceptin induced a strong and sustained antitumor activity *in vivo* (Damiano *et al.*, 2009). When CpG-ODN was administered to support photodynamic therapy to treat metastatic BC xenograft tumors, they synergized in tumor growth suppression and increase in survival. Neither treatment was individually better than the combination (Xia et al., 2014).

When $CD24^-/CD44^+$ BC stem cells were isolated from patients, they were shown to be very tumorigenic as 200 $CD24^-/CD44^+$ cells were enough to form tumors in mice, whereas 50 000 $CD24^+/CD44^+$ cells could not. Moreover, the $CD24^-/CD44^+$ tumors showed phenotypic heterogeneity of the original tumor (Al-Hajj *et al.*, 2003). BC stem cells replicate slowly, expel drugs and resist apoptosis, making them resistant to many therapies. In mammary tissue $CD24^-/CD44^+$ cells, TLR9 has a normal, endosomal location. Non-stem BC cells recognize oncolytic adenoviruses by TLR9 and have normal TLR9 trafficking whereas in stem cells, TLR9 and MyD88 traffick abnormally resulting in a malfunctional immune response. Normal breast tissue cells do not suffer from oncolytic viruses because their IFN signalling is intact (Ahtiainen *et al.*, 2010).

The research field of TLR9 and breast cancer begun when Merrell and coworkers published that both BC cells and clinical BC samples express TLR9. They also showed that CpG- and non-CpG-induced BC cell invasion was mediated by an increase in MMP-13 levels and a decrease in TIMP-3 levels (Merrell *et al.*, 2006). Later, normal breast epithelium was shown to have an apical TLR9 expression pattern, whereas tumor epithelial cells had a cytoplasmic expression pattern. In BC cells, CpG-ODN was shown to signal independently of MyD88 (Ilvesaro et al., 2008). This was the first instance when TLR9 was shown to signal without MyD88 implicating that the signalling cascades initiated by TLR9 can differ between immune and tumor cells.

Gonzáles-Reyes and coworkers showed that there is more TLR9 expression in samples from patients with a recurrent disease. Furthermore, they showed that TLR9 expression correlated with BC stage. Interestingly, the reduction of distant metastases was only associated with TLR9 expression in fibroblast-like cells, not in tumor cells or in mononuclear cells (for example monocytes and lymphocytes) (González-Reyes *et al.*, 2010). TLR9 activation can significantly inhibit BC estrogen responses by downregulating ERα-mediated transactivation. This can occur via the suppression of the ERα target promoter element binding, thus regulating the estrogen-induced BC growth (Qiu *et al.*, 2009).

TLR9 expression in BC is very common. Jukkola-Vuorinen and coworkers showed that 98% of 141 studied clinical BC samples were TLR9-positive, and that TLR9 staining intensity inversely correlated with the ER staining intensity. Also, high-grade tumors exhibited increased TLR9 expression (Jukkola-Vuorinen *et al.*, 2009). In another study, 63% of patient samples were TLR9-positive (Qiu *et al.*, 2011). It was later confirmed that in BC cell lines, non-neoplastic BC tissue and in BC specimens TLR9 mRNA levels are inversely correlated with both the ER and PR status. TLR9 expression associated with poor differentiation in BC specimens (Berger *et al.*, 2010). Further, patients with larger tumor size, lymph node metastases, or advanced pathological stage had

significantly higher TLR9 expression. Moreover, in this study ER and TLR9 inversely correlated, as ER-negative tumors had increased immunohistochemical TLR9 expression. Patients with TLR9-positive BC had a significantly shorter 5-year PFS time than the TLR9-negative patients. Also, OS was shorter in TLR9-positive patients (Qiu et al., 2011). The results above indicate that TLR9 expression correlates with bad prognosis. Moreover, the more TLR9, the more severe outcome.

The research field of TLR9 versus BC is still young. Currently, not much is known about the pathophysiological role of TLR9. We have circumstantial evidence of its role, but the direct evidence lacks. Further studies will reveal whether TLR9 is an innocent bystander or an active factor in the natural history of BC.

3. AIMS OF THE STUDY

At the time when this project was initiated there were 10 PubMed hits on TLR9 and breast cancer. As most interest on TLR9 had risen from an immunotherapy point-of-view, our approach was to focus on the role TLR9 in tumors, especially breast tumors. Therefore, this thesis project was initiated to further clarify the role of TLR9 in breast cancer.

The specific goals of the four subprojects were:

1) To characterize the relationship between sex hormones and TLR9 regulation
2) To characterize the prognostic significance of TLR9 in breast cancer
3) To study whether the antitumoral effects of chloroquine are dependent on TLR9 status
4) To characterize the role of tumor TLR9 in treatment responses to chemotherapy in breast cancer

4. MATERIALS AND METHODS

The more detailed methods are described in the Manuscripts I-IV.

Clinical samples (I, II)

The use of all clinical samples was approved by the local ethical committees and the Finnish Authority of Medicolegal Affairs. Approval numbers: Pohjois-Pohjanmaan sairaanhoitopiirin kuntayhtymän (North Osthrobotnian hospital district, date 17.10.2005) and National Supervisory Authority for Welfare and Health (Dnro 6050/32/300/05). The patient cohort in manuscript I has been published earlier (Jukkola-Vuorinen et al., 2009). For manuscript II, 196 BC samples were collected from patients treated at the University Hospitals of Oulu and Kuopio. HR and HER2 statuses were analyzed by hospital pathologists. Hif-1α and TLR9 stainings were blindly scored by MD Katri Vuopala and MD Katri Selander.

Cell culture and transfections (I, II, III, IV)

Only human-derived cell lines were used. Cells were cultured in an incubator at 37^{o}C and 5% CO_2/95% air. For experiments examining the effect of hypoxia, cells were cultured with a pO_2 of 5%.

CELL LINE	CELL TYPE	GROWTH MEDIUM	MANUSCRIPT
MDA-MB-231 (SA)	TNBC	DMEM	I, II, III, IV
MCF-7	ER^+ BC	RPMI	I
T47D	ER^+ BC	RPMI	I, II, IV
Hs578T	TNBC	DMEM	II
D54MG	glioma	DMEM	III, IV
U373MG	astrocytoma	DMEM	III
Caco-2	colon carcinoma	DMEM	III
AGS	gastric carcinoma	DMEM	III

Oligonucleotide transfections (human TLR9 Smart pool or a non-targeting pool) were performed with Lipofectamine RNAimax (Invitrogen).

Plasmid transfections were carried out with Mirus TransIT-LT1 transfection reagent (Mirus Bio LLC).

Plasmid	Vendor	Manuscript
pSG5-ERα	Agilent*	I
pcDNA3.1-zeo	Invitrogen	I
pNF-κB-hrGFP	Agilent	I
pSUPER-EGFP TLR9 siRNA	Oligoengine	II
pSUPER-EGFP CTRL siRNA	Oligoengine	II

*The vector backbone is from Agilent. ERα-expressing vector was a kind gift from professor Pierre Chambon.

Animal models (II, III, IV)

All animal studies were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC 091109001). Tumor cells were orthotopically inoculated into mammary fat pads of athymic nude mice. Animals were treated as indicated in manuscripts, and tumor sizes were measured at least twice a week. Animal welfare was monitored daily and the animals were maintained in a controlled pathogen-free environment. Tumors were allowed to grow for 21 or 22 days. After sacrificing the mice, tumors were prepared for histological and immunohistochemical analysis using standard methods.

DNA isolation (IV)

To study the uptake and invasion-inducing effects of intact and doxorubicin-treated cell DNA, DNA was isolated according to manufacturer's (Qiagen) instructions. DNA concentrations were measured using NanoDrop (Thermo Scientific).

Quantitative real-time PCR (I, II, III, IV)

Total RNA was isolated and purified. cDNA was synthetized from 0.2 µg of total RNA and a standard amplification program was used. Each cDNA was normalized against the ribosomal protein L15 (RPLPO). The relative quantifications were performed using $2^{-\Delta\Delta ct}$ values. All reagents were purchased from Applied Biosystems.

Reagents (I, II, III, IV)

Reagent	Vendor	Manuscript
Insulin	Fisher	I
Bicalutamide	Sigma	I
Testosterone enanthate	Endo Pharmaceuticals	I
17β-estradiol	Sigma	I
17β-estradiol pellet	Innovative Research of America	II
Chloroquine	Sigma	II, III
GM6001	Enzo Life Sciences	II, IV
MMP-9 inhibitor	Calbiochem	IV
MMP-13 inhibitor	Calbiochem	IV
MMP inhibitor III	Calbiochem	IV
Cathepsin K inhibitor I	EMD Millipore	IV
Doxorubicin	Sigma	IV
Taxol	Sigma	IV
Cis-platinum	Sigma	IV
LL-37	Anaspec	IV
CpG oligodeoxynucleotide	Midland Certified Oligos	I

Next-generation sequencing (IV)

Total RNA was isolated and its quality was checked. Then, rRNA was depleted and samples were concentrated. After RNA-Seq library preparation and validation, the samples were sequenced. The data analysis was performed as indicated in the manuscript.

Zymography (II, III, IV)

Cells were incubated for 24h in normoxia or hypoxia (II), or 24-48h in normoxia (III) in serum-free media. The supernatants were concentrated and equal amounts of proteins were loaded per lane of 10% gelatin gels (BioRad). The gels were ran, renaturated, developed and stained.

Reporter gene assay and flow cytometry (I)

Cells were transfected with a GFP-expressing NF-κB reporter plasmid and treated with CpG-ODNs with or without testosterone. To detect NF-κB activation, flow cytometry analysis was performed using a FACScan flow cytometer (BD Biosciences). Data analysis was performed with Flowing Software (www.flowingsoftware.com).

Western blotting and antibodies (I, II, III)

Cells were lysed and samples were run onto 10% or 4-20% gradient gels (BioRad) and transferred onto nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk in TBS-Tween and incubated with primary antibodies o/n. After secondary antibodies, the proteins were visualized with an enhanced chemiluminescence (ECL) kit (Pierce).

ANTIBODY	SOURCE	PRODUCT CODE	VENDOR	MANUSCRIPT
α-TLR9	rabbit	IMG431	Imgenex	I, II, III
α-ERα	rabbit	HC-20	Santa-Cruz	I
α-Actin	rabbit	A-2066	Sigma	I, II, III
α-TIMP-3	rabbit	AB8106	Millipore	II
α-Hif-1α	rabbit	NB100-134	Novus Biologicals	II
α-β-tubulin	mouse	D66	Sigma	II
α-MMP-13	rabbit	PC542	Oncogene Research Products	II
α-rabbit-HRP	rabbit	NA934V	GE Healthcare	I, II, III
α-mouse-HRP	mouse	NA931	GE Healthcare	II

Cell viability and proliferation assays (II, III, IV)

Cells were seeded on 96-well plates and treated as indicated in the manuscripts. The viability and proliferation of the samples was studied either with the MTS assay (Promega) or with Trypan blue using an automatic cell counter (BioRad).

Invasion assays (I, II, IV)

Cells were plated on 24-well invasion inserts (BD Biosciences) and treated as indicated in the manuscripts. Next, the cells were allowed to invade through Matrigel and the membrane (BD Biosciences). Membranes were stained and invaded cells were counted under a microscope.

Statistical analyses (I, II, III, IV)

The data were analysed with mean±SD, mean±SEM, Student's t-test, Mann-Whitney U test or 2-sided Fisher's exact test, as shown in the manuscripts. Survival was analyzed using the Kaplan-Meier curve with log-rank test. The survival data were analysed by Dr. Renee A. Desmond (University of Alabama at Birmingham, USA). SPSS 17.0.2 and GraphPad Prism software were used for data analysis.

5. RESULTS

5.1 ERα and sex steroid hormones regulate TLR9 expression and TLR9 ligand-induced invasion in BC cells

In Manuscript I, we investigated the effects of ERα expression, and the influence of sex steroid hormones estradiol and testosterone on TLR9 mRNA and protein expression. The basal TLR9 mRNA expression levels were significantly lower in the ER-positive cell lines than in the ER-negative cell line. Transfection of ERα into MDA-MB-231 cells resulted in a downregulation of TLR9 mRNA and protein expression. While sex steroids had no effect on TLR9 mRNA expression in MCF-7 cells, testosterone induced TLR9 mRNA and protein expression in T47D and MDA-MB-231 cells. The invasive effects of synthetic TLR9 ligands were augmented by testosterone in MDA-MB-231 cells. This effect was lost in TLR9 siRNA MDA-MB-231 cells and decreased by ERα overexpression. The results show that sex steroid hormones and ERα can regulate TLR9 expression, and that the relationship between ERα and TLR9 has functional significance.

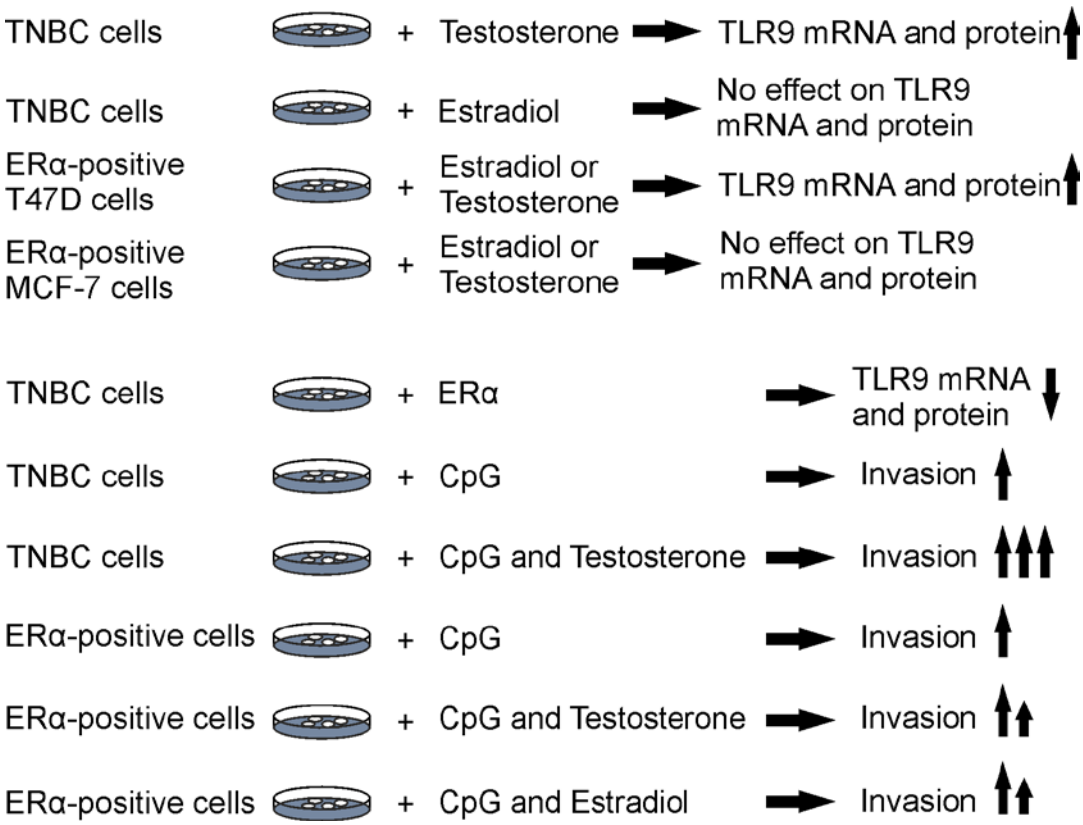

Figure 7. Main findings from Manuscript I

5.2 TNBC patients with low tumor TLR9 expression have significantly shorter disease-specific survival

In Manuscript II we discovered that low tumor TLR9 expression is associated with significantly shortened BC-specific survival in patients with TN but not with $ER^+/PR^+/HER2^-$ BC. Our preclinical studies indicated that while hypoxia upregulated TLR9 expression, low TLR9 expression had different effects on TN and ER-positive BC invasion in hypoxic conditions. In normoxia, the invasive capacity of TLR9 siRNA MDA-MB-231 cells was much lower than the invasive capacity of control siRNA cells. Hypoxia-induced invasion was augmented by TLR9 siRNA in TN, but not in ER-positive BC cells. This is possibly due to differential TLR9-regulated TIMP-3 expression, which remains detectable in ER-positive cells, but disappears from TN TLR9 siRNA cells in hypoxia. The results suggest that TLR9 expression may be a novel marker for TNBC patients that are at a high risk of relapse.

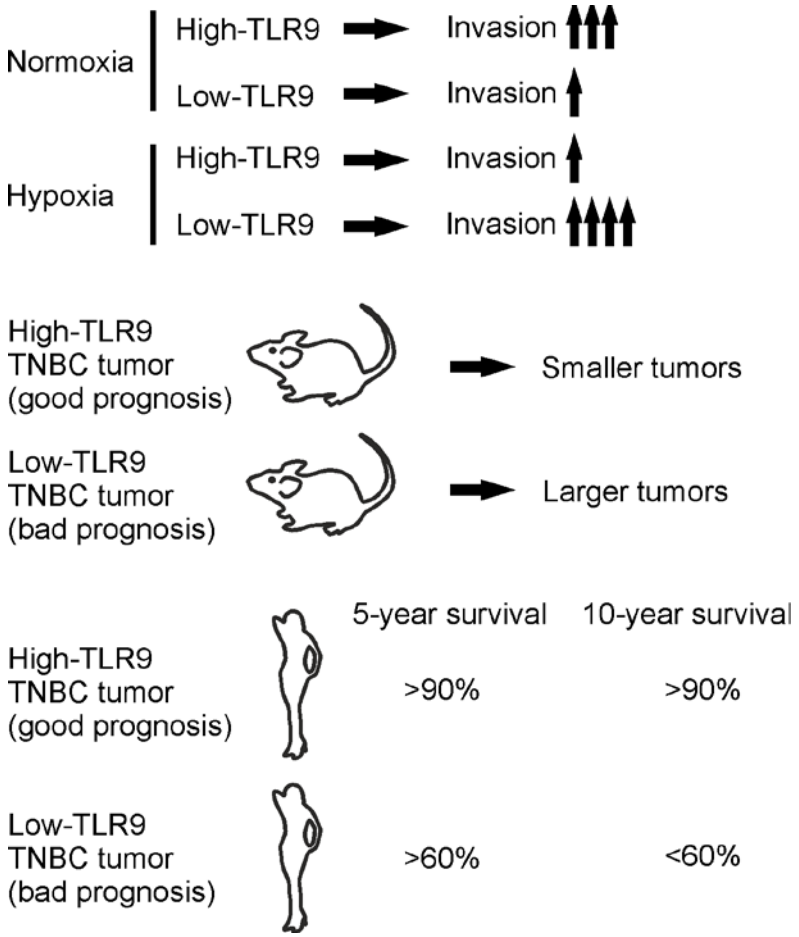

Figure 8. Main findings from Manuscript II.

5.3 Chloroquine has a dual role in TNBC

Chloroquine (CQ) is an autophagy and endosomal acidification inhibitor, and consequently, a TLR9 inhibitor. The study aimed to investigate the effects of CQ on TNBC cells having low or high expression of TLR9 *in vitro* and *in vivo*. MDA-MB-231 cell TLR9 mRNA levels were upregulated by CQ in both normoxia and hypoxia. Despite enhancing TLR9 mRNA expression, CQ suppressed TLR9 protein expression *in vitro*. CQ suppressed MMP-2 and MMP-9 mRNA expression and protease activity, whereas MMP-13 expression and proteolytic activity increased in both control and TLR9 siRNA MDA-MB-231 cells. Despite the favourable anti-tumor *in vitro* effects of CQ on TNBC invasion and viability, particularly in hypoxic conditions, CQ did not prevent the growth of orthotopic high or low TLR9-expressing MDA-MB-231 tumors.

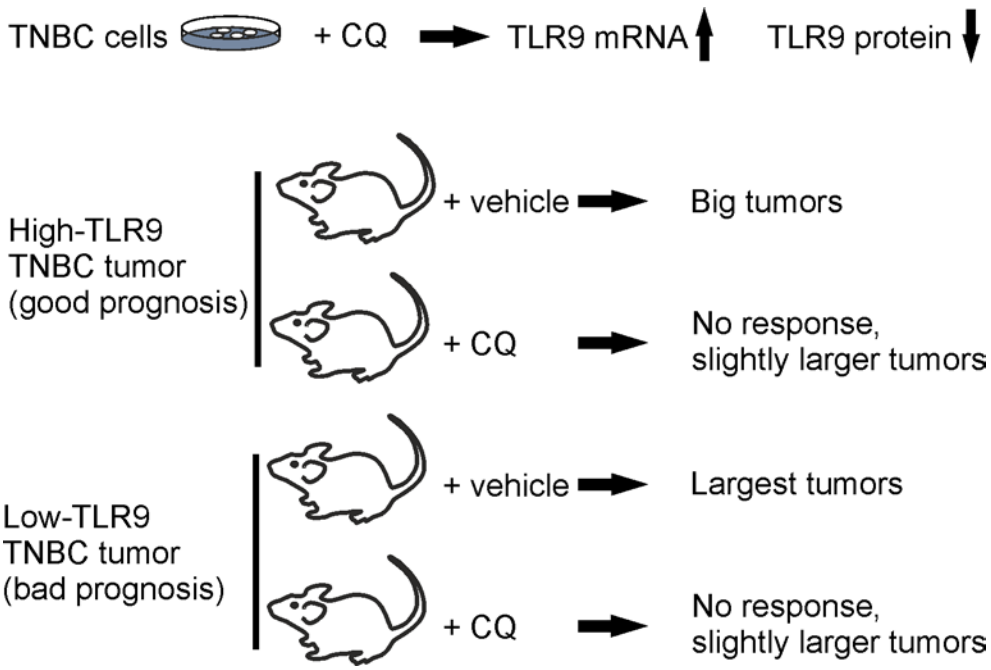

Figure 9. Main findings from Manuscript III.

5.4 DNA from chemotherapy-killed cells induces TLR9-mediated cancer cell invasion

Since self-DNA is known to act as a TLR9 ligand, we wanted to study whether self-DNA from chemotherapy-killed cells could activate TLR9. Indeed, we discovered that DNA from chemotherapy-killed cells induces TLR9- and cathepsin-mediated cancer cell invasion. To study whether this phenomenon contributes to treatment responses, we inoculated low or high TLR9-expressing TNBC cells orthotopically into nude mice and treated them with vehicle or the chemotherapeutic drug, doxorubicin. The tumors in both groups exhibited an equal decrease in tumor size in response to doxorubicin. However, while the body weights of the vehicle-treated mice were similar, mice bearing high-TLR9 tumors became significantly more cachectic in response to doxorubicin. This suggests a TLR9-mediated inflammation at the site of the tumor, as systemic inflammation is a hallmark of cancer cachexia. These findings suggest that DNA from chemotherapy-killed cells has a significant influence on TLR9-mediated biological effects in living BC cells. Through these mechanisms, tumor TLR9 expression may affect treatment responses to chemotherapy.

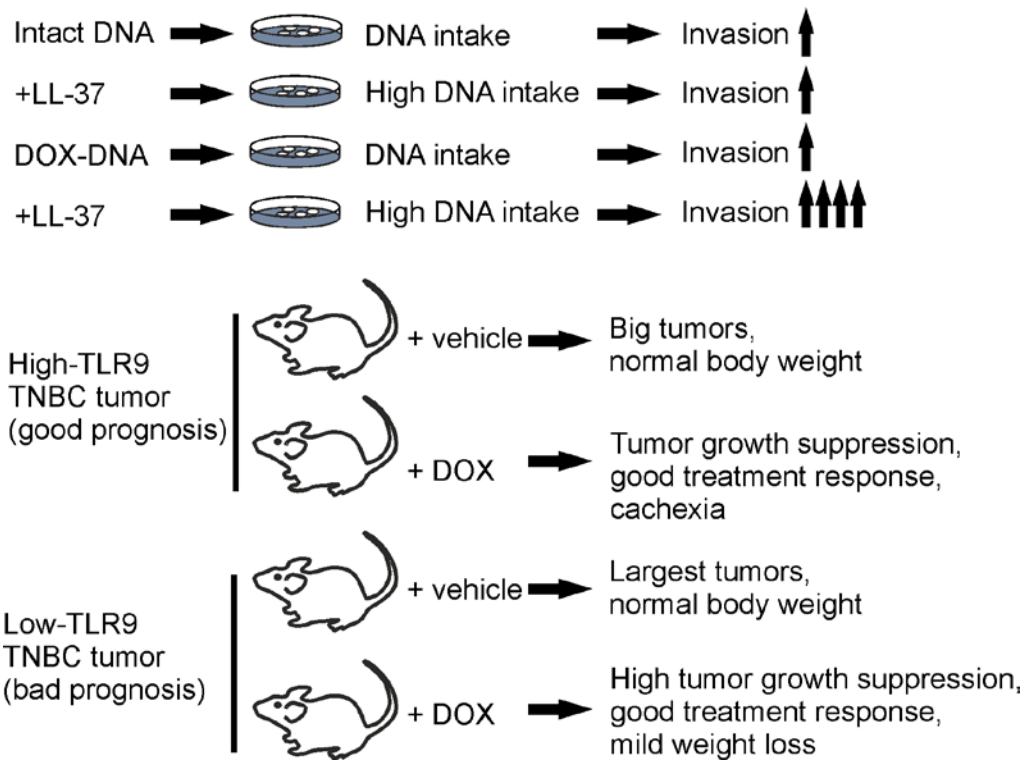

Figure 10. Main findings from Manuscript IV.

6. DISCUSSION

6.1 The relevance of tumor TLR9 expression

When discussing the role of TLR9 in cancer, one should always consider whether the emphasis is on TLR9 in resident or infiltrating immune cells, in tumor cells, or in tumor-related cells, such as fibroblasts. The reason why non-immune cells express TLR9 is unclear. Notably, it has been shown that non-immune cells, for example glioblastoma cells, can activate the adaptive immunity (Shibahara *et al.*, 2015) and tumor cells have also been shown to induce T cell apoptosis (Kovács-Sólyom *et al.*, 2010). Chiron and coworkers speculate (with caution) that bacterial DNA could trigger TLR9 signalling in various tumor cell lines, leading to immune activation (Chiron *et al.*, 2009). Whether this happens with self-DNA is not known. If tumor cell TLR9-mediated inflammation or T cell death exists in BC, it could account for the prognostic significance of TLR9.

One should bear in mind that tumors are very heterogenous within the tumor and between tumors. What is the source of the variation of TLR9 protein and mRNA expression in tumors? Whether the down or upregulation comes through epigenetic promoter modulation is of great interest. Therefore, the TLR9 epigenome should be studied using healthy donor and BC patient samples. However, regulation of TLR9 is at least partially transient, governed by endogenous transcription factors (section 2.8.3), or by DNA virus-induced downregulation (section 2.9.1). It can be that high or low TLR9 expression is a selected phenotype bringing advantage during tumor evolution. It is also possible that in some cases TLR9 activation might contribute to the prognosis (Gay et al., 2014). This would mean that the activity and not the expression level, is important. Currently, there is no evidence of that.

The inverse correlation between ER and TLR9 probably bears some significance (Jukkola-Vuorinen et al. 2009, Berger et al. 2010 and Qiu et al. 2011, Manuscript I). In Manuscript I, we showed that TLR9 is upregulated by testosterone and estradiol. Interestingly, ERα downregulated, whereas its ligand, estrogen, upregulated TLR9 expression. All estrogens are synthesized from androgen precursors by the aromatase enzyme. Healthy breast stroma expresses low levels of aromatase, whereas BCs have high levels of aromatase, which induces the production of large amounts of estrogen (Stocco, 2012). This suggests high TLR9 expression levels in tumors, specifically in tumors with low ERα expression. As mentioned in section 2.10, a high TLR9 level is bad, when BC subtypes are not separated, which could stem from the sex hormone effects. Yet, we showed in Manuscript II that a high TLR9 level is beneficial in TNBC. Interestingly, Jukkola-Vuorinen and coworkers showed a slightly higher TLR9 level in TNBC than in HR-positive or triple-positive tumors (Jukkola-Vuorinen et al., 2009).

TLR9 has different prognostic significance in different tumors. High tumor TLR9 expression is protective in TNBC (Manuscript II), renal cell carcinoma (Ronkainen et al., 2011) and mucoepidermoid salivary gland carcinoma (Korvala *et al.*, 2014), but associates with poor prognosis in prostate (Väisänen et al., 2013), brain (Leng et al., 2012) and esophageal (Sheyhidin et al., 2011) tumors. In our *in vitro* experiments, low-TLR9 TNBC cells were more aggressive than high-TLR9 cells in hypoxia (Manuscripts II and III). Consistently, in the *in vivo* experiments, low-TLR9 TN tumors grew bigger than high-TLR9 tumors (Manuscripts II, III and IV). This shows the compatibility between our *in vitro*, *in vivo* and patient data, and strengthens the clinical significance of our findings. Our data in Manuscript II suggest that the critical factor is hypoxia, which both upregulates TLR9 expression and induces low-TLR9 TNBC cell invasion. In Manuscript II, we showed that the transcription factor Hif-1α mediates the hypoxia effects. However, we did not show the direct interaction between Hif-1α and the TLR9 promoter. The interaction could be verified by chromatin immunoprecipitation sequencing (ChIP-Seq), followed by the analysis of a patient cohort TLR9 promoter methylation status, which would tell whether the Hif-1α binding site is methylated in the low-TLR9 TNBC patient group. If being the case, methylation therapy could be a potent therapy modality if applicable in the future.

Importantly, tumor TLR9 expression does not associate with prognosis in HR-positive BC. Also, in our experiments, there was no TLR9 level-dependent difference in HR-positive tumor aggressiveness *in vivo*, again showing the comparability of our *in vivo* and clinical data (Manuscript II). Immunohistochemical analysis of the tumor TLR9 level would be a straightforward and affordable addition to current BC analyses. Due to the results in Manuscript II, the staining should be taken into clinical use in TNBC patients.

As prognosis stems from the metastastic efficiency of the tumors, could TLR9 have metastasis-promoting effects in PCa and non-TNBC BC, and metastasis-inhibiting effects in TNBC? In the first, the effect could be understood by the invasion-promoting effect of hypoxia, estrogen, testosterone, or circulating cell-free DNA (cfDNA), which is increased by chemotherapy in both BC (Lehner *et al.*, 2013) and PCa (Kwee *et al.*, 2012). In Manuscript IV, we showed that chemotherapy-induced cfDNA induced TLR9-mediated TNBC cell invasion *in vitro*. Whether that is the situation *in vivo* or in patients requires further research. Consequently, the relationship of TLR9 level, hypoxia, cfDNA, and sex steroid hormones should be studied. Also, testosterone should be included in the studies as approximately 80% of BCs are androgen receptor-positive. There were higher TLR9 levels in higher-grade (higher Gleason score) PCa tumors (Väisänen et al., 2010), which are often castration-resistant, *i.e*. they do not respond to anti-androgen therapy. Both TNBC and castration-resistant PCa are often hypoxic and aggressive. In

Manuscript I, we showed that testosterone upregulates TLR9 expression and invasion in MDA-MB-231 cells, which express AR in low levels (Lehmann et al., 2011). Testosterone is the main driver of PCa, but protective in BC, as mentioned earlier, unless aromatized into estrogen. Interestingly, in our experiments, testosterone increased invasion. It is possible that TLR9 signalling has diverse pathways and endpoints in different tumors, which could also explain the dissimilarity between TNBC and HR-positive tumors.

It is possible that TLR9 is just a surrogate marker for the real factor. Several proteins have been shown to affect TLR9 levels. One of the interesting ones is CD73, a 5' ectonuclotidase which converts AMP to adenosine. The role of CD73 in tumorigenesis is controversial. In a small BC cohort, high CD73 immunostaining correlated with a poor outcome, independently of the HR or HER2 status (Leth-Larsen *et al.*, 2009). However, in another BC cohort, positive CD73 staining correlated with longer DFS (Supernat *et al.*, 2012). It has been proposed that CD73 induces tumor growth and metastasis formation *in vivo* by creating an anti-inflammatory environment via adenosine production. CD73 has an inverse relationship with TLR9 in the colon epithelium during inflammatory bowel disease, as CD73-negative mice have increased TLR9 expression (Bynoe *et al.*, 2012). In TLR9-deficient T cells, CD73 expression is increased leading to protection against diabetes (Tai *et al.*, 2013). It is tempting to speculate that in low-TLR9 TNBC, the upregulated CD73 would produce an anti-inflammatory protumorigenic shield through enhanced production of adenosine. Likewise, this hypothesis requires further research.

How to attack the poor prognosis low-TLR9 TNBC tumors? Immune cell-targeted direct TLR9 ligand injection could be detrimental for the patient, as it might induce tumor TLR9-induced tumor spread. Also, most low-TLR9 tumors have some TLR9 expression (Manuscript II) thus challenging the direct injection with those tumors also. Systemically given TLR9 ligands are strong immune-boosters, as known already from Coley's toxin, which contains heat-killed bacteria from erysipelas (Coley, 1891, Wiemann & Starnes, 1994). The immunomodulation should be targeted as locally effective, and also systemically to target disseminated cells. When thinking of treating the patients, systemic immunotherapy, where tumor antigens are presented to the immune cells *ex vivo* seems like the most plausible method. Then, the tumor's exposure for possible invasion inducing TLR9 ligands would be minimized.

6.2 Old drugs with new indications

By the words of Nobel laureate James Black, the most fruitful basis for the discovery of a new drug is to start with an old drug. It makes sense both cost and speed-wise (Chong

& Sullivan, 2007). Aspirin is perhaps the most discussed non-cancer drug, which has antitumor activity. Others include statins, bisphosphonates and CQ.

Aspirin has been shown to give a survival benefit in BC, perhaps stemming from the fact that aspirin users have lower estrogen levels (Holmes & Chen, 2012). Statins have been shown to decrease BC recurrence (Kwan *et al.*, 2008). BC and TNBC cell lines express beta-adrenergic receptors (Holmes & Chen, 2012). Melhen-Bertrand and coworkers suggested that TNBC patients with metabolic syndrome could have adrenergic dysregulation hinting that they could benefit from a beta-blocker treatment. Indeed, TNBC patients were shown to have a clearly lower relapse risk when they used beta-blockers (Melhem-Bertrandt *et al.*, 2011). Bisphosphonates are osteoporosis drugs, which are also in use in bone metastasis treatment. The bisphosphonate zoledronate has been shown to have antitumoral benefit in BC (Gnant *et al.*, 2015, Coleman *et al.*, 2014).

While trying to kill the tumor cells, many cancer drugs induce autophagy, where tumor cells use their own organelles as an energy source. In a sense, they shut the hostile outside world off to survive. Autophagy induces genetic instability by preventing inflammation and necrosis. However, autophagy can be either anti- or protumoral (Janku *et al.*, 2011). The TLR9 inhibitors CQ and HCQ are well-known autophagy inhibitors, and many studies have shown the significance of CQ in BC *in vitro* and *in vivo*.

Amaravedi and coworkers showed that autophagy promotes HMGB1 release, which can increase immunotherapy efficiency (Amaravadi *et al.*, 2011). HMGB1 together with DNA strongly activates TLR9-mediated immune response. *In vivo*, CQ induced an increase of immunigenicity of irradiated BC xenografts and drived antitumoral Th1 immunity (Ratikan *et al.*, 2013). Importantly, CQ has been shown to have autophagy-independent antitumoral effects. In a preclinical melanoma model, CQ induced tumor vessel normalization (functionality) and, therefore, tumor oxygenation and perfusion (drug delivery), and immune cell infiltration *in vivo*. CQ also impaired metastasis formation *in vivo* (Maes *et al.*, 2014), and many clinical trials have shown promising results for CQ and HCQ in late-stage cancers (Zhang *et al.*, 2015).

In Manuscript III, despite the promising anti-tumor effects *in vitro*, CQ had no effect on tumor growth *in vivo*, regardless of the tumor TLR9 status. Interestingly, CQ induced TLR9 mRNA expression in all 5 studied cell lines, and CQ treatment caused an increase in tumor growth, however, without statistical significance. This could be due to CQ supporting vessel functionality, as mentioned in the previous chapter. On the other hand, if the antitumoral effects of CQ are independent of the effects on autophagy and endosomes, the role of TLR9 in the CQ antitumorigenicity might be negligible. However,

if CQ would be administered simultaneously with the tumor cell-killing, autophagy-inducing, and DNA-releasing therapy, it might prevent TLR9 or TLR7-mediated protumorigenic signalling. Alternatively, if we would have used a syngeneic model with an intact immune system, there might have been TLR9-dependent differences in responses to CQ. Finally, it is possible that the CQ dose used in the study was not sufficient.

6.3 Further approaches

In the future, public databases, such as METABRIC and The Cancer Genome Atlas should be thoroughly studied to decipher, which pathways are under TLR9 regulation in cancer. The public KM plotter (kmplot.com) resource uses published gene expression data to create Kaplan-Meier curves of correlations between gene(s) of interest and RFS or OS, or other endpoints (Győrffy *et al.*, 2013). The KM plotter data supports our findings that TNBC patients with low TLR9 expression have a worse survival. This is important as it is common that mRNA results poorly correlate with histological data.

The relationship between TNBC and BLBC in reference to TLR9 is of interest. The significance of TLR7 is also intriguing, as the TLR7 and TLR9 downstream signalling is identical in immune cells. Whether that is the situation in tumor cells needs research. Could TLR7 have prognostic significance in BC? Our preclinical experiments with low-TLR9 and high-TLR9 expressing TNBC cells should be repeated with other TNBC cell lines with different TLR9 levels to see the coverage of the findings. The studies should also be expanded to murine TN tumors. Then, syngeneic low-TLR9 and high-TLR9 TN models, with an intact immune system, could provide information about the significance of the immune response, and the importance of TLR9. Also, the clinical findings should be validated with other patient cohorts. For example, analyzing the TLR9 status vs. the outcome in African-American TNBC patients is of great interest due to the high prevalence of TNBC in that patient population.

Eventually, when the coverage of the data is sufficient, it will reveal whether we can start calling TLR9 a drug target, instead of a biomarker or a prognostic factor.

7. CONCLUSIONS

Collectively, our results clearly demonstrate that TLR9 has prognostic significance in TNBC. We have identified a new TNBC subtype, more specifically, TNBC patients with a low tumor TLR9 expression, which have a significantly worse prognosis in comparison to high tumor TLR9-expressing TNBC patients. Our studies further suggest that by affecting the TNBC cells' ability to invade, TLR9 may also contribute to TNBC pathophysiology. Further research on the role of TLR in TNBC is strongly justified. Such research might especially benefit patients with low tumor TLR9 TNBC. Approximately 200-300 such tumors are diagnosed annually in Finland alone (Carey *et al*., 2010; Manuscript II).

According to our results, TLR9 should be considered as a clinical prognostic marker in TNBC to identify the individuals, which are at a higher risk of relapse following chemotherapy, and who might benefit from enhanced surveillance.

As mentioned in the end of chapter 2.10, we do not know much of the role of TLR9 in the natural history of BC. Therefore, we have to put effort in TLR9 research to clarify its role.

8. ACKNOWLEDGEMENTS

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