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The Toll-Like Receptor 9 — From Immune Cells to Cancer

Is TLR9 in tumours a cause or a consequence?

Tamiko Ishizu

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
SARJA – SER. D OSA – TOM. 1917 | MEDICA – ODONTOLOGICA | TURKU 2025

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“Courage is not having the strength to go on;
it is going on when you don’t have the strength”

-Theodore Roosevelt-

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ABSTRACT

Toll-like receptor 9 (TLR9) is an endosomal innate immunity receptor that plays a vital role in the first line of defense, primarily in immune cells, but in varying degrees, also in other non-immune tissues. Once TLR9 encounters its ligand, a single-stranded DNA, it activates the MyD88-dependent canonical NF-κB pathway, resulting in proinflammatory cytokine production. Besides its imperative role in defense against pathogens and resolving a tissue injury, the aberrant expression of TLR9 in multiple cancers has raised wide interest in investigating its role also in carcinogenesis.

This thesis presents new puzzle pieces for the ever-growing picture of TLR9 and its relevance to head and neck squamous cell carcinoma (HNSCC) and breast cancer (BC). The studies showed that (1) nitrogen-containing bisphosphonates (n-BPs) inhibit cell growth and viability in BC cells with suppressed TLR9-expression, (2) neither hypoxia nor TLR9 in non-immune malignant cells (i.e. tumour-originating TLR9) are essential for the HNSCC cell lines-induced alterations in uncommitted macrophage (MΦ) activity (polarisation) and functionality (cytokines), and that (3) TLR9-ligands (short cytidine-guanosine-rich oligonucleotides, CpG-ODNs) have an impact on cell migration and cell signalling on FaDu and FaDu^{TLR9def} cells.

Although no common denominator explaining the actions of the innate immunity receptor TLR9 in BC and HNSCC was discovered, these studies demonstrate that the functions of TLR9 in cancer are often capricious and convoluted. While TLR9 is associated with the viability of BC cells upon n-BP exposure, its endogenous expression in HNSCC appears insignificant to the production of immunomodulatory molecules relevant to MΦ maturation. However, the unpublished results demonstrate that stimulation of HNSCC cells with TLR9-ligands modulates their cell physiology as well as signalling. Since the outcome of TLR9-mediated alterations can be pro- or anti-tumorigenic depending on the context, its utility as a biomarker needs more research. These findings warrant clinician awareness when assessing and interpreting outcomes after novel cancer treatments, such as TLR9-binding oligonucleotides as cancer vaccine adjuvants.

KEYWORDS: TLR9, innate immunity, hypoxia, macrophage, bisphosphonates, CpG-ODN

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TIIVISTELMÄ

Tollinkaltainen reseptori 9 (TLR9) on luontaisen immuniteetin reseptori, jolla on tärkeä rooli ensisijaisesti sekä immuunisoluissa että myös muissa kudoksissa. Tunnistaessaan yksijuosteista DNA:ta, TLR9 aktivoi MyD88-riippuvaisen kanonisen NF-κB-signalointireitin sekä proinflammatoristen sytokiinien tuotannon. Vaikka TLR9 ilmentyminen on välttämätöntä mm. kudosvaurion korjaamisessa sekä immuunipuolustuksessa patogeenejä vastaan, sen ilmeneminen eri syövissä on herättänyt laajaa kiinnostusta tutkia sen roolia myös tämän sairauden yhteydessä.

Väitöskirjani valottaa TLR9:n merkitystä pään- ja kaulanalueen syövissä (HNSCC) sekä rintasyövässä (BC). Tulokset osoittivat, että (1) BC- solut, joiden TLR9:n ilmeneminen on geneettisesti hiljennetty ovat herkempiä bisfosfonaattien kasvua estäville vaikutuksille kuin villityypin rintasyöpäsolut, (2) HNSCC- solulinjat vaikuttavat sekä makrofagien polarisaatioon että toiminnallisuuteen riippumatta hypoksiasta ja syöpäsolujen TLR9-statuksesta, ja että (3) TLR9-ligandit (lyhyet sytidiini-guanosiinirikkaat oligonukleotidit, CpG-ODN:t) vaikuttavat pään- ja kaulanalueen FaDu- ja FaDu^{TLR9def}-solujen funktionaalisuuteen (soluliike) sekä solusignalointiin.

Huolimatta siitä, ettei yhteistä nimittäjää, joka selittäisi TLR9:n aikaansaamat efektit tutkituissa syövissä, löydetty, tulokset osoittavat, että TLR9-välitteiset toiminnot ja säätely syövässä eivät ole suoraviivaisia. Vaikka TLR9 on yhteydessä BC-solujen elinkykyyn n-BP-altistuksen yhteydessä, sen ekspressio ei vaikuta HNSCC-solujen immuunimodulatooristen molekyylien tuotantoon. Julkaisemattomat tulokset kuitenkin osoittavat, että TLR9-ligandit muokkaavat solujen fysiologiaa sekä signalointia. Koska muutokset voivat kontekstista riippuen olla joko pro- tai antitumorigeenisiä, TLR9-statuksen hyödyntäminen biomarkkerina vaatii edelleen lisätutkimuksia. Väitöskirjan havainnot kannustavat kriittiseen arviointiin esim. tulkittaessa kokeellisten syöpähoitojen tuloksia, joissa tutkitaan CpG-ODN:ien adjuvanttisia ominaisuuksia.

AVAINSANAT: TLR9, sisäsyntyinen immuniteetti, hypoksia, makrofaagi, bisfosfonaatit, CpG-ODN

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Abbreviations

AKT/PKB	Protein kinase B
ALN	Alendronate
AMPK	Adenosine monophosphate-activated kinase
APC	Antigen-presenting cells
ApppI/ApppD	Cytotoxic ATP-analogues
ATP	Adenosine triphosphate
BP	Non-nitrogenous bisphosphonate
BRCA1	BRCA1 DNA Repair Associated
BRCA2	BRCA2 DNA Repair Associated
BrdU	Bromo-2'-deoxyuridine
$CM^{NOX/HOX}$	Conditioned media under normoxia or hypoxia
CpG-ODN	Short cytidine-guanosine-rich oligonucleotide
CTLA-4	Cytotoxic lymphocyte-associated protein 4
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DMAPP	Dimethylallyl diphosphate
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ER	Endoplasmic Reticulum
ER^{+}	Oestrogen receptor-positive breast cancer
fl-TLR9	Full-length TLR9 (unprocessed)
FPPS	Farnesyl pyrophosphate synthase
GSK3	Serine/threonine kinase glycogen synthase kinase 3
HER2	Human epidermal growth factor 2
HIF-1	Hypoxia-inducible factor 1
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papilloma virus
HRE	Hypoxia-responsive element
IFN	Type I interferons
IKKβ	IkappaB kinase beta
IL-1β	Interleukin 1 beta

iNOS	Inducible nitric oxide synthase
IPP	Isopentenyl pyrophosphate
IT	Intratumoural injection
ITH	Intratumoural heterogeneity
LDH-A	Lactate dehydrogenase A
M1	Proinflammatory, type 1 macrophages
M2	Anti-inflammatory, type 2 macrophages
MAMP	Microbe-associated molecular pattern
MAPK	Mitogen-activated protein kinase
MHC II	Major histocompatibility complex II
mTOR	Mammalian target of rapamycin
MyD88	Myeloid differentiation primary response 88
MΦ	Monocyte-derived macrophages
NA MΦ	Non-activated macrophage control
n-BP	Nitrogen-containing bisphosphonate
NF-κB	Nuclear factor-kappa B
NLRP3	NLR family pyrin domain containing 3
NO	Nitric oxide
OS	Overall survival
pDC	Plasmacytoid dendritic cells
PD-1	Programmed cell death protein 1
PD-L1	Programmed death ligand 1
PD-L2	Programmed death ligand 2
PHD	Prolyl hydroxylase
PI3K	Phosphatidylinositol 3 kinase
PIK3Cα	Phosphatidylinositol 3 kinase catalytic subunit alpha
PRR	Pattern-recognising receptor
PTM	Post-translational modification
pVHL	Von Hippel-Lindau tumour suppressor
RAS	Rat sarcoma virus proteins
ROS	Reactive oxygen species
RT	Radiotherapy
sc-RNA-seq	Single cell-RNA-sequencing
SRE	Skeletal-related events
TAM	Tumour-associated macrophages
TIR	Toll/IL-1 receptor domain
TLR9	Toll-like receptor 9
TME	Tumour microenvironment
TNBC	Triple-negative breast cancer
TNF-α	Tumour necrosis factor-alpha

TP53	Tumour suppressor protein p53
UNC93B1	Uncoordinated 93 homolog B1
U.S. FDA	United States Food and Drug Administration
VEGF	Vascular endothelial growth factor
ZOL	Zoledronic acid

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Jouko Sandholm, Jaakko Lehtimäki, Tamiko Ishizu, Sadanandan E. Velu, Jeremy Clark, Pirkko Härkönen, Arja Jukkola-Vuorinen, Aleksi Schrey, Kevin W. Harris, Johanna M. Tuomela, Katri S. Selander. Toll-like receptor 9 expression is associated with breast cancer sensitivity to the growth inhibitory effects of bisphosphonates in vitro and in vivo. *Oncotarget*, 2016; 7(52): 87373-87389. doi.org/10.18632/oncotarget.13570.

II Tamiko Ishizu, Dominik Eichin, Artur Padzik, Sanni Tuominen, Reidar Grénman, Marko Salmi, Tove J. Grönroos, Johanna M. Tuomela. Head and neck squamous cell carcinoma cell lines have an immunomodulatory effect on macrophages independent of hypoxia and toll-like receptor 9. *BMC Cancer*, 2020; 21:990. doi.org/10.1186/s12885-021-08357-8.

The original publications have been reproduced with the permission of the copyright holders. In addition, the thesis contains unpublished data.

1 Introduction

The annoyance and discomfort of a small paper cut is well known to all. Fortunately, our immune system is always ready to heal minor wounds. As part of innate immunity, sentinel toll-like receptor 9 (TLR9) detects not only possible microbes but also various cellular stress signals caused by external damage. In consequence, it induces an inflammatory response to assist the adaptive immune system in addressing the danger.

While a tiny paper cut is not a long-term threat, persistent tissue injury and chronic inflammation are known risk factors for malignant transformation. Studies have linked dysregulated and heterogeneously expressed TLR9 with the pathophysiology of multiple cancers, including breast cancer and head and neck squamous cell carcinoma (HNSCC) (Droemann et al., 2005; Huang et al., 2005; Ilvesaro et al., 2008; Lai et al., 2019; Rosa et al., 2011; Tanaka et al., 2010). Intrinsic and extrinsic factors, such as a reduced molecular oxygen concentration termed hypoxia, viral infection by human papilloma virus (HPV), and molecules released by stressed and dying cells commonly burdening tumours, modulate tumoural TLR9 expression (Baruah et al., 2019; Sandholm et al., 2012; Tuomela et al., 2012). While research has indicated that the actions of TLR9 are impactful also outside the immune system, TLR9-induced changes in non-immune cells appears multifaceted, resulting in diverse and highly context-dependent outcomes. The challenge to understand more about TLR9's role in cancer has sparked great interest in the field. Despite years of investigation, researchers have not reached a clear agreement regarding the roles and actions of TLR9 expressed in non-immune malignant cells (i.e. tumour-originating TLR9), hence creating an obscure zone with more questions than answers.

To introduce the current knowledge concerning the relevance of TLR9 in cancer, the literature review begins by introducing themes tumour microenvironment (TME) and tumour immunity; conditions and features which are associated with aberrant expression of TLR9. It provides a synopsis of the current understanding of TLR9's association with the established hallmarks of cancer relevant to this thesis. Furthermore, the prospect of utilising bone resorption inhibitors, nitrogen-containing bisphosphonates (n-BPs), as anti-tumourigenic agents in soft tissue tumours is briefly discussed, before the essentials regarding breast cancer and HNSCC are presented.

This thesis focused on the prospect of tumour-originating TLR9 as a potential biomarker and target for anti-cancer therapy in HNSCC and breast cancer. The first

project (I) examined the roles of TLR9 and n-BPs in triple-negative breast cancer (TNBC). The second project (II) concentrated on determining whether soluble mediators produced by hypoxic HNSCC cancer cells with variable baseline expression of TLR9 altered macrophage polarisation. Finally, this thesis contains unpublished data characterising the effects of two TLR9-ligands on the HNSCC cell lines FaDu and TLR9-deficient FaDu ($FaDu^{TLR9def}$).

2 Review of the Literature

2.1 The Do-It-Yourself survival kit of a malignant cell

While 'liquid' cancers, such as leukaemia, circulate through the body via the vasculature, 'solid' tumours, including breast cancer and HNSCC, sprout as a mass of growing cells from a specific location. Therefore, the tissue of origin assigns the initial set of characteristics of the developing tumour. Several other shared perturbations among cancers further complicate the tumultuous nature of this disease. All these elements are set in motion during a process called tumourigenesis. This stepwise malignant transformation is more precisely characterised by the accumulation of disrupted gene expressions and progressive molecular perturbations. Extensive research has shown that once unlocked, malignancies utilise these perturbations to survive and even thrive. A widely accepted perspective by Hanahan and Weinberg conceptualises ten fundamental **hallmarks of cancer** (Fig. 1). Eight of them describe novel functions acquired during carcinogenesis: (i) *sustained proliferative signalling,* (ii) *evasion of growth suppressors*, (iii) *resisting cell death,* (iv) *inducing angiogenesis,* (v) *activating invasion and metastasis,* (vi) *enabling replicative immortality,* (vii) *avoiding immune destruction,* and (viii) *deregulating cellular energetics.* The remaining two characteristics, (ix) *tumour-promoting inflammation,* and (x) *genomic instability,* enable pathogenesis and survival of the tumour cells (Fig. 1) (Hanahan, 2022; Hanahan & Weinberg, 2011). Building on recent research, Hanahan has suggested four novel emerging hallmarks of cancer: (xi) *unlocking phenotypical plasticity*, (xii) *cellular senescence,* (xiii) *non-mutational epigenetic reprogramming,* and (xiv) *polymorphic microbiomes* (Hanahan, 2022). The first two are attributed to neoplastic progenitor cells that are either recruited into the tumour or transformed from differentiated tumour progenitor cells, adding new dimensions to intratumoural heterogeneity (ITH). While the areas of epigenetics and microbiota have only recently come into focus, compiled data have shown that they play an unequivocal role in tumourigenesis.

Thus, describing cancer as a uniform disease is oversimplified. A tumour typically uses more than one of the abovementioned hallmarks for its survival, and these patterns vary depending on factors, such as mutational landscape and sex. In addition, strong evolutionary pressure from the TME, the immune system and therapeutic interventions all sculpt tumours, generating subpopulations with different

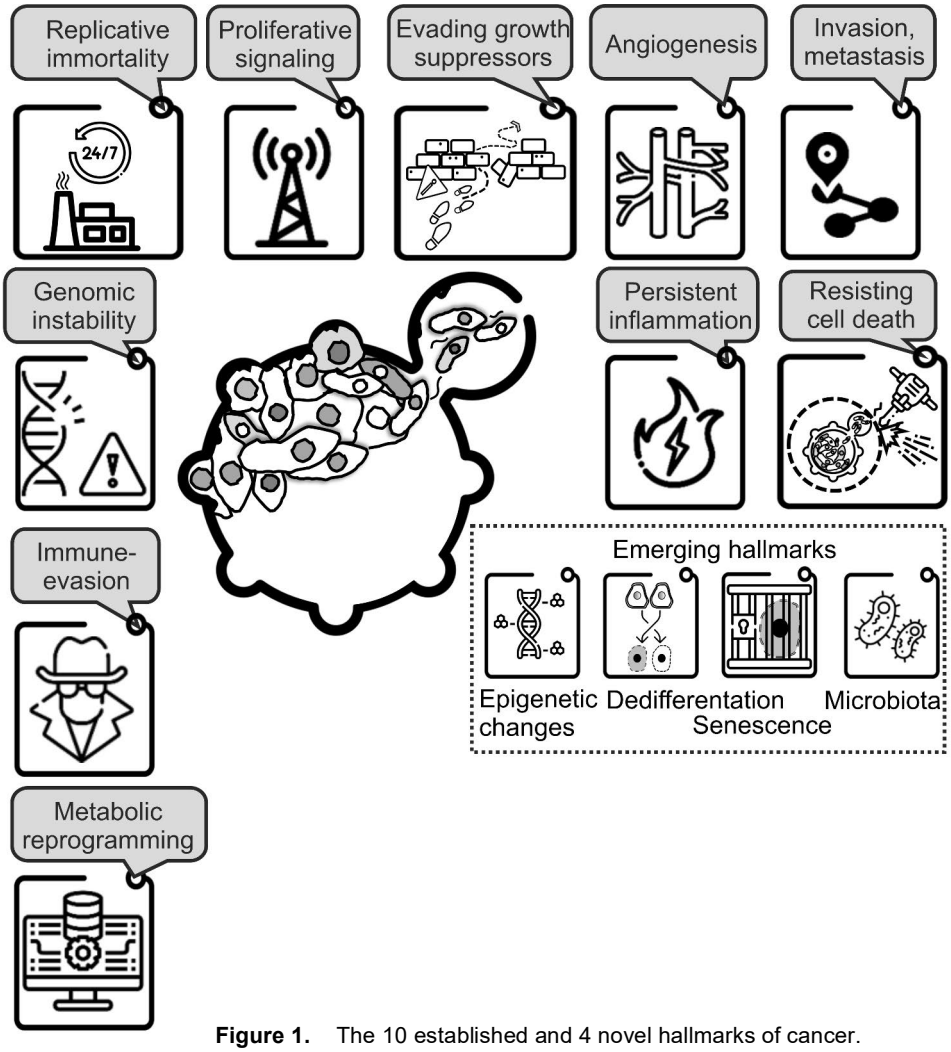

Figure 1. The 10 established and 4 novel hallmarks of cancer.

characteristics. When challenged, these clonally variable populations respond to a signal to varying degrees or use a disparate set of hallmarks, creating the basis for ITH, a bona fide state in multiple tumours (Hlubek et al., 2007; Lawrence et al., 2013; Vitale et al., 2021). ITH is also important in the context of tumour-infiltrating immune cells, including tumour associated macrophages (TAMs) and their immune responses, as single-cell sequencing has revealed that the heterogeneous tumour ecosystem unbalances the cellular architecture (Bindea et al., 2013; Zou et al., 2022).

Moreover, the complexities defining the disease are not restricted only to ITH but extend further to tumour interheterogeneity (Lawrence et al., 2013). Modern analysis methods like single-cell transcriptome analysis (sc-RNA-seq) have revealed thrilling new insights into the molecular landscape in the context of intertumoural

heterogeneity. For example, Wu et al. applied sc-RNA-seq to stratify a large breast cancer cohort into nine new molecularly defined subcategories termed ‘ecotypes’ with each characterised by unique cellular compositions and clinical outcomes (Wu et al., 2021).

A novel perspective attested by a sizable volume of the literature suggests, that toll-like receptors of innate immunity, such as TLR9, network with and contribute to numerous pathways and cellular functions fundamental to the hallmarks of cancer thereby adding nuances to the already erratic nature of solid tumours (Appel et al., 2005; Cai et al., 2014; De Dios et al., 2020; Lim et al., 2016; Liu et al., 2020). Although intuition would associate TLR9 with hallmarks such as tumour-promoting inflammation and polymorphic microbiomes, recent research has revealed novel ‘molecular crossings’ where TLR9 and previously unrelated cellular functions coincide and interact either directly or indirectly to dysregulate tumourigenesis. Besides introducing its biological function and relevance in healthy tissue, the following chapters will concentrate on TLR9 in the context of the TME and anti-tumour defence, delineate novel findings regarding molecular cooperation among TLR9 and hallmarks of cancer and describe why this receptor has raised interest as a potential target for multiple clinical trials over the years.

2.2 The TME manipulates the ecosystem-wide dialogue

Besides the mosaic characteristics caused by ITH, the microenvironmental tumour ‘ecosystem’ comprises versatile factors that contribute to their fate. These include, e.g. hypoxia, influx of stromal and immune cells, biochemical signalling molecules, and variable elements from the extracellular matrix (Fig. 2). In practice, the multilateral interactions between tumour and peritumoural stroma co-create a strong physiological ‘mashup’ that shapes cancer progression and even patient prognosis. This dynamic composition of the TME often evolves with the disease over time, subsequently contributing to events such as drug resistance and metastasis (Quail & Joyce, 2013).

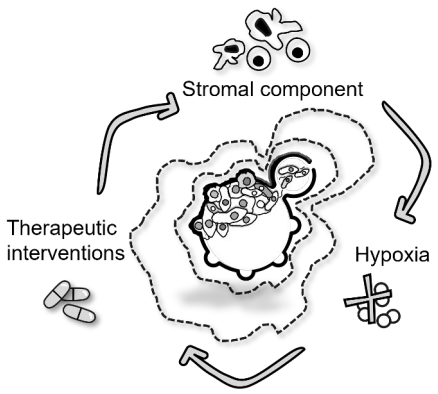

Figure 2. The evolution of solid HNSCC and BC tumour phenotypes ensues external factors rising from TME, such as hypoxia, immune cells, and variable treatment modalities.

Building on this understanding of the TME, the role of tumour-originating TLR9 in this dynamic environment warrants further exploration. Some earlier studies

have demonstrated functional ramifications of reduced tumour-originating TLR9 expression in stromal components (Tuomela et al., 2012), while others have suggested a causal relationship between TLR9 expression and hypoxia (Sandholm et al., 2014). Since hypoxia is a very common feature in tumours, its association and consequences influencing TLR9 signalling are introduced further in the following section. Understanding how TLR9 interacts with its surroundings is pivotal, as it may assist in developing novel therapeutic strategies to treat cancer.

2.2.1 The challenge of hypoxia: the survival of the fittest

A short-term lack of molecular oxygen is a normal physiological phenomenon during, e.g. embryonic development, wound healing, myeloid cell biology, and epithelial barrier function. In contrast, tumours often experience significant consequences due to chronic hypoxia (Cramer et al., 2003). In general, the haem-bound O_2 diffuses from erythrocytes into the tissues according to the O_2 concentration gradient (Fig. 3a). While individual organs usually maintain variable tissue O_2 levels (Fig. 3b), researchers have reported that on average, O_2 concentration is around 6 % in peripheral tissues under normoxia (Carreau et al., 2011; McKeown, 2014). Tissue O_2 homeostasis is regulated in a precise manner by several factors. They include Von-Hippel Lindau protein (pVHL), hypoxia-inducible transcription factor 1 (HIF-1) and prolyl hydroxylase domain-containing enzymes (PHD) in the HIF regulatory pathway. Under normoxia, pVHL recognises and binds the PHD-hydroxylated HIF-1, subsequently labelling the generated protein multicomplex for degradation. Yet, inside a hypoxic tumour, unhydroxylated HIF-1 escapes recognition by pVHL and enters the nucleus (Jaakkola et al., 2001; Marxsen et al., 2004). In the nucleus, HIF-1-triggered expression of the hypoxia-responsive element (HRE)-containing genes produces proteins that generate, e.g. inflammatory mediators such as reactive oxygen species (ROS), nitrogen species (NO), and inflammatory cytokines (Denko et al., 2003). These, in turn, activate adaptive immunity and generate both local and systemic responses (Dehne & Brüne, 2009). In addition, various physiological cues, such as cytokines, TLR-ligands, NO and hormones, can stabilise HIF-1 dimers independent of hypoxia (Cramer et al., 2003; Jantsch et al., 2011; Nizet & Johnson, 2009; Sandau et al., 2001; Zhou et al., 2003).

As it happens, current insights into HIF-1-induced biological outcomes suggest that HRE genes play distinct roles under normoxia and hypoxia (Nizet & Johnson, 2009). A chaotic tumour infrastructure responsible for hypoxia is typically based on poor vasculature and uncontrolled cell growth. As a hypoxic gradient exacerbates closer to the tumour core, it stimulates both pro-angiogenic and pro-inflammatory factors and causes significant alterations affecting cell metabolism, stemness and survival (Carreau et al., 2011; Denko, 2008). In addition, hypoxia promotes gene instability and generates resistance to various treatment modalities (Flamant et al.,

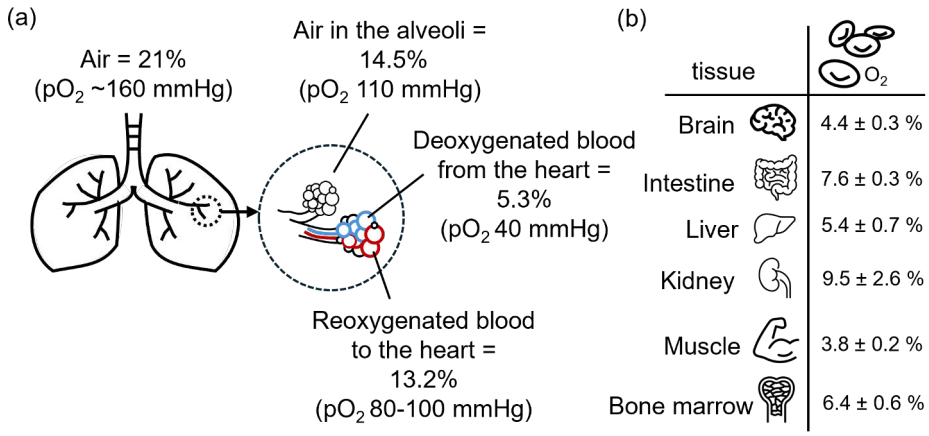

Figure 3. The median partial pressure of O_2 (pO_2) in (a) alveolar tissues and (b) in periphery. Fig. 3 inspired by McKeown *et al.* (2014), Carreau et al. (2011).

2010; Rice et al., 1986; Thienpont et al., 2016). This means that the clonal cells that adapt to chronic hypoxia will gain an advantage over the rest of the population, thereby increasing ITH.

Despite its ramifications, cells have mechanisms to withstand hypoxia. As an acute response to a limited amount of nutrients and a lack of O_2, neoplastic cells typically arrest their cell cycle. If hypoxia persists, cells activate extra cytoprotective measures to either adapt, enter quiescence, or die by apoptosis or necrosis. Cells on the path to adapt to the changed microenvironment produce a wide variety of bioactive and immunoregulatory molecules, such as type I interferons (IFN), growth factors, and adenosine triphosphate (ATP), to support their survival and promote local immunosuppression. In contrast, dying, injured, and stressed cancer cells release damage-associated molecular patterns (DAMPs) and soluble mediators that attract circulating myeloid cells, such as neutrophils, natural killer cells, and monocytes, to the site (Mantovani & Sica, 2010).

Interestingly, research has found that increased TLR9 expression coincides with HIF-1 stabilisation, positioning it at the crossroads of cellular hypoxia and inflammatory signalling. While some investigations have concluded that HIF-1 stabilisation precedes the increased TLR9 expression (Sandholm et al., 2014; Tuomela et al., 2012), others have reported that HIF-1 is stabilised post-TLR9 activation (Liu et al., 2015; Zinkernagel et al., 2012). In addition, Rius et al. have indicated that mice deficient in IκB kinase beta (IKKβ) downstream of TLR9 were unable to effectively upregulate HRE-target genes, revealing new avenues into the molecular mechanisms between HIF-1 and TLR9 (Rius et al., 2008). However, taken as a whole, these perceived inconsistencies highlight that there is still a large degree of ambiguity in the causal relationship between HIF-1 and TLR9 expression.

2.2.2 Stroma: a society of cells endeavouring in the turbulent frontier

Various cell populations, the extracellular matrix and a cocktail of paracrine signalling molecules surround a solid tumour. The prevailing architects in the tumour stroma are the abundant and heterogeneous populations of cancer-associated fibroblasts, joined by immune cells, mesenchymal stromal cells, adipocytes, pericytes and endothelial cells. In the healthy stroma, integrin-mediated anchoring of the epithelial and mesenchymal cells to the extracellular matrix maintains epithelial polarity and prevents tumour initiation. Abundant evidence has, however, demonstrated that altered stromal-epithelial interactions are essential for tumour initiation and progression (Costa et al., 2018; Hanahan, 2022; Kuperwasser et al., 2004; Moinfar et al., 2000). Indeed, several investigations have contemplated that a tumour-stroma ratio could be a useful prognostic biomarker alongside the tumour-node-metastasis classification already applied to several cancers in patient care (Almangush et al., 2021; Hagenaars et al., 2022). Moreover, the evaluation of tumour-associated immune cells using novel classification parameters, such as immunoscore, immune contexture or immunophenoscore, has emerged as a valuable tool for improving patient prognosis and tumour eradication (Charoentong et al., 2017).

The current understanding of cancer progression highlights the importance of taking the TME-originating pro-tumourigenic factors into account when striving for complete tumour elimination. For example, many clinical trials have reported improved patient responses to combinatorial chemo- and radiotherapy (RT) treatments with short cytidine-guanosine-rich oligonucleotides (CpG-ODNs) acting as TLR9 agonists (Burn et al., 2020; Younes et al., 2021). This is because the overall efficacy and improved patient responses are contingent not only on hypoxia but also on immune components like antigen-presenting cells (APCs), altering the immunosuppressive TME. While most of the in vivo evidence obtained after intratumoural injections (IT) of the CpG-ODNs suggests that the abundant tumour-associated APCs may be the primary responders to these TLR9-agonists, a growing amount of data has indicated that the actions are not solely restricted to this cell type but can also be mediated by neutrophils and cytotoxic CD8+ T lymphocytes (Humbert et al., 2018; Sagiv-Barfi et al., 2018).

Much of the knowledge about TLR9's influence on the TME is based on the adjuvant effects of CpG-ODNs interacting with the immune cells. Additionally, aberrantly expressed tumour-originating TLR9 has been shown to modify the expression of certain matrix-degrading metalloproteinases in prostate and breast cancer cells (Merrell et al., 2006; Tuomela et al., 2012). Such alterations can catalyse serious consequences as these enzymes can support tumour cell invasion in the peritumoural space. Apart from extracellular stimuli, recent investigations have suggested that the tumour-originating mitochondrial DNA can induce TLR9-mediated

nuclear factor-kappa B (NF-κB)-signalling and complement C3 maturation (Haldar et al., 2020). This paracrine tumour-stroma crosstalk has been reported to activate cancer-associated fibroblasts, enhance drug resistance and result in prostate cancer progression. A similar interaction with free circulating DNA has been hypothesised to be the cause of de novo resistance in B-cell malignancies. Indeed, Jayappa et al. have verified that treatment with CpG-ODN together with IL-10 and CD40L generated near complete resistance to combinatory treatment with inhibitors of B cell receptor-signalling and Bcl-2, specifically ibrutinib and venetoclax (Jayappa et al., 2017). Interestingly, this phenotypic agonist-induced drug resistance differed from the traditional drug resistance arising from mutations and was largely induced by the alternative NF-κB signalling.

2.3 Innate vs. adaptive immunity

Histological analyses of human tumours consistently reveal that infiltrating immune cells, albeit heterogeneous in both number and type, are regularly present in the TME. Variability in infiltrates partly stems from the immune system's dual nature. In compliance with its speed and specificity, our immune system is separated into innate and adaptive arms (Fig. 4). This distinction arises out of distinguishable cell populations: while the patrolling phagocytic immune cells, such as monocyte-derived macrophages (MΦ), dendritic cells (DC), neutrophils, and natural killer cells, are the swift actuators of the innate immunity, the responses by the adaptive immunity are gradual and largely carried out by the subsets of T and B lymphocytes (Mantovani et al., 2008). In contrast to the rapid response of innate immunity, the actions of the adaptive immune system are more complex and prolonged, focusing on educating lymphocytes to recognise specific antigens and generate a long-lasting immunological memory. Many clinical trials attempting to improve tumour eradication are prospecting to rebalance the typically dysregulated innate and adaptive immune cell populations in tumours. These pioneering efforts to unravel the intricate crosstalk and interdependencies among various factors in the TME pave the way for novel immunotherapies.

2.3.1 The attractiveness of the tumour is reflected by the tumour immune landscape

From cancer's perspective, its initiation, existence, and even death would optimally occur without being detected by patrolling immune cells. To achieve this, malignant cells harbour adaptive changes in the proteome, initiate persuasive immunosuppressive crosstalk with stromal components and opt for a type of cell death that creates an immunologically mild and tolerogenic imprint. Consequently, APCs responsible for $CD4^+$ and $CD8^+$ T cell activation remain insufficiently primed, which

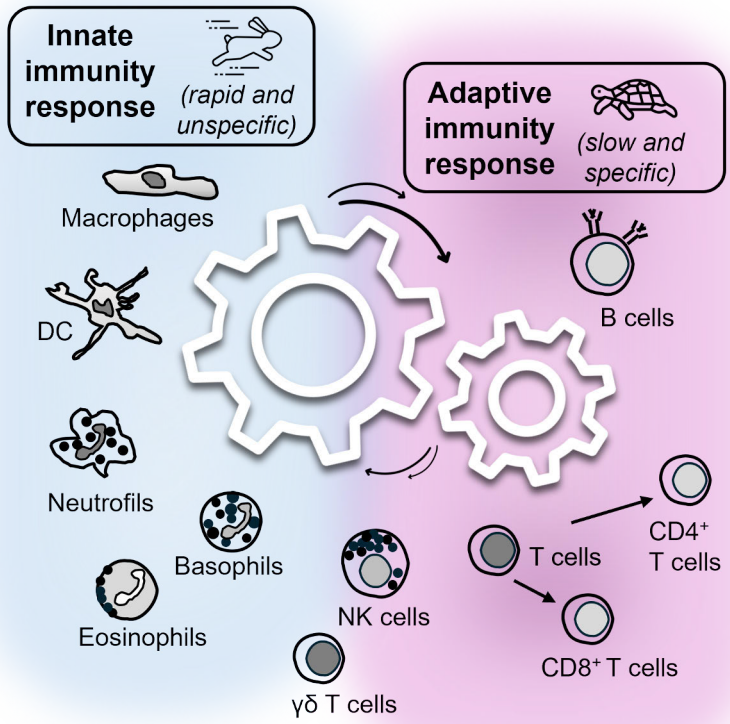

Figure 4. The bilateral immune system is composed of both rapid and slow responses. When recognizing danger, the cells of innate immunity, including monocyte-derived macrophages (MΦ), dendritic cells (DC), neutrophils, basophils, and eosinophils react quickly to resolve the harmful situation. If the danger persists, the T- and B-cells of the adaptive immune system get primed by the innate immunity response, thereby developing a fully matured immune response. Unlike innate immunity, which treats every threat as novel, adaptive immunity develops an "immunological memory" which boosts and refines the specificity of actions. Interestingly, the natural killer cells and regulatory γδ T cells exhibit properties from both systems.

markedly limits anti-tumour activation of adaptive immunity (Garg & Agostinis, 2017). Several factors, including stress-related small molecules, an immunosuppressive TME, neoantigens, hypoxia, treatment modalities, and mode of death (apoptosis/necrosis), generate variable tolerogenic, inflammatory or immunogenic signals subsequently defining tumour immunophenotype (Casares et al., 2005; Garg & Agostinis, 2017; Limagne et al., 2022). A tumour with high immunogenicity possesses an appropriate combination of these parameters and emerges as 'hot', consequently attracting a high number of immune cells. At the other

end of the spectrum are the ‘cold’, less immunogenic tumours with largely restoring, diminutive or absent immune cell infiltration.

It is well established that the quality and quantity of the immune cell influx correlate with patient prognosis. In general, high densities of cell populations such as myeloid-derived suppressor cells, regulatory T cells and anti-inflammatory type 2 (M2) MΦ correlate with poor patient prognosis (Hou et al., 2014; Shiao et al., 2015). In contrast, tumours harbouring cytolytic $CD8^+$ T cells have been associated with increased patient survival in many cancers (Mella et al., 2015; Tumeh et al., 2014; Zhang et al., 2022). Therefore, clinical studies have explored ways to expose tumours to the immune system by reversing the immunosuppressive TME and enhancing the adaptive immune response. The pharmaceutical revolution initiated by the first United States Food and Drug Administration (U.S. FDA)-approved T cell checkpoint inhibitors ipilimumab targeting CTLA-4 and nivolumab targeting PD-1 marked a new era in the field of targeted immunotherapy. The success of immunotherapies derives from their characteristics, including specific anti-tumourigenic actions, less intense cytotoxic side effects, and durable survival benefits for patients, in contrast to traditional and nonspecific cancer treatments that inevitably cause collateral damage within healthy tissues. Today, the list of U.S. FDA-approved combined immunotherapies is over 40 drugs long, and they have a steady foothold besides the traditional arsenal of therapies, including surgery, cytotoxic chemotherapy, RT, and targeted therapy (Subbiah et al., 2022).

So far, preclinical studies of TLR9 agonists have shown promising anti-tumour effects in several types of cancer (Damiano et al., 2009; Sato-Kaneko et al., 2017; Yu et al., 2025). TLR9 has been implicated to support the production of pro-tumourigenic factors and repress the expression of co-stimulatory molecules, subsequently impacting e.g. immune cell maturation (Huang et al., 2005). In murine models, IT injections of CpG-ODN and chemokine ligand 20 boosted $CD8^+$ T cell maturation and resulted in significant tumour regression and therapeutic immunity against melanoma and colon xenografts (Furumoto et al., 2004). This shows that TLR9 agonists may effectively convert ‘cold’ tumours to become more immunogenic, especially when used in combination with other treatments. This cumulative effect was also demonstrated in the phase I study testing PD-1 inhibitor pembrolizumab together with a TLR9 agonist. Here, combinatorial treatment boosted a broad immune activation and demonstrated an improved immunogenicity with an increased influx of lymphocytes in the TME (Ribas et al., 2018). In another study, IT administration of TLR9 agonists induced a favourable shift from immunosuppressive stroma to an influx of reactive $CD8^+$ T cells and sensitised tumours to anti-CTLA-4 and anti-PD-1 blockage (Reilley et al., 2019). Without activation by its ligand, however, TLR9 expression appears to be an independent factor in terms of $CD8^+$ T cell infiltrates in naïve TNBC and renal cell carcinoma patient cohorts (Mella et al., 2015).

2.3.1.1 TAMs as influencers in tumours

The current leading theory denotes that anti-tumourigenic actions of TLR9 agonists in tumours are mediated by immune cells. The monocyte-derived MΦ are a probable target population in this response, as they are typically abundant in the TME and have abundant TLR9 expression. A substantial body of evidence has linked TAMs with both pro- and anti-tumourigenic characteristics such as angiogenesis, remodelling of extracellular matrix, and immunosuppression (Mantovani et al., 2022). In contrast to the unstimulated MΦs, which maintain the homeostatic clearance of cell debris and invading pathogens, TAMs are typically exposed to abnormal tissue hypoxia, a chemical gradient of soluble mediators, as well as intense cell-to-cell engagements in the peritumoural area. These harsh and intense interactions make them susceptible to physiological reprogramming. Along with a cocktail of DAMPs released by cell stress and necrotic cell death, the disproportionately inflammatory TME subsequently sculpts the phenotype, or polarisation, of tumour-infiltrating MΦs. This proceeding concerns both the monocyte-derived MΦ and the tissue-resident MΦ originating from embryonic precursors.

MΦ polarisation is an important feature not only in terms of tumour eradication but also in terms of tumour immune evasion. While phagocytic MΦs are vital for co-stimulatory T and B cell activation and anti-tumour immunity, their compliant and versatile phenotype contingent on the TME-derived stimuli can dexterously become pro-tumourigenic during carcinogenesis (Mantovani et al. 2022). In general, MΦ polarisation fluctuates unrestrictedly from inflammatory, anti-microbial and tumouricidal M1 MΦ (referred to as a classic activation), to anti-inflammatory, scavenging and tissue-repairing M2 MΦ (referred to as an alternative activation (Cassetta et al., 2019; Shu et al., 2020). The M2 phenotype, which consists of several subcategories (M2a, M2b, M2c, M2d) with distinct cytokine profiles and variable roles in resolving tissue injury, partly explains why TAMs are typically unpredictable partners in the fight against cancer (Mantovani et al., 2022).

M1 MΦ with pro-inflammatory responses and M2 MΦ linked to anti-inflammatory processes generate a complex balance in tumour immune regulation. Inflammatory and phagocytic M1 MΦ support the Th_1 response by producing pro-inflammatory cytokines, such as interleukin-1 beta (IL-1β), tumour necrosis factor-alpha (TNF-α), and IL-12. They also express proteins important for antigen presentation and T cell co-stimulation, including CD80, CD86, MHC II, and inducible nitric oxide synthase (iNOS) (Dehne et al., 2017). In contrast, non-opsonic markers, such as CD163, CD200R, CD206, and programmed death ligand 1 (PD-L1), combined with anti-inflammatory cytokines, such as IL-4 and IL-10, are linked with the regulatory M2 MΦ phenotype. Moreover, paracrine exposure to inflammatory factors, such as IFN-γ, TNF-α or DAMPs, polarise MΦs towards M1, providing anti-tumoural stimuli, whereas cytokines, including IL-4, IL-10, M-CSF, and GM-CSF,

induce an alternative and immunosuppressive M2 MΦ phenotype. Besides soluble mediators, other factors such as HIF-1 stabilisation, tumour acidosis, interaction with tumour-originating integrins, as well as immunogenic cell death-inducing therapies, modify MΦ polarisation (Shu et al., 2020).

However, the physiological context in which MΦ activation occurs cannot be overlooked. The study by Celhar et al. fittingly underscores this, as their study found that activation of MΦ by specific DAMPs, such as TLR7 and TLR9 ligands, caused immunogenic tolerance, not an inflammatory response, possibly to prevent autoimmunity (Celhar et al., 2016). On the other hand, although M1 MΦ are generally considered anti-tumorigenic, the secreted inflammatory factors, when persistent, have been shown to generate adverse side-effects and promote invasive tumour phenotype via the NF-κB pathway (Kainulainen 2022). Therefore, given their rigorous regulatory roles, intratumoural abundance, and ability to repolarise, it is vital to consider the physiological spectrum of MΦ when developing anti-cancer therapies.

2.3.2 Non-mutational, yet tumour-promoting inflammation boosts oncogenesis and facilitates tumour immune evasion

Paradoxically, the very mechanism which intends to save us from cancer may end up exacerbating the situation. The association between inflammation and cancer was postulated by many researchers already a century ago, but the hypothesis was not verified until Martins-Green and colleagues reported some decades ago that persistent inflammation caused by the Rous sarcoma virus induced in situ tumours; an outcome, which was negated by administration of an anti-inflammatory drug (Martins-Green et al., 1994). Hereafter, multiple factors in the contemporary environment, such as inhaling tobacco smoke or other particles/impurities, *Helicobacter pylori* infection, and obesity, have been shown to contribute to carcinogenesis via unresolved low-level inflammation (Qiang M., 2020; Varon et al., 2022; Harris et al., 2022). While normal acute inflammation is self-limiting, *tumour-promoting inflammation* does not resolve in a dysregulated environment; in fact, tumours could be portrayed as wounds that never heal (MacCarthy-Morrogh & Martin, 2020). The crosstalk between tumour, mesenchymal and immune cells accelerates oncogenesis by persistently supplying the TME with growth factors, inflammatory cytokines, proangiogenic molecules, atypical adhesion molecules and matrix-modifying and stress-related enzymes, promoting inflammation further. Such a convoluted environment even impairs anticancer treatments (Hanahan & Weinberg, 2011; Kim et al., 2015; Shiao et al., 2015).

NF-κB-associated activation of anti-apoptotic and hypoxia-responsive genes is positioned at the epicentre of the innate and adaptive immune systems operating in the TME (Rius et al., 2008). As TLR9 activity associates with signalling pathways such as NF-κB and mitogen-activated protein kinase (MAPK), it promotes an

inflammatory milieu and influences the expression of cytokines, including IL-1β, IL-6, IL-12, IL-23, TNF-α, and MCP-1, in multiple autoimmune and cancer models (Krug et al., 2001; Li et al., 2017; Tang et al., 2022).

Interestingly, some reports have suggested that TLR9 polymorphism can worsen NF-κB-mediated inflammation; for example, the single-nucleotide polymorphism rs5743836 has been associated with premalignant changes in the gastric mucosa following *H. Pylori* infection (Ng et al., 2010). Moreover, defects in TLR9 function have been associated with inadequate type I IFN production, particularly by plasmacytoid dendritic cells (pDC) and B-cells (Cunningham-Rundles et al., 2006; Escobar et al., 2010).

2.4 Identifying the molecular motifs by the pattern-recognising receptor families is an ancient defence system

Much is known about how innate and adaptive immunity intercepts, interacts and even interlopes with abnormal cells. The innate anti-tumour defence is primarily based on the recognition of various molecular patterns, including 'self to non-self' and 'missing self' to 'altered self' tumour-associated antigens (Garg & Agostinis, 2017). In addition, alarmins, collectively called DAMPs, promptly amplify the inflammatory response. The array of variable endogenously derived danger signals consists of, e.g. molecules of cellular damage and degradation products, and soluble mediators secreted by the immune and non-immune cells (Fig. 5).

One crucial aspect of the innate immune response involves receptors that detect cellular stress, such as TLRs. TLRs are gatekeepers expressed throughout the body on innate and adaptive immune cells as well as on non-immunological cells, such as adipocytes, myocytes, fibroblasts, epithelial, cervical and cancer cells. The role of TLRs as gatekeepers and coordinators at the crossroads of innate and adaptive immunity is well-recognised today. However, the history of human toll-like receptors is relatively short as their identification within our genome occurred less than 30 years ago (Gunn, 1996). Before the discovery of TLRs, immunologists considered that the actions of innate immunity were needed primarily to prime and boost adaptive immunity. Since then, research has shown that innate immunity not only primes the adaptive arm of immunity but also takes part in resolving the threat and supports healing by, e.g. producing chemokines and cytokines, such as IL-1, IL-6, type I IFN and TNF-α (Mantovani et al., 2008).

TLRs are part of the evolutionarily conserved pattern-recognition receptor (PRR) family with several roles as managers of tissue homeostasis. These vigilant sentinel receptors not only detect microbial-associated molecular patterns (MAMPs, previously known as pathogen-associated molecular patterns) and toxins but can also be triggered by DAMPs, including molecules such as self-DNA or RNA, heat-shock

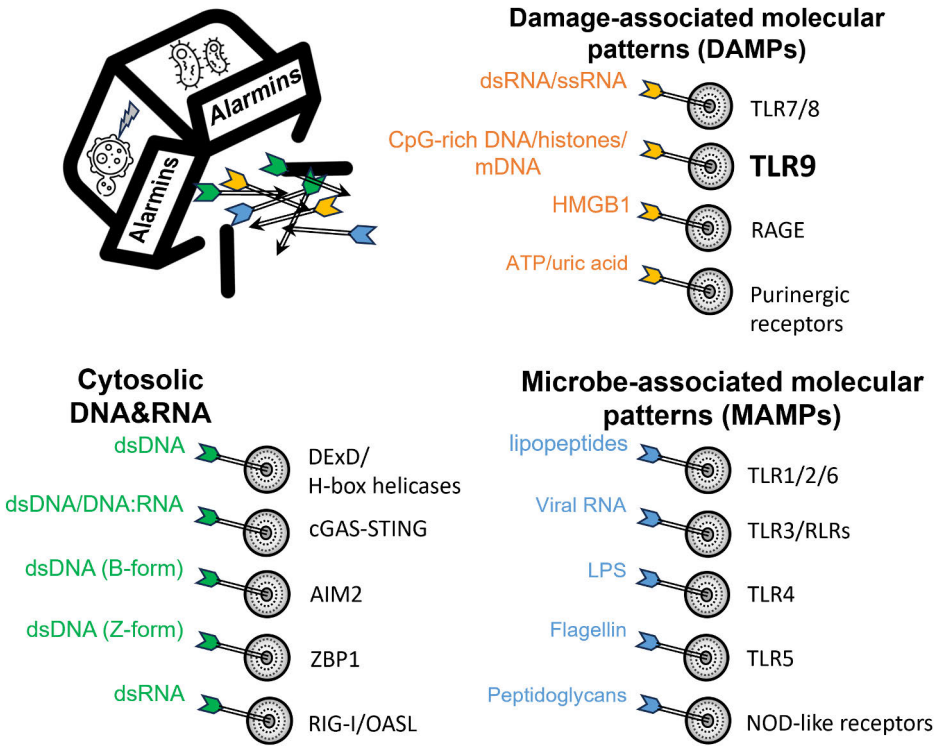

Figure 5. Various danger signals recognised by innate immunity PRRs.

proteins, extracellular ATP, and breakdown products of the glycosaminoglycans in the plasma membrane or extracellular matrix. The type of PRR ligand influences how the immune system responds: it may elicit a defensive response against foreign pathogens, a reaction to internal stress signals, or initiate healing to restore tissue homeostasis (Garg & Agostinis, 2017; Matzinger, 2002). MAMPs and DAMPs act as ligands for several PRRs, such as TLRs, C-type lectin receptors, NOD-like receptors, Rig-I-like receptors, AIM2-like receptors, and oligoadenylate synthase-like receptors (Garg & Agostinis, 2017; Takeda & Akira, 2005). Typically, activation of a PRR triggers the transcription factor NF-κB or interferon regulatory factor 3 or 7, generating either proinflammatory cytokines or type I IFNs, respectively. However, distinct PRRs share some internal signalling pathways and communicate and work dynamically in concert; hence, the outcome of the activation of one PRR is often pleiotropic (Takeuchi & Akira, 2010).

2.4.1 No trespassing: surveillance by the Toll-like receptor family keeps you healthy and maintains tissue homeostasis

Following the discovery of the Toll receptor's vital role in resisting infections in *Drosophila*, researchers have identified orthologs in various species. Owing to their similarity to the *Drosophila* Toll, these receptors have been named Toll-like receptors. In humans, TLRs located on the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10) detect bacterial, viral, and fungal MAMPs, such as tri- and diacetylated lipoproteins, viral envelope glycoproteins, and bacterial flagellin, whereas intracellular receptors (TLR3, TLR7, TLR8 and TLR9) in the endosomal compartment are responsible for pattern-recognition of double-stranded RNA, single-stranded RNA, and single-stranded DNA as well as other cyclic compounds, respectively.

TLRs from 1 to 9 are conserved between humans and mice, whereas TLRs from 10 to 13 have species-specific features. Although not much is known about the human orphan receptor TLR10, studies have suggested that it mainly antagonises TLR2-mediated inflammatory responses (Oosting et al., 2014). Moreover, while human TLR11 is without a known function, TLR11 in mice has been shown to play a specific role in fighting against uropathogenic bacteria (Takeda & Akira, 2005). The least studied murine TLR12 and TLR13 have also been linked with specific threats, such as recognition of *Leishmania* or a conserved bacterial 23S ribosomal RNA sequence (Oldenburg et al., 2012; Shukla et al., 2018). Although comparative genomic analysis has revealed more ancient family members (TLR21, TLR22, TLR23) in other vertebrates, including birds, amphibians, and fish, these are only pseudogenes in humans (Roach et al., 2005).

2.4.2 Self-DNA or foreign-DNA, that is the question

Since the molecular structure of DNA and RNA is universal throughout biology, it is crucial to distinguish the ‘self’ from the foreign. Multiple intrinsic factors responsible for ligand-receptor interactions have been identified, suggesting that structural features like DNA methylation could provide selectivity between self- and foreign-DNA (Kandimalla et al., 2005). However, TLR9 can bind DNA regardless of factors such as the DNA backbone or post-translational modifications (PTMs). This broad binding capacity of TLR9 not only contributes to autoimmune diseases but also raises questions about the evolutionary role of TLRs in responding to injury (de Jong et al., 2010; Kandimalla et al., 2005; Noack et al., 2012). To limit unintended activation by self-nucleotides, cells have evolved several physical and biochemical adaptations. These include enhancing the degradation of free nucleic acids, shielding them by nucleocapsid proteins, compartmentalising their recognition into the intracellular

endosomal compartment, and improving ligand-binding in acidic conditions (Cao, 2016; Takeuchi & Akira, 2010).

Another intriguing perspective examines the role of TLRs as protectors against danger. Researchers have suggested that TLRs, with their strong affinity for DAMPs, may have evolved to sense tissue injury cues, in addition to hunting down microbial patterns (Matzinger, 2002). It is plausible that living organisms, whether a plant, a fruit fly, or a mammal, would prioritise general cell stress and injury-related alarmins over signals originating specifically from a foreign source. Some scientists consider that such co-evolution could additionally have granted microbes an advantage to exploit these receptors for their survival (Garg & Agostinis, 2017).

Research has also highlighted the vital role PRRs have as regulators of tissue homeostasis. Although PRR-related signalling is, in general, considered inflammatory, commensal bacteria induce immunogenic tolerance through TLRs. Studies on intestinal diseases have shown that concomitant exposure to TLR-binding ligands fully rescued commensal-depleted mice from severe dextran sulfate sodium-induced colitis and morbidity and restored tissue homeostasis by downregulating pro-inflammatory cytokine production from intestinal DCs in vitro and in vivo, respectively (Owyang et al., 2012; Rakoff-Nahoum et al., 2004). Since at least TLR2, TLR4, TLR5, and TLR9 have been associated with anti-inflammatory roles in the gut, this suggests that TLRs play a significant role beyond targeting pathogenic invaders.

2.4.2.1 TLR9 recognises single-stranded CpG-rich DNA

Many molecular determinants, such as the length of the oligonucleotide and the sequence and location of CpG motifs, contribute to the accuracy of ligand recognition and consequential changes in cell physiology by TLR9 (Kandimalla et al., 2003; Vollmer et al., 2004; Yasuda et al., 2006). Regardless of whether the ligand is a MAMP or an immune-complexed DAMP, it must first be degraded to single-stranded DNA (Sinha et al., 2016). While natural DNA is short-lived inside endolysosomes, the synthetic CpG-ODNs resist nuclease degradation as they contain modifications such as variable sugar and base moieties, phosphorothioate backbone, and secondary or tertiary structures. The resulting longer half-life makes them suitable for studies investigating TLR9-mediated responses. Based on their different structure-related immunostimulatory effects, CpG-ODNs are categorised into three main classes: CpG-A ODNs, which promote natural killer cells and IFN-α production, CpG-B ODNs, which activate B-cells and boost proinflammatory Th1-response, and CpG-C ODNs, which have the combined characteristics of both CpG-A and -B ODNs (Abel et al., 2005; Kandimalla et al., 2005; Vollmer & Krieg, 2009).

TLR9 signalling is tightly controlled to prevent extensive tissue damage. This control occurs at multiple parallel levels during an immunological response. Besides the positive feedback loop, which mediates a temporary blockage of the transcription

from the TLR9 promoter by the complex p65/HDAC3, the endolysosomal proteolytic N-terminal fragment has been shown to bind the full-length receptor, thereby inhibiting TLR9-signalling (Sinha et al., 2016; Takeuchi & Akira, 2010; Zannetti et al., 2014). In addition, several transcription factors, such as CREB1, C/EBP and Ets families, and Elk1 have been reported to suppress TLR9 promoter activity following CpG-ODN treatment (Takeshita et al., 2004). Internalisation of CpG-ODN induces upregulation of inflammatory factors, including cyclooxygenase 2, NO synthase, indoleamine 2,3-deoxygenase, prostaglandin-2, NO, and IL-10, in murine DCs. It also activates parallel pathways like STAT3 to restore homeostasis (Leleux et al., 2017). Moreover, multiple intracellular proteins, such as IL-1 receptor-associated kinase-M (IRAK-M), mitogen-activated protein kinase phosphatase, and rat sarcoma virus (RAS)-related protein Rab7b, downregulate TLR9 signalling (Vollmer & Krieg, 2009; Yao et al., 2009).

2.4.3 TLR9 activates the NF-κB pathway

Regardless of their cellular localisation, TLRs share a common molecular structure. They pass through the membrane once, making them type I transmembrane proteins, with an N-terminal ligand-binding motif consisting of leucine-rich repeats (LRRs) and a C-terminal signalling domain known as Toll/IL-1 receptor domain (TIR). TIR enables activation of the same signalling cascade that is used by the IL-1 receptor, and depending on the TLR, it recruits the appropriate adaptor proteins for downstream signalling (Takeda & Akira, 2005).

The activation of TLR9 results in the production of antimicrobial proteins, proinflammatory cytokines or type I IFNs contingent on the cell type and context (Takeuchi & Akira, 2010). Figure 6 depicts the typical activation route as well as the main cofactors needed for TLR9-mediated NF-κB activation and proinflammatory cytokine production. In a resting state, an inactive unprocessed TLR9 (fl-TLR9) dimer is stored in the endoplasmic reticulum (ER) (Fig. 6a). In this conformation, the C-terminal TIR motifs responsible for signal transmission remain spatially far apart, preventing downstream signalling (Latz et al., 2007). When a cell encounters a ligand, fl-TLR9 exits the ER, assisted by the uncoordinated 93 homolog B1 (UNC93B1). In addition, ubiquitination of TLR9 facilitates the selective trafficking of TLR9-containing vesicles with the endosomal sorting complex required for transport to fuse with ligand-containing early endosomes (Fig. 6b) (Chiang et al., 2012). The incorporation of endosomes into lysosomes generates acidic lysosomal-associated membrane protein 1-positive late endolysosomes, where pH-sensitive enzymes, such as cathepsins, facilitate the degradation of the cargo (Fig. 6c) (Lee et al., 2013). Following proteolytic cleavage, the TLR9 dimer undergoes a conformational change that brings TIR domains together for recruitment of the adaptor protein myeloid differentiation primary response 88 (MyD88) (Latz et al., 2007). Oligomerisation of

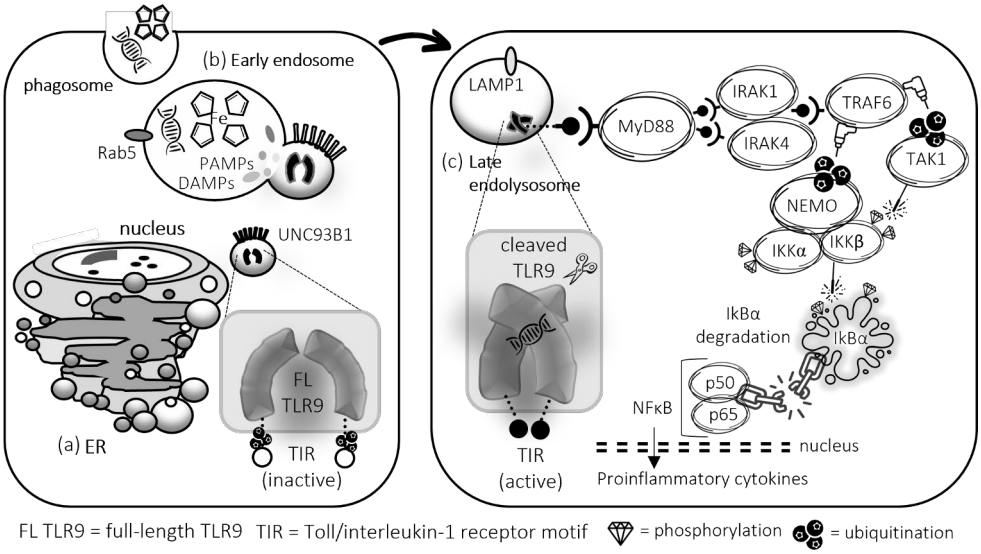

Figure 6. Canonical activation of TLR9 promotes Th1-milieau and production of proinflammatory cytokines in immune cells.

MyD88 through its death domains with IL-1 receptor-associated kinase 4 (IRAK4) forms a signalling complex, 'Myddosome', that sequentially interacts with other adaptor proteins, such as IRAK1. Myddosome- phosphorylated IRAK1 then interacts with E3 ubiquitin ligase tumour necrosis factor associated factor 6 (TRAF6), which in turn ubiquitinylates transforming growth factor-β activated kinase 1 (TAK1). Following activation by ubiquitination, TAK1 phosphorylates the trimeric IKK consisting of catalytic (IKKα and IKKβ) and regulatory (IKKγ) subunits. In resting cells, IKK canonical targets, transcription factor NF-κB dimers p50 and p65, are confined to the cytosolic compartment by the IκB protein. Once the inhibitory NF-κB-binding protein IκB is labelled by IKKβ for degradation, NF-κB can enter the nucleus and activate the transcription of NF-κB-responsive genes (Chiang et al., 2012; Combes et al., 2017).

Besides the classic inflammatory response, the NF-κB pathway can also be activated non-canonically, which regulates other durable immunological functions such as the development of lymphoid organs, control of immune tolerance, maturation, and homeostasis of APCs, B-cells, as well as effector and memory T-cells. While the canonical pathway is managed by TAK1, non-canonical activation is controlled by the NF-κB-inducing kinase (NIK1) and the NF-κB2 protein p100, a precursor of p52 with an inhibitory motif. In resting cells, NIK1 remains inactive when bound to the TRAF3-TRAF2-cIAP E3 ubiquitin ligase complex. Ligand-binding to one of the TNF-receptor superfamily members induces TRAF3 ubiquitination and its subsequent degradation, releasing NIK1 into the cytosol. NIK1

then phosphorylates IKKα, which in turn phosphorylates p100. Following activation, the inhibitory motif in p100 is cropped off, releasing the active p52 to translocate into the nucleus together with another NF-κB protein, RelB (Sun, 2012).

Apart from proinflammatory signalling, detection of viral material or DAMPs bifurcates TIR-induced signal downstream from MyD88 assisted by cofactors such as interferon regulatory factor 7, phosphatidylinositol 3P 5-kinase (PI3K), and adaptor protein-3 to produce type I IFNs in specialised pDCs (Combes et al., 2017).

2.5 The actions of TLR9 can diversify the survival kit for cancer

Besides its well-known functions in eliciting immune activation, non-immunological studies have associated aberrant TLR9 signalling with intracellular processes including cell proliferation, survival, cell locomotion, metabolic switch and resistance to apoptosis (Ilvesaro et al., 2007; Luo et al., 2015; Sester et al., 2006). Although many studies have assessed molecular changes following TLR9 activation by environmental factors or its ligands, some have argued that endogenous TLR9 expression alone has an impact on cell survival and proliferation (Tuomela et al., 2013). The supportive data in cancers associated with oncoviral origins, such as cervical cancer and HNSCC, indicate that virus-induced downregulation of TLR9 correlates with concomitant cell proliferation (Parroche et al., 2016). Although similar results have been reported in TLR9 siRNA-silenced breast cancer cells, enhanced cell proliferation was not detected in the TLR9-silenced prostate cancer cells (Luo et al., 2015; Tuomela et al., 2013). Herein, the following paragraphs will give a short synopsis of the current knowledge of connections and crosstalk TLR9 has with cancer hallmarks relevant to this thesis.

2.5.1 TLR9 and evasion of growth suppressors and resistance to cell death

Apoptosis is fundamental to complete tissue regeneration during wound healing and to eliminate abnormal cells with disturbed molecular integrity or DNA damage. Neoplastic cells typically outwit this critical process by inactivating several tumour suppressor genes. Molecular events including the corruption of proapoptotic tumour suppressor protein p53 (TP53) and von Hippel-Lindau stress sensor pathways or overexpression of the survival-promoting insulin-like growth receptor 1, the specific ‘don’t eat me’ signal proteins, such as PD-L1 and CD24, as well as the anti-apoptotic Bcl-2, and similar proteins, support the cancer hallmarks such as *resistance to cell death, evasion of growth suppressors* and *cellular senescence* (Barkal et al., 2019; Hanahan & Weinberg, 2011; Liu et al., 2019; Yuen et al., 2007). The last was recently suggested to become a novel hallmark of cancer by Hanahan (Hanahan, 2022). While

certain in vitro studies have reported CpG-ODN-induced reduction in cancer cell viability and apoptosis (Brignole et al., 2010; Damiano et al., 2009; Natarajan & Ranganathan, 2020; Noack et al., 2012; Olbert et al., 2015), other research highlights the potential for immunostimulatory agonists to trigger an opposing response (Kennedy et al., 2021; Tarnani et al., 2010; Xu et al., 2010; Zhang et al., 2014). These alterations have been connected to several molecular operators, including cyclin-dependent kinases and TP53 (Holm et al., 2017; Xu et al., 2010). On the other hand, depolarisation and damage of the mitochondrial membrane, as well as TLR9 single-nucleotide polymorphism, have been listed as potential elements modulating the expression of critical gatekeeping proteins related to apoptosis signalling following treatment with CpG-ODNs (Brignole et al., 2010; Damiano et al., 2006; Noack et al., 2012). Hence, we are still without a comprehensive understanding of the TLR9-mediated molecular events in cancer cell survival and apoptosis.

2.5.2 TLR9 and senescence

Many malignancies, including HNSCC and breast cancer, can resist cell death due to a mutated TP53 (Ku et al., 2007; Ungerleider et al., 2018). Without functional TP53, the intrinsic apoptotic pathway becomes inoperative, providing these cells with resistance against treatments like drug-induced cell cycle arrest. In addition to its role in apoptosis, TP53 also interacts with the NF-κB pathway. While TP53-induced apoptosis is potentiated by NF-κB activation, defects in the NF-κB pathway can abrogate TP53-induced cell death. This highlights the critical, interdependent relationship between these stress-sensitive transcription factors (Ryan et al., 2000).

If cells are unable to commit ‘suicide’ by apoptosis, they can alternatively enter senescence. Although these cells are irreversibly blocked from continuing the cell cycle beyond stages G1 or G2, they remain alive and metabolically active (Acosta et al., 2013; Biran et al., 2014). In certain cases, CpG-ODNs have been shown to support such senescence. Studies in virally and non-virally induced cancers in the head and neck, as well as the cervix, have indicated that while apoptosis was not increased in these cells, increased $p16^{INK4}$ stability reduced cell growth by extending the S-phase during the cell cycle (Parroche et al., 2016). Treatment with TLR9 agonist in endothelial cells induced a similar cell cycle arrest at the G0/G1 phase, suggesting that while the TLR9 agonist was not cytotoxic, it induced c-Jun N-terminal kinase activation and inhibition of the MAPK pathway (Li et al., 2022).

2.5.3 TLR9, cell proliferation and proliferative signalling

After unlocking its replicative potential, a neoplastic cell needs extra stimuli to thrive. This is typically provided by growth hormones and other soluble mediators originating primarily from the TME. In addition, neoplasms commonly overexpress

growth receptors while the proteins responsible for the inhibitory negative feedback loops are mutated (Hanahan & Weinberg, 2011). Consequently, the general signalling homeostasis is disturbed and overwhelmed by aberrant crosstalk among pathways and permanently active growth receptors. Such examples are loss-of-function mutations in the apoptosis-inducing phosphatase and tensin homolog or the mammalian target of rapamycin (mTOR); two disturbances, which consequently can boost PI3K- and protein kinase B (AKT/PKB)-mediated signalling (Maehama & Dixon, 1998; O'Reilly et al., 2006). Moreover, point mutations typically around the ATP-binding pocket can turn growth receptors, such as epidermal growth factor receptor (EGFR) and RAS, permanently on, resulting in enhanced PI3K signalling (Kim et al., 1994; Yang et al., 2012).

TLR9 agonists have been shown to stunt cancer cell growth, especially as part of a combinatorial treatment, albeit the exact molecular mechanism is yet unclear. As demonstrated by Damiano et al., CpG-ODNs reduced the survival of colon carcinoma and inhibited several growth receptors and activators, such as EGFR, vascular endothelial growth factor (VEGF), the human epidermal growth factor-2 (HER2), MAPK, and AKT/PKB, synergistically with anti-VEGF mAb bevacizumab (Damiano et al., 2007).

Although it is not known what contextual variables determine if exposure to TLR9 agonists inhibits or supports paracrine proliferative signalling, cancer cells themselves participate in the process via auto- and paracrine signalling. For instance, altered expression of cytokines and growth factors, such as IL-8, TNF-α, INF-β, VEGF-A, placental growth factor, leukaemia inhibitory factor, and IL-6, have been reported post-TLR9 agonist treatment (Natarajan & Ranganathan, 2020; Olbert et al., 2015; Won et al., 2017). Furthermore, sex hormones, together with the TLR pathway activation, influence paracrine signalling. Research has shown that exposure to TLR7/9 ligands together with 17β-oestradiol enhanced IFN-α production from pDCs (Seillet et al., 2012). Similar synergy exists in hormone-responsive cancers, such as breast and prostate, in which sex hormones contribute to the regulation of TLR9 expression and its downstream activity (Cunningham et al., 2014; Natarajan & Ranganathan, 2020; Sandholm et al., 2012).

2.5.4 TLR9 and metabolic reprogramming

Proliferating cells need a great amount of energy and molecular building blocks, in contrast to normal, quiescent cells. After Otto Warburg discovered 'aerobic glycolysis', the unusual way cancer cells utilise glucose under aerobic conditions, researchers have further explored the Warburg effect and metabolic reprogramming in cancer. As opposed to Warburg's original hypothesis, which surmised that oxidative phosphorylation under normoxia was inhibited by malfunctioning mitochondria, the classic cancer cell metabolism model describes that malignant cells

prioritise their general metabolism over ATP production during a chronic lack of resources (Kilburn et al., 1969). The Warburg effect, which produces e.g. glycerol, sugar derivatives, nucleotides and non-essential amino acids and other precursor molecules, favours de novo lipid biosynthesis and manages the generation of reducing equivalents needed for biosynthetic reactions (Fig. 7). By boosting anabolic pathways and enhancing the production of precursors and especially nicotinamide adenine dinucleotide phosphate, proliferating cells utilise the Warburg effect to concomitantly repress mitochondrial oxidation and production of ATP and nicotinamide adenine dinucleotide (Denko, 2008). Re-routing accumulating pyruvate towards lactate production by the lactate dehydrogenase A (LDH-A) instead of mitochondrial oxidative phosphorylation reinforces this dysregulation pattern, as ATP and nicotinamide adenine dinucleotide are negative regulators of glycolysis. Furthermore, extracellular conditions such as hypoxia suppress the tricarboxylic acid cycle but via different mechanisms. Under hypoxia, HIF-1 stabilisation protects the cells from an energy crisis and promotes the Warburg effect by upregulating many metabolic enzymes, including pyruvate dehydrogenase kinase 1. As it happens, inactivation of pyruvate dehydrogenase by HIF-1–upregulated pyruvate dehydrogenase kinase 1 reduces ATP production and leads to decreased aerobic metabolism and utilisation of alternative energy sources in favour of production of anabolic substrates for biosynthesis.

Accumulating evidence has linked chronic inflammation in cancer with metabolic reprogramming, further complicating the big picture. For example, TLR9–induced cytokines, such as IL-6 and TNFα, emanating from both immune and malignant cells, intensify the tumour energy expenditure in a paracrine manner (Wang & Ye, 2015). In immune cells, the dynamic de novo production of cytokines and other bioactive molecules by e.g. PI3K/AKT/mTOR, serine/threonine kinase glycogen synthase kinase 3 (GSK3) and TLR pathways requires a considerable amount of energy. A good example of unmanageable energy expenditure occurs during an unresolved viral infection, when the associated persistent inflammation typically generates exhausted and dysfunctional immune cells (Dominguez-Villar et al., 2014). On the other hand, enzymes, such as GSK3, can also have a non-metabolic purpose in the tenaciously inflammatory milieu. While the two isoforms of GSK3, GSK3α and GSK3β, often dysregulated in HNSCC, manage glucose homeostasis, they have additionally been shown to take part in cellular functions like cell survival, immune responses and cytokine production (Martin et al., 2005; Schulz et al., 2018; Turnquist et al., 2010). Unlike most kinases, which are typically activated by post-translational modifications, phosphorylation of the constitutively active GSK3 at its residue Ser 9 downregulates its activity (Cross 1995). While GSK3 is found at the crossroads of several signalling pathways, including PI3K/AKT/mTOR, its interaction with the NF-κB pathway is imperative when considering its co-operation with TLR9 signalling in the dysregulated inflammatory TME (Demarchi et al., 2003; Hoeflich et al., 2000).

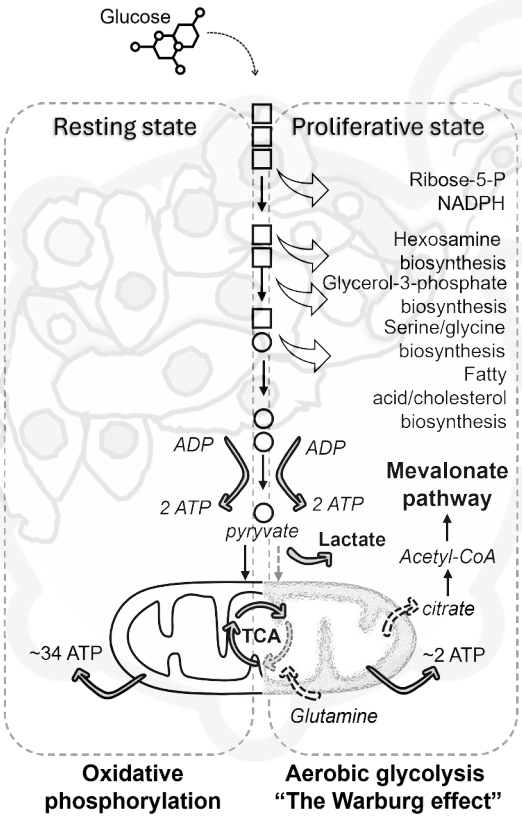

Figure 7. A simplified illustration describing the basic metabolism in resting and neoplastic cells. Distinct environmental and physiological conditions as well as dysregulated signalling drive cells to utilize either the mitochondrial oxidative phosphorylation or the Warburg effect, respectively, in their energy production.

In different circumstances, TLR9 in non-immune cells, such as cardiomyocytes and neurons, supports non-canonical energy metabolism instead of inflammatory signalling. It does this by modifying the ratio of intracellular adenosine monophosphate/ATP, which activates the AMP-activated kinase (AMPK) (Shintani et al., 2013). The AMPK activation downstream of TLR9 rewires metabolic consumption by inducing autophagy to recycle organelles and cytoplasm. Such self-cannibalisation is a vital part of TLR-related early innate immunity responses in APCs, evoking an enhanced antigen presentation and type I IFN production (Henault et al., 2012). However, such functional autophagy is not limited to immune cells, as exposure of colon carcinoma tumours and cervical cancer cell line HeLa to CpG-ODN revealed similar global changes associated especially with metabolic functions and autophagy (Bertin et al., 2008; Lim et al., 2016). Therefore, a context-dependent TLR

signalling can both corrupt and protect cells by modulating the energy expenditure and the AMPK-mediated energy stress response.

Overall, the metabolic reprogramming in cancer is typically governed by multiple oncogene-driven signalling pathways, such as PI3K/AKT/mTOR, HIF, AMPK and growth receptors such as transforming growth factor-beta (TGFβ) and EGFR (Hiraki et al., 1988; Kim et al., 2006; Lee et al., 2014; Zheng et al., 2015). Furthermore, the rate-limiting enzymes within branching pathways which manage the efflux of indispensable glycolytic intermediates are typically upregulated. As a result, de novo lipogenesis is typically boosted via the mevalonate pathway, generating an increased pool of acetyl-CoA from mitochondrial citrate (Fig. 7). As malignant cells generally depend on mevalonate metabolism, control of the mevalonate pathway has emerged as a potential target for cancer therapies as protein prenylation inter alia enhances the mitogenic and migratory signalling as well as cell size and autophagic flux by small GTP binding proteins (Kamata & Feramisco, 1984; Miettinen & Björklund, 2015; Mulcahy et al., 1985).

2.5.4.1 Nitrogen-containing bisphosphonates in cancer treatment: the old remedy with new tricks?

N-BPs are a class of drugs known to modulate the mevalonate pathway. As analogues of natural inorganic pyrophosphates, they have a high affinity for the exposed sites of active remodelling in the bone (Compston, 1994). Both the non-nitrogenous bisphosphonates (BPs) and n-BPs have been used to reduce the loss of bone mineral density and skeletal-related events, including osteopenia, osteoporosis, hypercalcemia, and bone-related pain in advanced breast cancer (Burstein et al., 2019).

The mechanism of action of both BPs and n-BPs in bone is, however, divergent. While the first-generation BPs, such as clodronate, induce intracellular accumulation of ATP-like, non-hydrolysable metabolite of clodronate to promote osteoclast apoptosis, the n-BPs, such as zoledronate (ZOL) or alendronate (ALN), act mainly on the mevalonate pathway by blocking the farnesyl pyrophosphate synthase (FPPS) enzyme (Fig. 8) (Fisher et al., 1999; Frith et al., 2001). Inhibition of FPPS results in depletion of the downstream intermediates farnesyl pyrophosphate and geranylgeranyl pyrophosphate, which in turn prevents both the production of isoprenoid lipids needed for biosynthesis of, e.g. sterols, cholesterol, and ubiquinone, and the post-translational prenylation of target proteins, mainly the small GTPases. As a result, n-BPs promote the accumulation of the precursors isopentenyl pyrophosphate (IPP) and dimethylallyl diphosphate (DMAPP), which act as substrates for the biosynthesis of cytotoxic ATP-analogues, ApppI and ApppD, respectively (Mönkkönen et al., 2006). Eventually, these metabolites contribute to impaired osteoclast function and subsequent apoptosis. Besides their primary

mechanisms of action, these metabolites also possess immunomodulatory functions, making n-BPs an interesting prospect regarding tumour immune surveillance. N-BPs boost not only cytokine IL-6 production in murine peritoneal macrophages and human peripheral blood mononuclear cells in response to LPS but also trigger the activation and release of cytokines from a specific subgroup of γδ T cells, explaining the typical acute flu-like symptoms following n-BP infusion (Kunzmann et al., 2000).

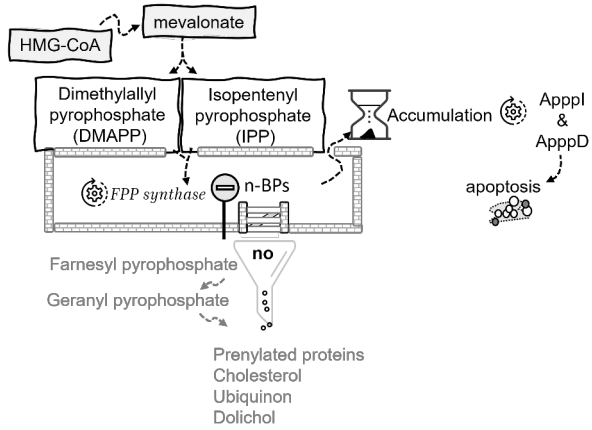

Figure 8. Inhibition of the mevalonate pathway by nitrogen-containing bisphosphonates results in cytotoxic ApppI production and blocks the synthesis of many precursor isoprenoids.

2.6 Breast Cancer

While the estimates are that 1 female out of 20 will develop breast cancer during their lifetime, the GLOBOCAN statistics indicated that in the year 2022, this risk totalled to nearly 2.3 million cases worldwide (Kim et al., 2025). During the same year, almost 700000 lives were lost to breast cancer, making breast cancer unquestionably the most diagnosed and lethal cancer in women in the modern era. Yet, the patient outcomes for breast cancer have steadily improved over the past decades, thanks to the deeper knowledge about breast cancer genetics, molecular mechanisms, subtype-specific surrogate markers and improved multidisciplinary drug and therapy modalities. Despite the numerous advances in diagnosis and treatments, TNBC, a hormone receptor and HER2-negative subtype of breast cancer, is still a severe problem for patient survival (Dent et al., 2007).

2.6.1 Aetiology & pathophysiology

The epidemiology and risk factors for breast cancer are well documented. It is known that factors including age, female sex, genetic background, and the age of onset of menarche/menopause are significantly associated with one's risk for breast cancer. In addition, environmental and lifestyle choices, including childlessness, hormonal treatments, physical activity/obesity, nutrition, alcohol, and tobacco consumption, increase the risk of breast cancer (Kim et al., 2025).

Currently, the clinical diagnosis and pathological characterisation of breast cancer recognise several stages, such as localised, locally advanced, and disseminated disease, with molecular subcategories of luminal A and B, HER2-overexpressing, and basal-like TNBC (Iacopetta et al., 2023). In addition, the variable expression of nuclear hormone receptors oestrogen and progesterone further expands the convoluted molecular nature of breast cancer. Notably, TNBC lacks the expression of all the above-mentioned receptors. Further to receptor positivity, the expression of the proliferation marker Ki-67 can be used for diagnosis as it indicates the aggressiveness of cancer (Goldhirsch et al., 2013). Moreover, the recently updated Saint-Gallen International guidelines have suggested including tumour-infiltrating lymphocytes as a biomarker due to their prognostic value for TNBC diagnosis (Burstein et al., 2019).

The heterogeneous nature and unique mutational landscape of breast cancer have been studied by modern technologies, including microarray analyses and next-generation sequencing. Interestingly, out of hundreds of known somatic disturbances in breast cancer, studies in patient cohorts have identified multiple driver mutations in oncogenes and tumour suppressors, such as TP53, PIK3Cα, BRCA1, and BRCA2, which form the basis for different genetic subcategories with variable responses to targeted therapies and patient survival (Ng et al., 2015).

2.6.1.1 The conceivable role of TLR9 in the aetiology & pathophysiology of breast cancer

Many investigations have focused on the question of the role of TLR9 in breast cancer. In one study, TLR9 mRNA overexpression was positively correlated with the clinicopathological prognosis factors and metastasis-free survival (Meseure et al., 2016). In contrast to that, one study found a link between the aggressive phenotype and the low TLR9 expression (Tuomela et al., 2012). The finding showing that sex hormones and transient overexpression of oestrogen receptor α can modulate TLR9 expression in TNBC MDA-MB-231 cells additionally suggests a specific role for TLR9 in hormonal cancers (Sandholm et al., 2012). In the list of many cellular functions, epithelial-mesenchymal transition (EMT) and cellular invasion have also been associated with TLR9. Indeed, Merrell et al. demonstrated that MDA-MB-231 cells treated with CpG-ODNs increased their invasive behaviour by boosting the production of matrix metalloproteinase 13. The authors proposed that the invasive behaviour observed in breast cancer could be TLR9-mediated (Merrell et al., 2006). In addition, NF-κB induces the production of variable matrix metalloproteinases, reinforcing the hypothesis that links TLR9 to EMT (Min et al., 2008).

2.6.2 Breast cancer therapies are founded on the multidisciplinary knowledge

Once breast cancer has been diagnosed, the treatment typically consists of surgery, post-surgical radiation for breast and optionally regional nodes, and variable selection of drug-based therapies (Burstein et al., 2019). With the increasing knowledge of molecular characterisation of the tumours, the progressive personalised and multidisciplinary therapy options for distinct subtypes in breast cancer are a reality today. After surgical treatment, adjuvant chemotherapy and RT, treatment options including endocrine therapy, selective oestrogen receptor modulators and aromatase inhibitors are effective for up to 70% of the patients with oestrogen receptor-positive breast cancer (ER^+). In general, patients with ER^+ breast cancer receive long-term systemic endocrine therapy encompassing selective oestrogen receptor modulators, such as tamoxifen, or androgen inhibitors, whereas patients with $HER2^+$ overexpression or TNBC are treated with DNA-damaging chemotherapeutics, such as taxanes, anthracyclines, and cyclophosphamides, combined with antibodies against HER2, including trastuzumab (Burstein et al., 2019; Iacopetta et al., 2023).

In the same spirit, factors such as the stage of breast cancer, histopathological characterisation and variable gene expression patterns contribute to the individualised choice regarding post-operative RT. While RT in general reduces the rate of disease recurrence and can be utilised regardless of age, clinical trials indicate that a proportion of patients could be effectively treated without RT, as it does not improve overall survival (OS) (Amini et al., 2022). Hence, the current standard of care recommends a careful assessment of the general benefits to the patient, as RT is relatively toxic for frail and aged patients with comorbidities (Burstein et al., 2019). However, if the disease progresses to the bone, a single- or multifractionated palliative RT is used to alleviate pain generated by pathological skeletal-related events (SRE) (Rich et al., 2018).

As with many other cancer types today, novel immunotherapies are also considered promising treatment options for breast cancer. Certain immune checkpoint inhibitors, such as pembrolizumab, nivolumab, and ipilimumab, targeting PD-1 and CTLA-4, respectively, have been accepted as a standard of care treatment for TNBC in combination with chemotherapy. However, tumours characterised either by $HER2^+$ overexpression or, in contrast, as hormone receptor-negative, are problematic to eradicate as these molecular subgroups typically represent the most aggressive cancers. To date, 30 drugs have been approved by the U.S. FDA for the treatment of breast cancer, but persistent side effects, insufficient efficacy and intrinsic/acquired drug resistance warrant the search for novel treatments, especially against the subtypes with the worst prognosis (Iacopetta et al., 2023).

2.6.3 Bisphosphonates in breast cancer treatment

BPs are safe, well-tolerated and effective multifaceted tools utilised already for decades to reduce the loss of bone mineral density in breast cancer. By coupling a nitrogen moiety into the structure, researchers have significantly improved the anti-resorptive potency of the first-generation non-nitrogenous simple BPs. Pamidronate was the first n-BP to effectively alleviate SRE morbidity in breast cancer when compared with a placebo (Theriault et al., 1999). Since then, many n-BP derivatives have changed the practice of how bone-related morbidities in different pathologies can be treated. Today, n-BPs, including ZOL and clodronate, can be utilised on top of the routine palliative care in the treatment of postmenopausal women, to alleviate SRE and bone loss in metastatic breast cancer (Coleman et al., 2020). In premenopausal women with ER^+ breast cancer, the use of ZOL is assessed case by case (Paluch-Shimon et al., 2022).

Apart from their eminent character as inhibitors of bone resorption and metastasis, n-BPs also possess anti-tumourigenic properties in soft tissue (Dumon et al., 2004; Tuomela et al., 2008; Virtanen et al., 2018). One theory suggests that n-BPs can impact other processes and cell types; in fact, several in vitro studies have reported profound effects on soft tissue tumour cell metabolism, growth, motility, and survival following treatment with n-BPs (Dumon et al., 2004; Karlic et al., 2017; Merrell et al., 2007). For example, previous data in the orthotopic mouse tumour model of prostate cancer revealed that daily administrations of ALN induced prostate cancer cell apoptosis and reduced the lymph node metastasis (Tuomela et al., 2008). Virtanen et al. have reported that exposure to ALN was able to both disrupt the organisation of the actin cytoskeleton and downregulate cofilin expression in PC-3 prostate cancer cells, causing inhibited cell motility and invasion (Virtanen et al., 2018).

2.7 Head and neck squamous cell carcinoma

HNSCC constituted nearly 900000 new cases and 500000 deaths worldwide in 2022, according to the global cancer statistics (Bray et al., 2024). A large portion of these cases occur in low- and middle-income countries, and based on several estimates, the overall incidence of HNSCC may rise by nearly 30% by the end of this decade (Patterson et al., 2020).

2.7.1 Aetiology & pathophysiology

Anatomically, cancers originating from the mucosal epithelium in the oral and nasal cavity, the areas of the larynx and pharynx, and salivary glands are classified as HNSCC. While exposure to a variety of carcinogenic substances, such as tobacco, alcohol, and air pollutants, elicits the majority of HNSCC cases, investigations have

found an additional causal link between viral infections and HNSCC (Johnson et al., 2020). Based on the epidemiological data, 13 strains out of over 200 different types of HPV have been identified as having a high oncogenicity, with HPV16 being by far the most common in HNSCC (Mirabello et al., 2018). Moreover, evidence shows that HPV^+ malignancies are a biologically distinguishable subgroup of HNSCC encompassing distinct clinicopathological features and favourable response to cancer treatments such as radiation therapy (Ang et al., 2010; Chen et al., 2017; Gillison et al., 2000; Lassen et al., 2009). Thus, considering differing anatomical locations, variable ethnicity and aetiological agents initiating malignant transformation, it is unsurprising that HNSCC consists of a highly heterogeneous group of cancers with distinct molecular and immunological profiles (Johnson et al., 2020; Partlová et al., 2015).

HPV-negative HNSCC typically has a poor patient prognosis. Several factors, such as frequent chromosomal aberrations, polymorphism in genes related to the immune system or metabolism, epigenetic changes resulting from aberrant methylation, and defects in DNA repair machinery, have been associated with the development and progression of HNSCC (Johnson et al., 2020). In addition, research has identified frequently mutated key modulators, such as TP53, cyclin-dependent kinase 2A, phosphoinositide 3-kinase, FAT atypical cadherin 1, TGFβ receptor 2, EGFR amplification, aberrant regulation of cell cycle, cytoskeletal reorganisation, and an elevated expression of several matrix metalloproteinases and cytokines, associated with HNSCC carcinogenesis (Erjala et al., 2006; Johnson et al., 2020; Pries & Wollenberg, 2006). What is more, immunoproteogenomic analysis has revealed that reduced immunogenicity in HNSCC tumours correlated with the loss of heterogeneity at site 3p21, encoding, for example, several chemokine/cytokine receptors as well as TLR9 (Lawrence et al., 2015). Furthermore, deep sequencing approaches have broadened our knowledge of the dysregulated molecular pathways in HNSCC, showing that among many disturbances, the PI3K/Akt/mTOR pathway is one of the most common perturbations in HNSCC lesions.

While HPV-negative HNSCC are difficult to treat, several case studies have suggested that HPV-positive cancers remain susceptible to different cancer treatment modalities and portray improved patient survival (Ang et al., 2010; Chen et al., 2017; Lawrence et al., 2015; Sartor et al., 2011). Besides genetic mutations, molecular events such as histone modification, DNA methylation and expression of a vast number of regulatory micro- and non-coding RNAs can turn the critical tumour suppressors off. As HPV-positive cancers lack genetic mutations in the key regulators, genome reprogramming arises from non-mutational HPV-induced epigenetic modifications (Sartor et al., 2011). Hence, the phenotype of such tumours is reliant on the E6 and E7 oncoproteins, which inhibit the tumour suppressors p53 and retinoblastoma protein, respectively (Dyson et al., 1992; Werness et al., 1990).

2.7.1.1 The conceivable role of TLR9 in the aetiology & pathophysiology of head and neck squamous cell carcinoma

HPV oncoproteins E6 and E7 suppress TLR9 expression by manipulating the transcriptional activity of the TLR9 promoter via enzymes such as histone deacetylases, histone demethylases, and DNA methyltransferases (Hasan et al., 2007; Sen et al., 2017). These findings have been corroborated in patient cohorts showing a positive correlation between persisting HPV infection and reduced TLR9 expression, for instance, in endocervical and oropharyngeal samples (Daud et al., 2011; Jouhi et al., 2015). Some investigations have assessed the engagement of TLR9 in HNSCC pathophysiology and patient survival. As an example, an increased TLR9 expression in a patient cohort of oral tongue squamous cell carcinoma has been shown to correlate with poor patient survival (Kauppila et al., 2015). On the other hand, two independent investigations utilising HNSCC tumour samples of the oral cavity, oropharynx, larynx, and hypopharynx suggested that infection with HPV suppresses TLR9 expression in comparison with HPV-negative oral squamous cell carcinoma (Jouhi et al., 2015; Wang et al., 2021). Moreover, the study by Wang et al. reported a positive correlation between HPV-positivity and better OS and disease-free survival (Wang et al., 2021).

Apart from patient cohorts, TLR9-related discrepancies are also evident in vitro as several studies have indicated that activation of TLR9 promotes tumourigenesis by increasing proliferation, cell migration and invasion in oral squamous cell carcinoma (Mäkinen et al., 2015; Min et al., 2012). A study by Baruah et al. has also suggested that the TLR9 pathway may contribute to an immunosuppressive TME by increasing the expression of PD-L1 and PD-L2 (Baruah et al., 2019).

2.7.2 Multifarious, combinatorial treatments in head and neck squamous cell carcinoma

As expected, multiple factors, such as anatomical location, stage of the disease, HPV status, and surgical accessibility, pose many challenges when combating HNSCC. At an early stage, an untreated malignancy is usually removed by surgery (Fig. 9). If the disease is unresectable or locoregionally advanced, adjuvant therapy, chemotherapy, RT, or the last two combined (=chemoradiation) are commonly used (Johnson et al., 2020). Studies have shown that chemoradiation based on a concurrent high dosage of platinum-containing cisplatin has so far been the most effective approach in patients with advanced disease, as cisplatin sensitises HNSCC tumour cells to radiation. Albeit efficient when combined with RT, cisplatin is particularly damaging to the renal, nervous, and olfactory systems, resulting typically in acute and late toxicities. As a result, a subgroup of patients is typically unable to continue this treatment. Moreover, many patients develop resistance to cisplatin over time.

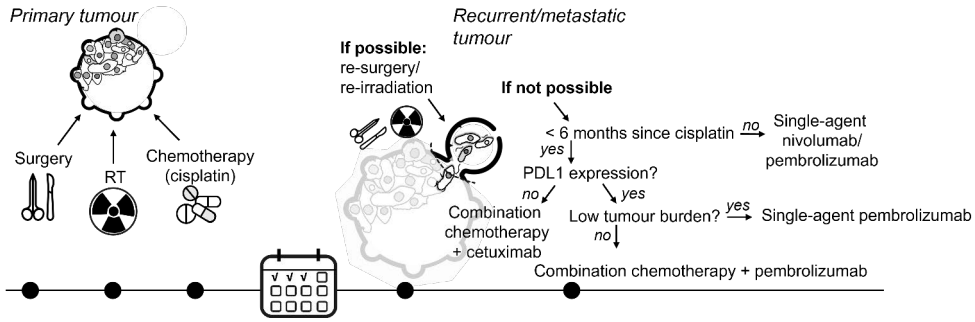

Figure 9. A short summary of decision-making for primary and recurrent/metastatic HNSCC. Figure 9 is customized from Johnson *et al.* (2020).

In general, RT has its shortcomings as its effectiveness emanates from an unpredictable plethora of anti-tumourigenic signals and immunosuppressive molecules impacting immunogenic cell death (Chakraborty et al., 2004; Garg & Agostinis, 2017; Sharma et al., 2011). However, when combined with immunomodulatory drugs, RT can elicit the production of type I IFNs, a release of danger signals representing an 'in situ vaccination' and subsequently boost the cross-priming of APCs and anti-tumorigenic responses (Burnette et al., 2011; Li et al., 2025). In recent years, specific combinatorial immunomodulatory treatments, such as EGFR-targeting antibody cetuximab and/or PD-1-targeting pembrolizumab, have already been introduced alongside the traditional chemotherapies to treat the recurrent or metastatic disease (Miyauchi et al., 2019; Vermorken et al., 2008).

As a novel approach, immunomodulating TLRs have also been suggested to synergistically transform both RT's direct as well as abscopal effects into an antitumoural immune response. While the preclinical and clinical studies have been promising in other contexts such as B-lymphoma, glioma and colon carcinoma (Brody et al., 2010; Li et al., 2016; Younes et al., 2021), the efficacy of TLR9 agonists in combination with RT in HNSCC is less studied. Some recent data, however, suggest that combining immunomodulatory TLR9 agonists with RT could elicit and support antitumourigenic immune responses in HNSCC (Moreira et al., 2021). In the same vein, promising in vitro results in malignancies such as colon and ovarian carcinoma indicate that TLR9 agonists can achieve good anti-tumour outcomes together with traditional chemotherapy, sparking an interest towards immunomodulatory combinatorial therapies (Sommariva et al., 2011). However, while the combinatory treatment with TLR9 agonist IMO-2055 together with cetuximab was well tolerated, it lacked cumulative efficacy in comparison with cetuximab monotherapy in recurrent/metastatic HNSCC. This indicates that in HNSCC, the clinical prospect has not yet reached the level of preclinical promise (Ruzsa et al., 2014). Some clinical studies have also been prematurely terminated due to adverse side effects, such as

myelotoxicity and hypokalaemia, suggesting that at least platinum-based chemotherapy may not be suitable in combination with TLR9 agonists (Machiels et al., 2013).

2.7.3 Hypoxia-modifying strategies

Chronically hypoxic tumours are notoriously tough to treat, and the correlation between tumour hypoxia and metastatic potential, as well as resistance to RT, has been well reported (Lehtiö et al., 2004; Pettersen et al., 2014; Yaromina et al., 2010). Although the physiological amount of oxygen in the tissues can temporarily reduce below 6 mm Hg without long-term consequences, from early on it was understood that chronically suppressed O_2 tension can prime cells to oppose RT-mediated destruction (Conger, 1956; Fowler et al., 1976). Many distinct mechanisms, including the hypoxic gene expression signature, hypoxia-driven selection of radioresistant clones, lack of ROS, reduced oxygen consumption, and cancer stem cells, have been identified as transmitters of such resilience in HNSCC (Bentzen et al., 2015; Chen et al., 2009; Dadgar et al., 2021; Kim et al., 2015; Toustrup et al., 2012). To overcome and abolish the hypoxia-induced resistance against RT, researchers have attempted to develop variable hypoxia-modulating strategies. These included approaches such as hyperbaric oxygen treatment, which forces excess O_2 into the tumours and chemical hypoxic radiosensitisers, that aim to overcome the hypoxia-induced protective radiobiological mechanisms in the tumours (Henk et al., 1970; Overgaard et al., 1982). While the modulation of hypoxia, in general, has been found to improve the loco-regional control of HNSCC tumours and patient survival in several clinical trials, interestingly its efficacy appears independent of the treatment modality (Overgaard, 2011).

In principle, radiosensitisers accelerate the radiation-induced DNA damage by releasing free radicals. In combination with RT, the coupling of reactive radicals to intracellular macromolecules, including DNA and proteins, damages them beyond repair (Wardman, 2007). However, the development of novel radiosensitisers and chemical modulation of hypoxia remains an ongoing challenge due to the dose-limiting toxicity of these compounds (Overgaard et al., 1982). Hence, better diagnostic tools are needed to non-invasively identify patients whose tumours respond to hypoxic modification. To tackle this problem, the DAHANCA 5 study, consisting of a Danish cohort of over 400 patients, prospected a genetic approach to identify HNSCC tumours which could be sensitised to RT by nimorazole (Toustrup et al., 2012). Researchers generated a genetic classifier consisting of 15 hypoxia-responsive genes and showed that tumours identified by using this classifier responded to nimorazole, resulting in improved loco-regional tumour management in comparison to patients receiving RT and placebo only. Despite the promising clinical trials,

treatment of oropharyngeal tumours via hypoxic modulation is only an option in the Danish healthcare system (DAHANCA, 2020).

3 Aims

This thesis aimed to assess possible novel functions TLR9 may possess regarding cancer cell survival, immune evasion and cell locomotion in HNSCC and breast cancer.

The specific aspects studied were as follows:

1. Assess the growth-inhibitory effects of n-BPs in TLR9-silenced breast cancer (I).
2. Investigate whether hypoxic HNSCC cancer cells and hypoxia-induced tumour-originating TLR9 are associated with immune modulation in macrophages (II).
3. Characterise cancer cell locomotion and signalling pathway activation in parental FaDu and genetically engineered TLR9-deficient $FaDu^{TLR9def}$ cell line following exposure to the TLR9 ligands CpG-ODN 2006 and GpC-ODN 2137 *(unpublished).*

4 Materials and Methods

4.1 Summary of methods

This chapter contains a summary of all materials and methods used in the original publications. For detailed information, please refer to the original publications. All the additional material, which was not included in the original publications, is found here and marked as *unpublished*.

Method	Publication
ApppI synthesis	I
Cell culture	I-II, *unpublished*
Cell culture: hypoxic treatment	II
Cell proliferation assay	I
Cell viability assay	I
In vivo: Bone density measurement	I
In vivo: O2 partial pressure measurement	I
In vivo: xenograft tumours	I
Cell culture: MΦ polarization	II
Flow cytometry	II
RNA isolation, cDNA synthesis and qPCR	II, *unpublished*
siRNA silencing	I-II
Western blot	I-II, *unpublished*
ODN treatments	*unpublished*
Live cell imaging: chemokinesis	*unpublished*

Table 1. Summary of the methods used in this thesis.

4.2 In vivo experiments (I)

4.2.1 Xenograft tumours (I)

1 x 10^6 TLR9 mock and TLR9 shRNA MDA-MB-231 cells were inoculated into the mammary fat pads of four-week-old female nude mice, n = 60 (athymic nude/nu Foxn1 mice, Harlan). After 3 days, the diameter and volume of the tumours were measured. On day 4, the mice were randomised into 4 treatment groups (15 mice/per group): two groups received the vehicle, and two groups received 0.3 mg/kg ZOL. Mice were treated 3 times per week, and tumours were dissected and measured at the end of the experiment on day 26. Furthermore, O_2 partial pressures in the representative tumours were measured on day 24. Mice were maintained under controlled pathogen-free environmental conditions with *ad libitum* access to chow and water. Animal experimentation was approved by the State Provincial Offices of Finland. The experiment was performed under the animal license ESAVI/3257/04.10.07/2014.

4.2.2 Bone densitometry (I)

Bone mineral density was assessed by a peripheral quantitative computer tomograph XCT 540 (Stratec). Animals were sacrificed, and the left tibiae were fixed in 4% formaldehyde and stored in 70% EtOH (n = 5 – 6 per treatment). A reference line was marked at the proximal end of the tibia, and three 0.25 mm cross-sections 1.8 mm from the reference line and 1 midshaft measurement were analysed. The manufacturer's software at the resolution of 70 mm^3, and density thresholds of 0.5 mg/cm^3 and 0.71 mg/cm^3 for trabecular and cortical bones, respectively, were used for analysis.

4.2.3 Intratumoural O_2 tension measurement (I)

On study day 24, the intratumoural pO_2 in two representative tumours in each study group was measured. The measurements were performed with flexible polarographic (Clark style) electrodes, Ø 0.47 mm (Licox® GMS). The electrode was guided into the tumour tissue with the help of an insertion needle catheter. The inserted oxygen-sensitive probe was stabilised intratumourally for 5 min before a 20-minute pO_2 measurement to obtain a stable pO2 level. Tumour tissue temperature was measured to amend pO_2 values as temperature-adjusted (mmHg). The gluteus muscle of the same animal served as a positive pO_2 measure control site.

4.3 In vitro experiments (I-II)

4.3.1 ApppI synthesis (I)

All chemicals for the ApppI synthesis were purchased from Sigma (St. Louis, MO). A 46.4 mM solution of anhydrous pyridine in isopentenyl alcohol was cooled on ice for 10 min before adding 23.3 mM p-toluene sulfonyl. Mixing was continued on ice for 15 min before the incubation was continued at room temperature (RT) for 4 h. The mixture was diluted with H_2O and extracted three times with diethyl ether (Et_2O). The combined organic layers were washed with sulphuric acid (H_2SO_4), a concentrated NaCl-solution (brine) and sodium sulphate (Na_2SO_4), filtered and concentrated in vacuo to give a colourless oil. The crude product was purified by flash column chromatography using 5-10% Et_2O in hexane as solvent. Next, ATP disodium salt was deionised by Dowex 50WX8-100-200 (H+) ion exchange resin and the eluent was titrated to pH 8 with tetrabutylammonium hydroxide (n-Bu_4NOH). The resulting tetra-butyl ammonium salt of ATP was extracted, concentrated, and lyophilised for 48 h. The lyophilised solid was dissolved in anhydrous acetonitrile (CH_3CN) and 0.46 mM isopentenyl tosylate under nitrogen gas. The solution was stirred at RT overnight and dried under a vacuum. The resulting oily residue was dissolved in a mixture of H_2O and CH_3CN (5:1, respectively) and purified by semi-preparative HPLC using 0.1M tetraethylammonium bromide ($C_8H_{20}N^+Br^-$) as eluent. The fractions containing pure product were combined and concentrated under a high vacuum. The residue was dissolved in H_2O and lyophilised for 48 h to yield the triethyl ammonium salt form of ApppI. The purity was measured by NMR, and the final product was dissolved in sterile d-H_2O, aliquoted and stored at -20°C.

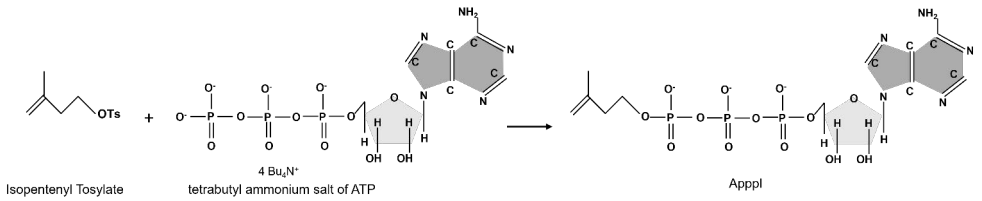

Figure 10. ApppI synthesis (I, Suppl.Figure S1).

4.3.2 Cell culture (I-II)

The human primary HNSCC cell lines UT-SCC-8 (II), UT-SCC-74A (II) as well as commercial HNSCC cell line FaDu (II) and in-house modified $FaDu^{TLR9def}$ (II), immortalised keratinocyte cell line HaCaT (II), Burkitt's lymphoma cell line Raji (II)

and human breast cancer cell lines MDA-MB-231 (I-II), T47D (I), MCF-7 (I), Cal-51 (I), and murine breast cancer 4T1 (I) were utilised in this study. All UT-SCC- cell lines were established from HPV-negative primary tumours. Apart from the UT-SCC-cells, cell lines were purchased from ATCC (Manassas, VA) or DSMZ (Braunschweig, Germany). The $FaDu^{TLR9def}$ cells were established by Turku Bioscience, Genome Editing Core. All commercial cell lines were maintained in growth media composed of Dulbecco's modified Eagle's medium (DMEM) with high glucose (Gibco) supplemented with heat-inactivated 10% foetal calf sera (FCS) (Gibco), 1 x MEM non-essential amino acids (Gibco), 2 mM l-glutamine GlutaMAX™ (Gibco) and 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco) in a humidified atmosphere at +37°C containing 5% CO2. Raji cells were cultured in RPMI 1640 medium (Gibco) supplemented with heat-inactivated 10% foetal calf serum (Gibco), 2 mM l-glutamine GlutaMAX™ (Gibco) and 100 U/mL penicillin and 100 µg/mL streptomycin. All experiments were performed before primary cell line passages reached 40 doublings to reduce cell transformations due to the culture conditions.

4.3.3 TLR9 silencing (I-II)

(I) A ready-made GFP-expressing pSuper vector with TLR9 short hairpin (shRNA) sequence (Oligoengine) was used to genetically silence TLR9 expression in MDA-MB-231 triple-negative breast cancer cells. An empty vector was used as a transfection control. Transfection was performed with TransIT-LT1 transfection reagent according to the manufacturer's instructions (Mirus Bio LLC). After a recovery period of 48-72h, positive clones for pSuper-vector were selected under 800 µg/ml of G418. To purify the generated single-cell clones with the highest GFP expression, the second round of selection was performed by GFP-guided cell sorting.

Conversely, a lentiviral approach was used to silence TLR9 in human CAL-51 breast and murine 4T1 mammary carcinoma cells (using Mission lentiviral transduction particles (Sigma). 1.6×10^4 cells were plated and left to adhere overnight. The following day media was changed to a treatment medium containing 8 µg/ml of hexadimethrine bromide and an appropriate volume of lentiviral particles. Cells were incubated overnight and replaced with fresh media the next day. Positive clones were selected and cultured under 6 µg/ml of Puromycin. Quantification of TLR9 expression following transduction was assessed with RT-qPCR as well as with western blot.

(II) A lentiviral two-component CRISPR-Cas9 system was used to generate TLR9-deficient HNSCC FaDu ($Fadu^{TLR9def}$) cells. The system consisted of separate lentivectors spCas9 (Addgene plasmid #52962) and sgRNA (Addgene plasmid #52963). The desired short guide-RNA sequences (sgRNA), seq#1-CACATCGAGCACACGCAGGG, seq#2-AGGTATCGGGATGTAGCTGG, seq#3-AGGCTGGTGACATTGCCACG, were designed by using the DeskGEN platform.

Lentiviral vectors were produced by transiently transfecting HEK 293FT cells and isolating the particles by using the calcium-phosphate precipitation method (for detailed information, refer to the original article). Next, 8 x 10^4 FaDu cells were plated for transduction in growth medium supplemented with 10% FCS, 1 x l-glutamine, 1 x penicillin/streptomycin, and 1 x MEM non-essential amino acids, and allowed to adhere overnight. The next day, 7.5 MOI of spCas9 was introduced to the cells. Following a recovery period of three days, cells positive for Cas9 expression were selected with 8 µg/ml Blasticidin. Next, Cas9-positive cells were transduced for the second time with the mix of TLR9 sgRNA lentiviral vectors (MOI 11). Cells were grown for three days before selecting the double-positive Cas9/Lenti sgRNA$^+$ cells by adding 1 µg/ml of Puromycin. Finally, the heterogeneous mix of Cas9/Lenti sgRNA$^+$ cells was single-cell sorted by Sony SH800 cell sorter (Sony Biotechnology Inc) to generate homogenous cell clones. Sanger sequencing of clones confirmed that only TLR9 heterozygote FaDu cells were successfully grown (FaDu^{TLR9def}).

4.3.4 Cell culture: cell viability assay (I)

MTS assay (I): 1 x 10^5 of stably transfected mock or TLR9 shRNA MDA-MB-231 and mock or TLR9 shRNA CAL-51 breast cancer cells were plated in DMEM supplemented with 10% FCS and ascending concentrations (1 - 100 µM) of BPs pamidronate and ZOL. Cell viability was measured at 24 h and 72 h time points with the CellTiter 96 Aqueous One Solution Cell Proliferation assay (Promega Corporation), following the manufacturer's recommendations.

Investigation of cell layer confluency (I): 2000 - 4000 stably transfected mock or TLR9 shRNA MDA-MB-231 and mock or TLR9 shRNA ER-positive T47D cells were plated in a 96-well plate with growth media supplemented with 1, 10 or 100 µM of BPs or vehicle. The list of used BPs is found in Table 1. An increase in cell confluency was measured using IncuCyte FLR and IncuCyte ZOOM® (Essen BioScience Ltd.) kinetic high-content live cell microscopes. 3-8 parallel wells per treatment were analysed with the IncuCyte 2010A or 2014A software (Essen BioScience Ltd.) to obtain the mean confluence ratio.

Bisphosphonates	
ZOL	zoledronate
PAM	pamidronate
ALE	aledronate
CLO	clodronate
RIS	risedronate

Table 2. A list of the bisphosphonates (I)

4.3.5 Cell culture: cell proliferation assay (I)

1 x 10^5 stably transfected mock or TLR9 shRNA MDA-MB-231 cells were plated in DMEM supplemented with 10% FCS and vehicle or n-BPs alendronate (100 µM) or

ZOL (100 μM) for 48 h. The incorporation of pyrimidine analogue 5-bromo-2'-deoxyuridine (BrdU) was detected and quantified with a commercial kit, according to the manufacturer's recommendations (Exalpha Biologicals).

4.3.6 Cell culture: hypoxic treatment (II)

To study the effects of hypoxia on cancer cells, reagents and required accessories were either kept under a normal atmosphere (normoxia) or exposed to 1% O_2 for up to 2 days. 3×10^5 cells were seeded onto $6cm^2$ Petri dishes with parallel control cells under normoxia. Hypoxia incubation took place in the Invivo^2 hypoxia chamber (Ruskinn Technology Ltd) at + 37 °C (Fig. 11). While the collection of conditioned media (CM) took place under hypoxia, the remaining steps were performed outside the hypoxia chamber. The CMs were centrifuged to remove cell debris before the supernatants were stored at -20°C. The resulting conditioned media (abbr. CM_{NOX} or CM_{HOX}, respectively) were used to polarise human primary MΦ in vitro. After the CM removal, hypoxic cells were washed with phosphate-buffered saline (PBS) before lysis for immunoblotting or quantitative real-time PCR (inside the hypoxia chamber). Experiments were repeated 7–10 times, except for MDA-MB-231 (n = 5).

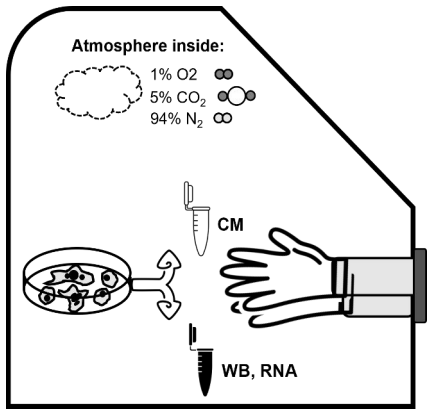

Figure 11. Cancer cells were kept under hypoxia (1% O_2) for up to 2 days before CM and WB sample collection (II).

4.3.7 Cell culture: human monocyte-derived MΦ polarisation (II)

Human monocytes from healthy donors were isolated by negative selection using the MACS Monocyte Isolation Kit II (Miltenyi Biotec). Isolated cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM, Gibco) supplemented with macrophage colony-stimulating factor (M-CSF, 25ng/μl, PeproTech) for 6 days to differentiate them towards uncommitted MΦ. On day three, the medium was replenished with fresh IMDM supplemented with M-CSF. After 6 days, 50% of the medium was replaced with $CM^{(NOX/HOX)}$ or $CM^{(FaDu/FaDuTLR9def)}$. Regular DMEM was used for non-activated MΦ control (marked onwards as NA MΦ). The control groups to represent M1, M2a and M2c polarisation were induced with LPS (100 ng/mL) + IFNγ (35 ng/mL); IL-4 (10 ng/mL) + IL-13 (35 ng/mL), or TGF-β (15 ng/mL) + IL-

10 (15 ng/mL), respectively. The MΦ polarisation was assessed by flow cytometry after 2 subsequent days in culture with CMs, and the cytokine expression was measured using qPCR. Experiments were repeated 7–10 times (except for MDA-MB-231, n = 5).

4.3.8 Flow cytometry (II)

The expression of MΦ surface markers was assessed with the LSR Fortessa flow cytometry (Becton Dickinson). MΦ were blocked with 100µg/mL human Ig (KIOVIG, Baxter) to block the unspecific binding of antibodies before staining with primary antibodies for 30 min on ice (list of antibodies in Table 2). The Fixable Viability Dye was used as a viability control to exclude the dead cells from analysis. Isotype-matched negative control antibodies were included to determine non-specific background staining.

4.3.9 Cell culture: ODN treatments (*unpublished*)

5 x 10^5 cells were plated in 1% DMEM for RNA and western blot sampling. Raji, FaDu and FaDu^{TLR9def} cells were treated with 5 µM CpG-ODN 2006 or GpC-ODN 2137 for 24 h in 1% DMEM. Cells in 1% DMEM acted as a negative control. Before sample collection for western blot or RNA, the cells were washed twice with PBS.

4.3.10 Live cell imaging: chemokinesis (*unpublished*)

To assess undirected single-cell movement, 1500 FaDu or FaDu^{TLR9def} HNSCC cells were seeded in a growth medium in 24-well plates treated with 1 µg/cm2 fibronectin-coating and left to adhere overnight at 37ºC and 5% CO_2. The next day, cells were washed with PBS, and a growth medium containing a reduced serum concentration (1%) and 5 µM of ODN 2006 or ODN 2137 was changed. Live imaging was performed in an incubation chamber (37°C, 5% CO_2) with an Eclipse Ti2-E wide-field inverted microscope (Nikon Corporation). Three areas per well were captured every 5 min for 18 h. For analysis, the distance advanced (µm) and the speed (µm/s) of 25 individual cells were measured in ImageJ-Fiji with a plugin MTrackJ. The experiments were repeated four times.

4.3.11 RNA isolation, cDNA synthesis, qPCR (II)

RNA extraction was done by RNeasy Plus Midi Kit (Qiagen), and concentration (ng/µl) was measured with a NanoDrop 2000 (Thermo Fisher Scientific). cDNA was synthesised by reverse transcription with a mix containing 1µg of purified RNA, Maxima Reverse Transcriptase (Thermo Fisher Scientific), dNTP (Thermo Fisher

Scientific), RiboLock RNase inhibitor (Thermo Fisher Scientific), and the oligo-dT mRNA primer (New England Biolabs).

100 ng of cDNA was amplified with DyNAmo HS SYBR Green (Thermo Fisher Scientific) on a CFX96 real-time PCR detection system (Bio-Rad Laboratories) at a final volume of 20 µl. The delta-delta Ct (ΔΔCt) method was used for the relative quantification of TLR9 and LDH-A gene expressions in cancer cells. Tata-box binding protein (TBP) was used as a reference gene to normalise the gene expression in both HS SYBR and TaqMan Gene Expression Assays.

Cytokine mRNA in CM polarised MΦ was quantified with qPCR. Ten nanograms of cDNA was amplified by quantitative TaqMan Gene Expression Assays using TaqMan Universal Master Mix II (Thermo Fisher Scientific) on a 7900HT Fast Sequence Detection System (Applied Biosystems). Finally, the delta-delta Ct (ΔΔCt) method was used for the relative quantification of gene expression in MΦ and cancer cells (n = 4–6, except for HaCat n = 2). Experiments were repeated 5 – 7 times, except in the control groups (n ≥ 2). The detailed primer information is provided in Figure 12.

SYBR Green

Gene	Sequence	Pub
LDH-A	5'-TCTCTGGCAAAGTGGATA-3' 5'-CCAGCCTTTCCCCCATTA-3'	II
TBP	5'-ACTTCACATCACAGCTCC-3' 5'-GAATATAATCCCAAGCGG-3'	II
hTLR9	qHSACED0003672	II

Taqman Assay

Gene	Cat#	Pub
IL6	Hs00985639_m1	II
IL12A	Hs01073447_m1	II
IL12B	Hs01011518_m1	II
TNFα	Hs00174128_m1	II
IL10	Hs00961622_m1	II
TGFβ	Hs00998133_m1	II
NOS2	Hs01075529_m1	II
TBP	Hs00427620_m1	II

Figure 12. Primers used in gene expression analysis (II).

4.3.12 Western blot (I-II, *unpublished*)

Cells were washed with PBS before lysis in radioimmunoassay buffer (RIPA) supplemented with Pierce protease inhibitors (Thermo Fisher Scientific). Following a short sonication, the remaining cell debris was cleared by centrifugation (10 min, 16,000 x g, + 4 °C). Protein concentrations were measured by using the bicinchoninic acid method to ensure equal loading of proteins on the TGX electrophoresis gel (SDS-PAGE) (Bio-Rad). Up to 80 µg of the sample was diluted with the Laemmli sample buffer supplemented with dithiothreitol, and samples were denatured at +95°C for 5 min. Samples were run on a gel for ~1 h at 100 V on the Mini-PROTEAN electrophoresis system (Bio-Rad). Proteins were transferred to a nitrocellulose filter (Santa Cruz Biotechnologies) at +4°C for ~ 2 h at 100 V. The filter was blocked with 5% skimmed milk or BSA in tris-buffered saline. Thereafter, the membrane was probed overnight at +4°C with primary antibodies (Fig. 13). After washing with tris-buffered saline containing 1% Tween-20, the membrane was incubated with secondary antibodies suitable for fluorescence detection. Signals were measured using the Odyssey CLx Imager (LI-COR Biosciences) and quantified with Image Lite Studio (v5.2, LI-COR Biosciences).

Flow cytometry

Antibody	Cat#	Pub
CD14 PE	BD #555398	II
CD163 PerCP Cy5.5	BD #563887	II
CD200R AF488	BR #MCA2282A488T	II
CD206 APC	BD #550889	II
CD40 BV 510	BD #563456	II
CD64 BV510	BD #563459	II
CD68 AF647	SC #sc-20060	II
CD80 PE	BD #557227	II
CD83 BV421	BD #562630	II
CD86 PerCP Cy5.5	BD #561129	II
HLA FITC	BD #555558	II
Viability Dye eFluor 780	eBio #65–0865-18	II

Western blot

Antibody	Cat#	Pub
anti-Rap1A	SC #sc-1482	I
Anti-Rap	SC #sc-65	I
phospho-p38	CST #9211	I
p38	CST #9212	I
AKT	CST #4691	*unpub.*
phospho-AKT (T308)	CST #4056	*unpub.*
phospho-AKT (S473)	CST #4060	*unpub.*
p44/p42 MAPK	CST #9102	*unpub.*
phospho-p44/42 MAPK (T202/Y204)	CST #4370	*unpub.*
iκBα	NB # MAB4299	*unpub.*
GSK3α/β	CST #5676	*unpub.*
phospho-GSK3α/β	CST #9323	*unpub.*
mTOR	Abcam #ab87540	*unpub.*
phospho-mTOR (S2448)	Abcam EPR426(2)	*unpub.*
Toll-like Receptor 9	BT #NBP2-24729	I-II
Vinculin	SC #sc-73614	*unpub.*
IRDye® 800CW Donkey anti-Mouse IgG	LB #926-32212	II-*unpub.*
IRDye® 800CW Donkey anti-Rabbit IgG	LB #925-32211	II-*unpub.*

Figure 13. Antibodies used in all studies (I-II, *unpublished*).

4.3.13 Statistical analysis (I-II, *unpublished*)

The data is expressed (I) as mean ± SD or ± SEM, or (II, unpublished) as median ± 95% CI. The quantitative PCR data were presented either with ΔCt or normalised

ΔΔCt values. Statistical analyses were conducted with GraphPad Prism software (version 7 and 9, La Jolla). The statistical significance was tested (I) with the student's unpaired t-test and the one-way ANOVA, or (II) the Wilcoxon signed-rank test or the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test. The effect of hypoxic CM treatment was analysed with the Mann-Whitney test. The unpublished data were analysed with the student's unpaired t-test and two-way ANOVA. $p \leq 0.05$ was considered significant.

4.3.14 Graphical resources and design

The figures and tables in this thesis are original, unless otherwise specified. All figures, except figs. 10 and 16 contain vectors from the icon and illustration platform Flaticon (https://www.flaticon.com/).

5 Results

5.1 Nitrogen-containing bisphosphonates modify cell viability and proliferation in TLR9-silenced breast cancer cell lines in vitro and in vivo (I)

While n-BPs originally have been developed to prevent bone resorption, these compounds have also been shown to prime and sensitise non-skeletal cells to inflammatory TLR-related signalling (Norton et al., 2011). Therefore, we studied the effects of n-BPs on proliferation and viability in genetically TLR9-silenced breast cancer cell lines. Following 2-day exposure to ZOL or ALN, incorporation of BrdU into the DNA of dividing cells was significantly reduced in TLR9 shRNA cells in comparison to the control shRNA cells (I, Fig. 1a). Besides proliferation, we assessed cell viability by using an MTS assay as well as live cell imaging. Results indicated that the bisphosphonates' inhibitory effect of limiting cell viability was time-, concentration- and TLR9-status-dependent, whereas the chemical structure was expendable (I, Fig.1b-d). The live cell imaging of the cell growth confirmed that human and murine TLR9 shRNA breast cancer cells were more sensitive to various BPs than the control shRNA cells (I, Fig. 2, Fig. 4, SFig.3-4).

Next, we investigated whether n-BP's cytotoxic effect, especially in TLR9 shRNA cells in vitro, was replicable in vivo. For that, we established a syngeneic mouse model by inoculating control shRNA or TLR9 shRNA MDA-MB-231 cells into the mammary fat pads of nude mice. Starting on day 4, mice received vehicle or ZOL injections three times per week until the end of the experiment, and the growth of the tumours was monitored regularly. On day 24, before the sacrifice, we measured the pO_2-content inside the representative tumours and verified that tumours in all groups were hypoxic (I, SFig. 6). The volumetric measurements of tumours throughout the experiment revealed that TLR9 shRNA MDA-MD-231 formed significantly larger tumours than their corresponding control shRNA cells (I, Fig. 8a-b). While ZOL treatment induced tumour growth in control shRNA tumours, the volume of TLR9 shRNA tumours treated with ZOL remained smaller (I, Fig. 8c). Finally, we verified the bioavailability of injected ZOL by measuring the mineral density at the cortical bone of tibiae by using peripheral quantitative computed tomography. As expected, the ZOL-treated groups had a higher mineral density than

the respective control groups, verifying that the drug had a pharmacological effect (I, Fig. 8d).

5.2 TLR9-silenced breast cancer cells are susceptible to BPs-induced changes in the mevalonate pathway (I)

Rigorous research has revealed that the mechanism of action inside the cells varies depending on the nitrogen content in the BP. As the n-BPs have been shown to inhibit the enzymes responsible for the biosynthesis of the hydrophobic prenyl groups, we measured the Rap1A prenylation as a surrogate marker of ZOL's inhibitory activity on the mevalonate pathway. Non-nitrogen-containing clodronate acted as a negative control. Curiously, our results showed a dose-dependent, yet TLR9-independent, increase of unprenylated Rap1A following exposure to ZOL in MDA-MB-231 and T47-D cells; a response, which was reversible by adding geranylgeraniol to the mix. As expected, clodronate had no cumulative effect on Rap1A prenylation (I, Fig. 6a). Furthermore, we inspected the phosphorylation of the MAP-kinase p38. We observed a comparable p38 phosphorylation irrespective of BP's chemical composition or TLR9 status (I, Fig. 6b, SFig. 5).

To characterise the n-BPs' mechanism of action further, we assessed whether TLR9 silencing converts breast cancer cells sensitive to the accumulation of the BP end-metabolite ApppI. We exposed TLR9 shRNA MDA-MB-231 and TLR9 shRNA Cal-51 cells to 1 μM ApppI and assessed the cell viability by MTS assay for up to three days. The two cell lines demonstrated varying sensitivity to ApppI exposure over time. A significantly decreased cell viability of Cal-51 cells at the first timepoint demonstrated that the cell line was hypersensitive to ApppI, irrespective of the TLR9-status. On the other hand, MDA-MB-231 cells became sensitised to ApppI after TLR9 silencing, as we measured significantly decreased cell viability after 48 h in TLR9 shRNA MDA-MB-231 compared with its respective vehicle control (I, Fig. 7). This data suggests that the inhibition of the mevalonate pathway and the subsequent production of the non-hydrolysable ApppI by ZOL have damaging properties particularly in breast cancer cells which lack TLR9 expression.

5.3 HNSCC induce a hybrid M1, M2a and M2c MΦ phenotype (II)

MΦ are a vital yet double-edged part of tumour immunology. Often abundant in tumours, their role as managers of tissue homeostasis is reflected in their polarisation; a functional status derived from the broad microenvironmental conditioning. Therefore, we aimed to uncover if MΦ polarisation in vitro could be modified by primary HNSCC cell lines (UT-SCC-8, UT-SCC-74A and FaDu) and breast cancer

cell line MDA-MB-231. The immortalised keratinocyte cell line HaCaT acted as a negative control. To study this, we isolated blood monocytes and educated them towards uncommitted NA MΦ for 6 days with growth media supplemented with M-CSF (25 ng/µl). Next, we exposed the NA MΦ to CM_{NOX} or the control polarisation inducers for 2 days. To generate the representative positive control groups for M1, M2a, and M2c polarised MΦ, we treated NA MΦ with established polarisation inducers 100 ng/ml LPS + 35 ng/ml IFNγ (M1), 10 ng/ml IL-4 + 35 ng/ml IL-13 (M2a), and 15 ng/ml TGF-β + 15 ng/ml IL-10 (M2c). Characterisation of these groups by flow cytometry verified distinct surface marker expressions of CD40, CD64, CD80, CD86 and MHC II in M1, CD200r and CD206 in M2a and CD14 and CD163 in M2c (II, SFig. 4). By using flow cytometry, we uncovered that MΦ polarisation was significantly modified by HNSCC and breast cancer cell CM_{NOX} in comparison with the immortalised keratinocyte cell line HaCat. In addition, results indicated that CM_{NOX} from different cell lines provoked variable activation of MΦ, often resulting in a hybrid inflammatory (M1), anti-inflammatory (M2a) and healing/'architectural' (M2c) phenotypes (II, Fig. 1b-d).

5.4 Hypoxia and TLR9 are dispensable for MΦ polarisation and functional phenotype (II)

Rapid tumour proliferation is typically encompassed by incomplete and perturbed tissue structure with poor vascularisation. The insufficient vasculature prevents the transport of molecular O_2 to the tissue, resulting in local hypoxia. Therefore, we investigated if the O_2-deficient, hypoxia-reprogrammed tumour cells can modify MΦ polarisation similarly to normoxic cancer cells. For this experiment, HNSCC cell lines, MDA-MB-231 and HaCat cell lines were cultured under 1% O_2 for 24h before the CM was collected. To verify that cancer cells had been exposed sufficiently to hypoxia, we measured the expression of the hypoxia-inducible gene LDH-A in cancer cells. A manifold increase in expression corroborated a sufficient exposure to hypoxia (II, Fig. 3). NA MΦ were then exposed to either CM_{NOX} or CM_{HOX} for 2 days. However, to our surprise, the comparison of surface marker expression in MΦ induced by CM_{NOX} or CM_{HOX} revealed no discernible differences, and hence, the soluble mediators released under hypoxia appeared dispensable regarding MΦ polarisation (II, Fig. 2).

To explore further if hypoxia-treated HNSCC cells can alter MΦ function in comparison with normoxic cells, we additionally assessed the production of relevant MΦ cytokine mRNA (IL-6, IL-10, IL-12A, TNFα, TGFβ and iNOS) following HNSCC cell line FaDu CM_{NOX} and CM_{HOX}-exposure. For the analysis, the expression profile in positive M1, M2a and M2c MΦ controls, as well as MΦ treated with FaDu CM_{NOX} and $CM_{HOX,}$ were all compared to the NA MΦ (II, Fig. 4a). Results in positive control groups were largely analogous to the well-established literature showing a

markedly elevated cytokine mRNA expression of IL-6, IL-12a, TNFα, and iNOS in the inflammatory M1 MΦ group. On the other hand, the anti-inflammatory groups M2a and M2c MΦ shared a trend towards increased IL-6 and iNOS expression, while the IL-12a expression was reduced. IL-10 expression was significantly suppressed only in M2a MΦ.

Surprisingly, FaDu CM_{HOX} was inefficient in modifying the functional phenotype of the NA MΦ beyond its normoxic equivalent. Yet, FaDu CM_{NOX}-treated MΦ ascertained an increase in IL-6, IL-10, and iNOS mRNA production while cytokines IL-12A and TNFα remained suppressed (II, Fig. 4b). Irrespective of the constant TGF-β expression, the functional alterations in cytokine expression indicated a robust and analogous anti-inflammatory MΦ phenotype under both microenvironmental conditions.

Previous findings associating TLR9 expression with changes in, e.g. cancer cell invasion in the hypoxic TME (Sandholm et al., 2014) sparked the interest to investigate if TLR9 in hypoxic cancer cells can adjust the repertoire of cancer cell-secreted soluble mediators. We hypothesised that hypoxia-induced altered cocktail of soluble mediators could impact MΦ polarisation or functional activation and subsequently support tumour immune evasion. To study this, we utilised a TLR9-deficient $FaDu^{TLR9def}$ clone which had a suppressed TLR9 expression under hypoxia in comparison to its parental control (II, Fig. 5a, Supplementary Materials and Methods). Our findings indicated that the surface marker expression generated with CM from hypoxic $FaDu^{TLR9def}$ corresponded to both its normoxic equivalent as well as hypoxic parental FaDu cells, excluding the possibility that the tumour-originating TLR9 independently influences tumour cell immune evasion by modifying MΦ polarisation under hypoxia (II, Fig. 5b). The exception to this was the surface marker iNOS, whose expression surged following FaDu CM_{HOX} exposure, whereas $FaDuTLR9^{def}$ CM_{HOX} had only a moderate impact on its expression (II, Fig. 5c). We also noted a more discernible plunge in the cytokine IL-12A expression in MΦ exposed to CM_{HOX} from $FaDu^{TLR9def}$ cells compared to CM_{HOX} from parental FaDu. Overall, however, these findings suggest that the tumour-originating TLR9 alone is not a sufficient immunomodulatory factor as regards TAM polarisation and activation.

5.5 TLR9 ligands induce variable cellular responses in Raji and HNSCC cells (*unpublished*)

While the previous study indicated that the hypoxia-induced endogenous TLR9 expression in malignant cells is dispensable in the context of MΦ polarisation, it was still intriguing to characterise the relevance of TLR9 in malignant cells themselves. Since TLR9 is abundantly expressed in lymphoid tissues, the B-cell line Raji was used as a reference to characterise the baseline TLR9-mediated responses associated with immune response (iκBα), cell survival (AKT/PKB and extracellular signal-regulated

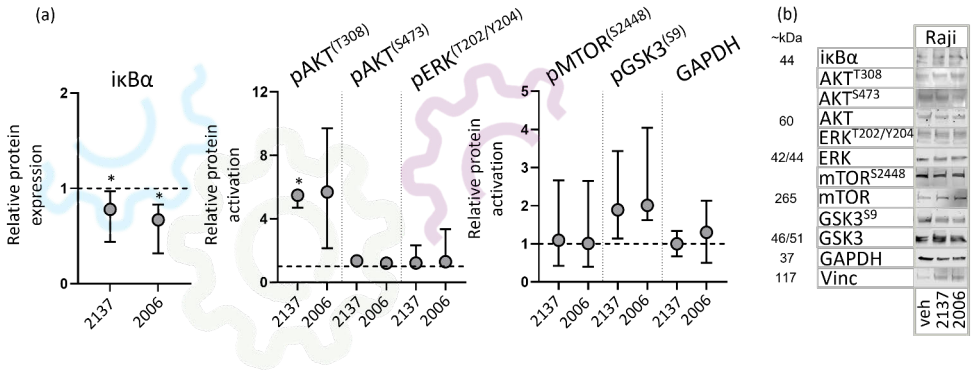

Figure 14. The response of B cell line Raji to TLR9 ligands differs from HNSCC cell line FaDu. (a) Raji were exposed to 5 μM ODN 2137 or ODN 2006 for 24 h before collecting samples for western blot analysis. The data represent relative ratio of median expression (iκBα, GAPDH) or activation against the corresponding vehicle control with 95% CI, biological replicates n = 2–3, vinculin was used as a loading control. The statistical significance to the vehicle was tested with the student's t-test and two-way ANOVA, respectively, $p \leq 0.05$ was considered significant (asterisk). (b) A representative immunoblot *(unpublished)*.

kinase 1/2 (ERK)), and metabolic adjustment (mTOR, GSK3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)). The exposure of Raji to short oligonucleotides ODN 2137 (GpC-rich) and ODN 2006 (CpG-rich) (collectively ODNs) induced a significant degradation of iκBα, substantiating that both ODNs, irrespective of their sequence, were activating the NF-κB pathway (Fig. 14a). While both ODNs induced a specific increase in phosphorylation of $AKT^{(T308)}$ but not $AKT^{(S473)}$, a few other changes in remaining markers were observed.

Next, parental FaDu and TLR9-deficient $FaDu^{TLR9def}$ cells were exposed to ODNs. For the analysis, the results obtained with $FaDu^{TLR9def}$ were compared to the vehicle-treated parental FaDu cell line. Interestingly, at a basal level, parental FaDu and $FaDu^{TLR9def}$ exhibited differences in the activation of proteins ERK, mTOR and GAPDH (Fig. 15a). Moreover, the assessment of ODN-induced alterations indicated that ODN 2006, but not ODN 2137, induced a significant iκBα degradation in the intact FaDu cells (Fig. 15b). As expected, exposure of $FaDu^{TLR9def}$ cells to the ODNs failed to generate a corresponding iκBα degradation.

Furthermore, alterations observed in the remaining markers affirmed that the responses to the same ODN by parental FaDu and $FaDu^{TLR9def}$ cells were distinct. Beyond a mere activation of the NF-kB pathway, notable ODN 2006- and TLR9-specific changes were observed in the MAPK (ERK), GAPDH, and mTOR pathways. Conversely, although the response was specific to ODN 2006, the modulation of GSK3 activity did not appear to be TLR9-mediated, as both cell lines yielded a corresponding response. Another distinct reaction to ODNs was generated on the AKT/PKB pathway as both ligands induced a sequence- and TLR9-independent

inhibition of the otherwise specific responses. It was additionally investigated if TLR9 deficiency influences HNSCC chemokinesis by using live cell microscopy to record random cell movement parameters of distance (µm) and mean velocity (µm/s). In comparison with the vehicle controls, the distance and mean velocity were similar in FaDu and FaDu^{TLR9def} cells, indicating that TLR9 *per se* was not modifying these parameters (Fig. 16a - b). However, a comparison of the ODN 2006-treated groups to their corresponding vehicle controls suggested that chemokinesis was markedly boosted in FaDu ($p_{[\mu m]} = 0.0014$, $p_{[\mu m/sec]} = 0.0002$), but not in FaDu^{TLR9def}. Hence, TLR9 deficiency hampered ODN 2006 boosted chemokinesis, consequently generating a significant difference across these cell lines ($p_{[\mu m]} = 0.0083$, $p_{[\mu m/sec]} = 0.0019$).

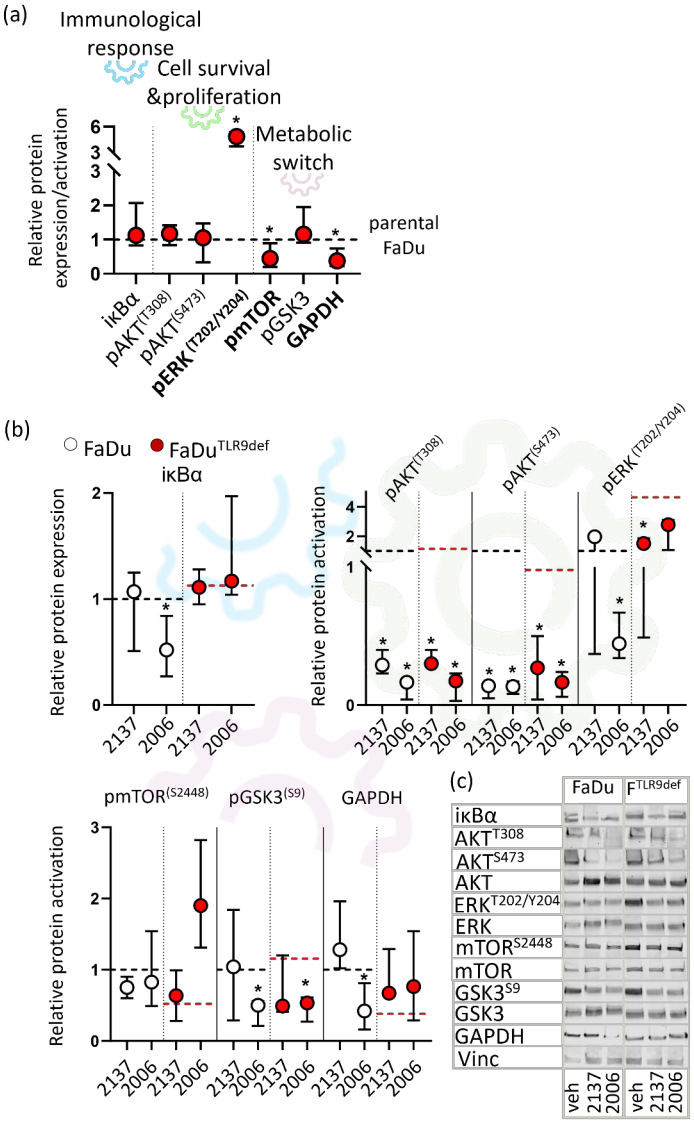

Figure 15. TLR9 deficiency appears to affect variable pathways responsible for immunological, cell survival or metabolic response. (a) To assess the baseline effect of TLR9 deficiency in FaDu, FaDuTLR9def cells were compared to the corresponding FaDu vehicle control (1% FCS/DMEM, dashed line). The data represent relative ratio of median expression (iκBα, GAPDH) or activation against the corresponding vehicle control (FaDu = black dashed line; FaDu^{TLR9def} = red dashed line) with 95% CI, biological replicates *n* = 3–4. The statistical significance compared to the vehicle was tested with the student's t-test, p ≤ 0.05 was considered significant (asterisk). (b) Both cell lines were exposed to 5 μM ODN 2137 or ODN 2006 for 24 h before collecting samples for western blot analysis. Biological replicates n = 3–4., vinculin was used as a loading control. The statistical significance to the vehicle and among groups was tested with the student's t-test test and 2-way ANOVA, respectively, p ≤ 0.05 was considered significant (asterisk). (c) Representative immunoblots of the studied markers (*unpublished*).

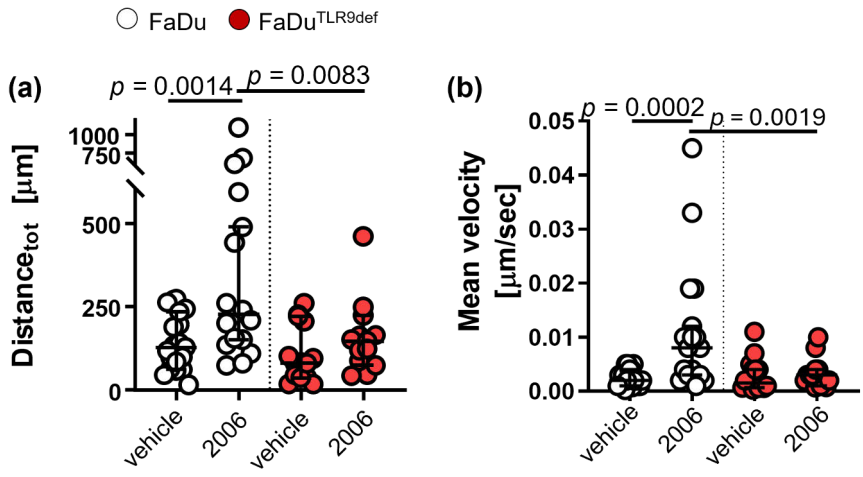

Figure 16. ODN 2006 boosted chemokinesis is TLR9-dependent in FaDu. FaDu and FaDu^{TLR9def} cells were treated with vehicle (1% FCS/DMEM) or 5 µM ODN 2006 for 18 h and their chemokinesis was monitored using live cell microscopy. Each point represents a single cell tracking (n = 20–25) of (a) distance in µm and (b) velocity (µm/sec). The experiment was repeated 2 times. The data is represented as median with 95% CI, two-way ANOVA was used for statistics. The p-values represent the significant change among FaDu and FaDu^{TLR9def} groups, p-value ≤ 0.05 was considered significant *(unpublished)*.

6 Discussion

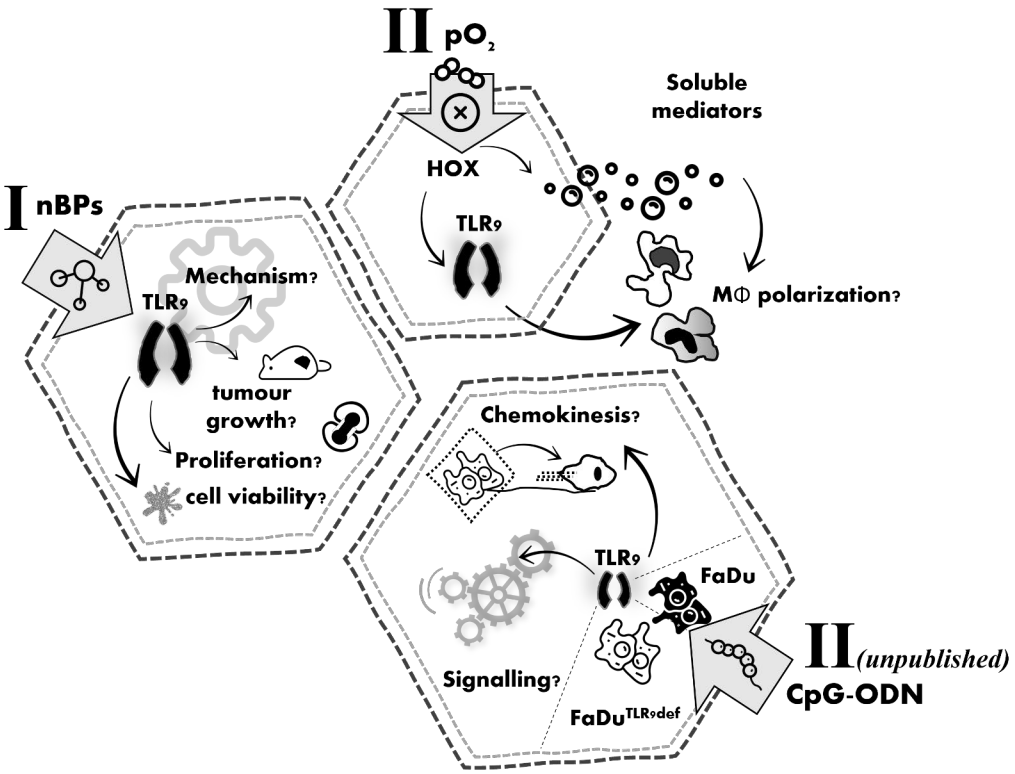

Figure 17. In summary, this thesis assessed the following study questions:(I) Do n-BPs impact cell survival and growth in TLR9-silenced breast cancer cell lines and (II) do hypoxia and TLR9 expressed in cancer cells have immunomodulatory effects in the context of MΦ polarization. The unpublished work extended the studies of the project (II) by characterizing the effects of TLR9-ligands regarding cancer migration and signalling pathway activation in FaDu and FaDu^{TLR9def} cells.

6.1 Are TLR9 and bisphosphonates joining forces? (I)

Adjuvant n-BPs are routinely prescribed to patients with breast cancer to inhibit SRE and improve bone-related pain control. Yet, the effects of n-BPs outside the bone have remained a controversial topic, albeit ample research has shown BPs' potency in modulating cell survival both in the bone and other tissues (Dumon et al., 2004; Merrell et al., 2007; Senaratne et al., 2000; Van Beek et al., 1999). We focused our investigations on the questions (1) do n-BPs impact the cell survival in TNBC in vitro, and (2) is the possible influence contingent on the tumour-originating TLR9. To do this, we used several methods that examined the viability and growth of the cancer cells from different angles with separate regulation mechanisms. While the incorporation of BrdU reflects the dynamics of the DNA synthesis, the MTS assay measures cells' metabolic activity, and the live cell imaging observes the apparent changes in proliferation as a function of time. Thus, the slight variations observed with different methods offered us a wider angle to information regarding the dynamic physiological responses to the n-BP exposure.

Our data revealed that the effects of n-BPs in breast cancer cell line propagation were largely inhibitory. The hampered cell growth by several n-BPs was observed by both a continuous live microscopy (I, Fig. 2a-j) and an endpoint measurement (MTS) (I, Fig. 1b-c), aligning with the previous reports (Dumon et al., 2004; Senaratne et al., 2000). In addition, the growth inhibitory effects were associated with the concentration, chemical structure, and duration of exposure. However, as the observed outcomes were not fully consistent across the breast cancer cell lines, this indicates that the effect of n-BPs on cancer cells is contingent on several factors, such as mutational burden and other signalling disturbances within individual cell lines. An example of this was the observation of ZOL-induced cell growth in the control shRNA CAL-51 cell line. While the result was slightly surprising, it was not unprecedented. Previously, daily administration of 4 μg of ibandronate to mice carrying orthotopic MDA-MB-231 tumours showed an increased, instead of inhibited, occurrence of adrenal metastases (Michigami et al., 2002).

As the NF-κB and p38 pathways are known to regulate cell cycle and growth, it was applicable to ask if the actions of these pathways interfere with each other. We found that the activation of the p38 pathway could not explain the sensitivity of TLR9 shRNA MDA-MB-231 to n-BPs. It is noteworthy, however, that the activation of the p38 pathway may represent an inconsistent mechanism to resist n-BPs' effects. This has been indicated before with other breast cancer cell lines, including MCF-7 and HCC38, which were inherently sensitive to n-BPs' growth inhibitory effects without a concomitant p38 pathway activation (Merrell et al., 2003). Likewise, the applicability of RAS GTPase superfamily proteins as biomarkers related to cell survival was challenged some years after our publication, as curious findings by Yu

et al. suggested that the antiproliferative effect of fluvastatin, another inhibitor of the mevalonate pathway, became significant to cell survival only when it correlated with the EMT phenotype (Yu et al., 2018). While these findings together point to the direction that TLR9 expression appears to be uncoupled from n-BP-induced reduction of cell survival, it does not answer the question of why TLR9-deficient cells emerged sensitised to n-BPs. While more information is still needed, it is plausible to speculate that some of the susceptibility may relate to the invasive phenotype of TLR9 shRNA MDA-MB-231 cells, which is enhanced especially under hypoxic conditions (Tuomela et al., 2012). To the same degree, an enhanced invasive phenotype could partly explain our in vivo results. While ZOL had a diminutive impact in preventing the tumour growth in the orthotopic animal model carrying the control shRNA MDA-MB-231 tumours, it inhibited the growth of TLR9 shRNA MDA-MB-231 tumours when compared with the corresponding vehicle-treated group. While the data does not reveal the underlying reason for the enhanced growth of TLR9 shRNA MDA-MB-231 tumours in vivo, it indicates that ZOL restored the TLR9-deficiency amplified invasive tumour growth to a comparable level with the control shRNA MDA-MB-231 tumours. What is more, the inhibition of the geranylgeranylation of the small GTP-binding protein Rab11 has previously been shown to hamper autophagic functions, subsequently resulting in an increased cell size (Miettinen & Björklund, 2015). Since TLR9 is involved with alternative energy metabolism and autophagic functions (Shintani et al., 2013), it is plausible to consider the possibility that TLR9-deficiency in MDA-MB-231 may result in enlarged tumour size due to dysregulated autophagy.

Furthermore, the fact that the hormone receptor expression *per se* appeared as an insignificant factor considering TLR9 shRNA MDA-MB-231 cells' susceptibility to n-BPs is not surprising. While TLR9 expression following ageing and post-menopausal hormonal changes has not been investigated in patient cohorts, a meta-analysis combining 26 clinical trials and nearly 20000 breast cancer patients reported a positive association between the treatment with adjuvant BPs and the subgroup of post-menopausal patients (Early Breast Cancer Trialists' Collaborative Group (EBCTCG), 2015). This study found that while n-BPs were generally efficient in preventing SREs in all patient groups, irrespective of the chemical structure, tumour status or concomitant chemotherapy, they prevented breast cancer recurrence and improved OS, particularly in the post-menopausal women's group. While the focus of the meta-analysis was not to define the underlying molecular processes, it nevertheless introduces an interesting possibility implying that BPs' involvement in regulating the mevalonate pathway could also extend to the malignant cells and the typically dysregulated lipid metabolism occurring after the menopause (Fisher et al., 1999).

Depending on the chemical structure, and in particular the nitrogen content, n-BPs modulate cholesterol metabolism and the subsequent protein prenylation or

induce apoptosis via the production of non-hydrolysable ATP-analogues. Since the n-BPs' main mechanism of action, inhibition of protein prenylation, did not on its own explain the sensitivity of TLR9 shRNA MDA-MB-231 to n-BPs, we considered other parallel or synergistic processes. One such mechanism involves the accumulation of IPP and DMAPP, which are converted into pro-apoptotic ATP-analogues of ApppI (triphosphoric acid 1-adenosin-5′-yl ester 3-(3-methyl-but-3-enyl) ester) and its stereoisomer ApppD (triphosphoric acid 1-adenosin-50-yl ester 3-(3-methylbut-2-enyl) ester, respectively (Mönkkönen et al., 2006). The exposure of breast cancer cells to the pro-apoptotic ApppI revealed that, once more, especially the TLR9 shRNA MDA-MB-231 cells showed markedly reduced cell survival when compared with the corresponding control. Apart from the harmful effects of the non-hydrolysable ApppI, the precursors IPP and DMAPP are involved in a negative regulatory feedback loop, which synergistically inhibits the activity of the HMG-CoA reductase and the protein prenylation even further upstream of FPPS (Mönkkönen et al., 2007).

Besides the invasive phenotype and the manifold actions of the n-BPs on the mevalonate pathway, it is worth considering the n-BP-induced pro-inflammatory effects regarding cell growth. N-BPs can induce diverse immunomodulatory responses ranging from altered cytokine production to reversal of the local tumour immune suppression (Derenne et al., 1999; Zang et al., 2019). Events such as reduced pro-IL-1β processing, changes in autophagic functions or impaired NLR family pyrin domain containing 3 (NLRP3) inflammasome formation by both ApppI and dysregulated TLR9 (Lim et al., 2016; Nakahira et al., 2011; Shintani et al., 2013) can result in sufficient cell stress and trigger apoptosis. Here, one option is that TLR9 and NLRP3-inflammasomes represent compensatory mechanisms that modulate a range of immunological responses arising from both extracellular and intracellular signals. Research has found that reduced amounts of autophagic proteins boost NLRP3/caspase-1-related inflammation (Nakahira et al., 2011). Following our article, such a compensatory mechanism between TLR9 and NLPR3-inflammasomes was found in TLR9-deficient mice, which were infected with *Salmonella typhimurium* (Li et al., 2017). In this study, TLR9 deficiency correlated with an intensified inflammatory phenotype in the intestines, comprising severe NF-κB pathway and NLRP3-inflammasome activation and subsequently boosted IL-1β production. While NLRP3 was not investigated in our study, we cannot rule out the possibility of tumour cell-derived auto- and paracrine responses to cytokines and DAMPs following n-BP treatments. Such an effect would ostensibly accumulate over time, particularly in those cell lines which express, e.g. interleukin-1 receptor.

Another hypothetical n-BP-mediated disruption involves the processing of procaspase-1 into active caspase-1. Since the procaspase-1 prenylation with geranylgeranyl is inhibitory (Montero et al., 2004), the n-BP-mediated lack of isoprenoids could thereby increase the amount of activated caspase-1. The stabilised caspase-1 could in turn process pro-inflammatory cytokines pro-IL-1β and pro-IL-18

into mature IL-1β and IL-18, which have been associated with multiple pathologies, including autoimmune disorders and cancer (Dujmovic et al., 2009; Hakelius et al., 2012). Considering all the findings by us and others, ApppI may have synergistic and cumulative effects with TLR9 deficiency.

Before concluding, some aspects of this study warrant careful evaluation. In general, n-BP's poor lipophilicity and non-complex pharmacological profile are essential factors when considering the soft-tissue bioavailability in the clinical context. Pharmacokinetic studies have shown that administered bisphosphonates, irrespective of their chemical structure, accumulate rapidly in the bone tissue before excretion from the body primarily through the kidneys (Shmeeda et al., 2013). This suggests that the free systemic concentration of n-BPs would remain relatively low. Yet, it is fair to argue that even the low concentration of n-BPs may be adequate in the context of tumours, since these compounds, such as ZOL, inhibit FFPS at nanomolar concentrations. In addition, the binding of ZOL to the hydroxyapatite in bone is strong but reversible, meaning that small amounts are gradually released back into the bloodstream over time. The low free systemic concentration has inspired attempts to improve n-BP soft-tissue absorption with novel lipid-based formulations. Although this approach resulted in increased soft-tissue concentrations, the formulations were unsuitable for clinical use due to general toxicity and a very narrow therapeutic window.

Nevertheless, these results may warrant further investigation into whether TLR9 expression correlates with the efficacy of adjuvant n-BPs in clinical patient cohorts.

6.2 Barking up the wrong tree: hypoxia and tumour-originating TLR9 are dispensable for MΦ polarisation (II)

The ability of tumours to hide in plain sight and avoid immune destruction is partly explained by the tumour-produced soluble mediators that confuse and persuade immune cells, including MΦ, to ignore the danger. Research has demonstrated that tumour-induced altered MΦ polarisation, which develops and fluctuates contingent on multifarious environmental stimuli, is one mechanism leading to immune evasion (Sousa et al., 2015). Although our data also confirmed that MΦ polarisation changed as a response to the HNSCC-secreted immunomodulatory factors, as expected, the hybrid M1/M2a/M2c phenotype remained unaltered, irrespective of the culture environment (CM_{NOX} vs. CM_{HOX}). This somewhat unexpected finding suggests that, although hypoxic, stressed cancer cells may prioritise e.g. metabolic switch or reduction of mitochondrial functions, over enhanced immune escape out of the available survival strategies. Other explanations for the MΦ polarisation similarity encompass elements such as cancer cell secreted secondary factors, including lactate, growth factors, and insulin, as well as other HIF isoforms, which are known to hold

distinct functions and tissue expression (Colegio et al., 2014; Odegaard et al., 2007; Takeda et al., 2010).

Furthermore, the functional cytokine profile of CM-polarised MΦ was both microenvironment-independent and largely analogous to the anti-inflammatory M2a/M2c controls. The elevated expressions of IL-6 and IL-10 mRNA agreed with previous reports associating these cytokines with pro-tumourigenic properties in several cancers (Costa et al., 2013; Duffy et al., 2008). Although IL-6 is generally considered a proinflammatory cytokine, it can also act as a pro-tumourigenic factor, and its abnormal concentrations have clinicopathological relevance in HNSCC (Duffy et al., 2008). In the same vein, the suppressed IL-12a and TNF-α expressions complemented the TAM-like phenotype with functionality to promote tissue remodelling, angiogenesis and regulation of homeostasis.

In essence, our in vitro data suggest that hypoxia is uncoupled from HNSCC CM-induced MΦ polarisation. Although MΦ phenotype and functionality in our setting were not altered by hypoxia, it does not exclude the mechanism of hypoxia and the HIF-signalling pathway having a direct impact on MΦ in vivo. According to the literature, both components have been reported to skew MΦ polarisation towards the alternative M2 phenotype and, e.g. arrest MΦ migration and chemotaxis (Grimshaw & Balkwill, 2001; Raggi et al., 2017). Furthermore, recent advanced methodologies like sc-RNA-seq have revealed distinct phenotypes of TAMs and tissue-resident microglia localising in differently oxygenated areas inside human gliomas (Müller et al., 2017). This is particularly relevant considering that physiological hypoxia is present also in wounds, where it triggers an inflammatory stress response, and transcription of angiogenic factors, as well as serves as a signal to recruit homeostatic immune cells to the site (Martins-Green et al., 1994; Moreira et al., 2010). While such corralling of MΦ by hypoxia is critical in promoting wound healing, in the TME it typically results in inadvertent and pro-tumourigenic consequences.

Interestingly, the flexible spatiotemporal spectrum of homeostatic and hybridised MΦ phenotypes exists in vivo, for example during prenatal development, obesity, and viral or parasitic infections (Gustafsson et al., 2008; Odegaard et al., 2007; Raes et al., 2005). However, in the TME, heterogeneous or alternative MΦ activation typically leads to adverse effects. For example, $MHCII^{low}$ MΦs are labelled as profoundly immunodeficient because these cells exhibit hampered antigen presentation, insufficient priming of $CD4^+$ Th cells, and reduced production of pro-inflammatory cytokines, such as TNF-α (Mengos et al., 2019). What is more, suppressive $MHCII^{low}$ MΦs have been shown to correlate with disease progression and tumour immune evasion in both mice and men (Meyer et al., 2014; Wang et al., 2011). Hence, the HNSCC-provoked downregulation of MHCII expression is likely calamitous to the induction of specific immune responses in the TME. The concomitant downregulation of T cell co-receptor CD86 further suggests that HNSCC-originating soluble mediators had an overall sedentary effect on the uncommitted MΦ.

Yet, some technical caveats in this study must be considered. While the stimuli needed to produce the control polarisation groups from the uncommitted MΦ were known, we did not assess the content of soluble mediators in the CMs, making our investigation partially limited. Therefore, the unbalanced production of both cancer cell-produced cytokines and immunomodulatory components, including immunoregulatory miRNAs and DAMPs, may contribute to the observed M1/M2a/M2c MΦ polarisation phenotypes. As it happens, the asynchrony to previous publications could also have been introduced by different laboratory practices, including protocols to isolate and maturate $CD14^+$ monocytes to MΦ (Benner et al., 2019).

The previous findings, which suggested that an increased expression of TLR9 may coincide with hypoxia in many cancers (Cai et al., 2019; Sandholm et al., 2014), led us to investigate whether suppressing TLR9 would alter the observed hybrid M2 MΦ polarisation. Our data with both parental FaDu and $FaDu^{TLR9def}$ CM_{NOX} indicated that tumour-originating TLR9 on its own had only a minor immunomodulatory function on MΦ in vitro. The outcome was recapitulated under hypoxia, irrespective of the increased TLR9 expression in FaDu.

However, when interpreting the results regarding TLR9-mediated alterations, a note of caution is due here since the model cell line ($FaDu^{TLR9def}$) was haplodeficient. It is possible that some effects may have remained undetectable on account of the functioning allele, and the interpretation of TLR9's role on its own affecting MΦ polarisation is limited. Despite the limitations in the study model, it is still possible that TLR9 can, in certain situations, have a specific influence on MΦ polarisation or their functional/costimulatory state. For example, a study in murine MΦ found that TLR9 is required for optimal iNOS2 expression (Pudla et al., 2018). Although we investigated only hypoxia-induced soluble mediators in conjunction with TLR9, our observation paralleled that of Pudla et al., as parental FaDu CM_{HOX} but not the $FaDu^{TLR9def}$ CM_{HOX} induced an increased iNOS2 expression in MΦ. While the identification of iNOS inducers was not the scope of this study, cytokines, such as TNF-α, IL-1β and IFN-γ, have previously been shown to influence the iNOS promoter activity (Pautz et al., 2010).

Since the maximal expression of TLR9 in HNSCC is dependent on variable and temporal signals, it is most likely that its role can be attributed primarily to specific situations, such as chronic hypoxia. Together, these findings additionally endorse a consideration of a TME-specific niche where the actions of TLR9 may become relevant in the context of MΦ activation.

6.3 Does the TLR9 pathway repurposing benefit the disease? (*unpublished*)

More than a century ago, William Coley, the 'Father of Cancer Immunotherapy', discovered that injecting patients' tumours with a heat-inactivated extract of bacterial DNA resulted in the shrinkage of tumours and regression of their disease (Decker & Safdar, 2009). At the heart of Coley's observation lies the TLR9-associated recognition of the short single-stranded DNA containing immunomodulatory sequence motifs, subsequent activation of the NF-κB pathway, and secretion of pro-inflammatory cytokines and type I interferons, actions priming the adaptive immune system to fight against pathogens (Takeda & Akira, 2005). The leading theory in the field suggests that CpG-ODN-induced anti-tumour activity is primarily orchestrated by the PRR receptors in innate immune cells (Camelliti et al., 2021; Sato-Kaneko et al., 2017). Yet, it is important to consider that Coley's injection of the DNA extract into an imperfectly fabricated tumour tissue presumably found its way also into the tumour cells. Therefore, the responses these short oligonucleotides may raise in cancer cells should not be overlooked. The unpublished data presented in this thesis support this notion by showing that ODNs can mount responses in both immune as well as non-immune cells (Figs. 14-16). Although the number of alterations in Raji Burkitt's lymphoma cell line seemed relatively small, this outcome was not necessarily surprising, as immune cells generally are under strict regulation to prevent unintended or disproportional immune responses. Typically, a costimulatory signal, such as CD40 or MHCII, from a separate source is needed before a full-scale immune cell activation, which could at least partly explain the modest activation in Raji (Frauwirth & Thompson, 2002).

In contrast, the data in HNSCC cells showed that ODNs prompted activation beyond the classical NF-κB pathway (Fig. 15). The ODN exposure altered the activity of several regulatory proteins previously linked with cellular processes such as inflammation, autophagy, cell cycle regulation and cell motility. These findings are in concert with previous studies, which have shown a synchronous stimulation of multiple pathways besides the NF-κB, such as the AKT/PKB, PI3K, MAPK, and mTOR (Damiano et al., 2006; Fukao et al., 2002; Li et al., 2016; Lim et al., 2006).

Some of the alterations seen in the present data may represent auto- or paracrine signalling, secondary responses to ER stress or even compensatory TLR9-independent reactions. Other synergistic mechanisms, including branched signalling through a common signalling node TRAF6 to activate cGAS-STING or activation of the DNA-dependent protein kinase to restrain the phosphorylation of AKT/PKB irrespective of TLR9 expression, are examples of reported alternative outcomes so far (Dragoi et al., 2005; Liu et al., 2023). While the alternative routes to TLR9 were not the focus of this study, the results would warrant further investigation into the extent of the parallel pathway activation.

While only partially elucidative and simplified, this data establishes a space to theorise what potential physiological gains or impediments ODN-prompted alterations may represent to malignant cells (Fig. 18). It is conceivable that some of the ODN-induced alterations in signalling could modify, e.g. tumour immune functions. For example, inhibition of mTOR by rapamycin is known to promote the production of proinflammatory cytokines in DC (Turnquist et al., 2010), whereas GSK3 activity can adjust the balance of pro- and anti-inflammatory cytokine production via the negative feedback loop with NF-κB (Cross et al., 1995; Martin et al., 2005). Several publications have indicated that this mechanism is reciprocal and intended to counter the NF-κB-induced proinflammatory response, and some of the observed changes in FaDu and FaDu^{TLR9def} cells could also point to this general direction. Therefore, the reduced inhibitory phosphorylation of GSK3 by ODN 2006 generates a relevant follow-up question of the ensuing cytokine profile, which could impact not only the in vivo MΦ polarisation but also other cell populations in a paracrine manner within the TME.

In addition, GSK3 is a component of a so-called 'destruction complex' that prevents cytosolic β-catenin from entering the nucleus by phosphorylating it in the Wnt/β-catenin pathway (Rubinfeld et al., 1996). When β-catenin enters the nucleus, it activates gene transcription associated with, e.g. proliferation, growth and cell migration. Research has revealed that GSK3 regulates β-catenin expression, nuclear localisation, as well as its destruction, by phosphorylating the adenomatous polyposis coli in the destruction complex. Investigating by what mechanism ODN 2006 induced an increased activity of GSK3 in the absence of Wnt-ligand would be an interesting subject in the future.

The altered GSK3 signalling could also impact cell proliferation, as e.g. the kinases ERK and GSK3 together regulate c-*myc* turnover (Cross et al., 1995). Furthermore, the TLR9/NF-κB pathway has been reported to modulate cell proliferation by increasing the EGFR growth receptor signalling (Damiano et al., 2006; Meseure et al., 2016). The study performed in vitro and in vivo colon cancer models showed that immunomodulatory oligonucleotides decreased EGFR expression by concomitantly suppressing mTOR, ERK and AKT/PKB activities. Hence, it would be worthwhile to explore the EGFR expression in FaDu^{TLR9def} on account of their distinct mTOR activity following the ODN 2006 exposure.

The data additionally suggest alterations in metabolic functions. TLR9-ERK-mTOR-induced self-cannibalisation as well as a direct suppression of mTOR-dependent metabolism are some published examples of CpG-ODN-induced changes in cell metabolism (Bertin et al., 2008; Li et al., 2016; Yazar et al., 2020). In addition, the AKT/PKB activity, independent of mTOR, has been shown to modulate e.g. autophagic functions as well as histone acetylation in energy-deprived situations (Lee et al., 2014; Wang et al., 2012). TLR9 has also been shown to activate the autophagy-related AMPK to improve stress tolerance during energy deprivation (Shintani et al.,

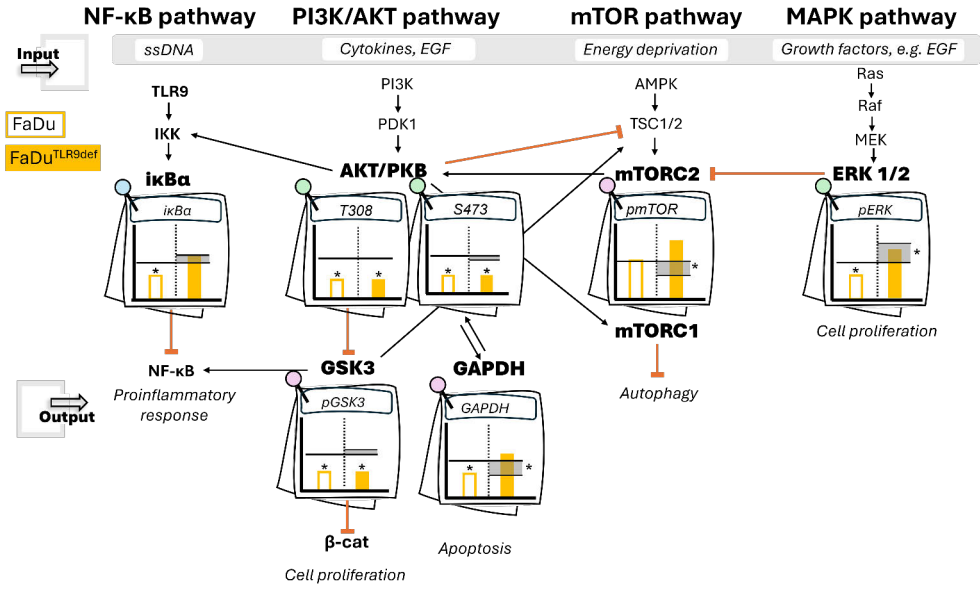

Figure 18. A simplified illustration of the ODN 2006-induced changes across several pathways and some hypothetical ramifications (output) to cellular functions in FaDu and FaDu^{TLR9def} (see also Fig.15). The activation of signaling pathways is represented with a hollow (FaDu) and solid (FaDu^{TLR9def}) bars. The grey box represents the altered base level expression in FaDu^{TLR9def} in comparison with parental FaDu (solid line). A significant change is marked with asterisk ($p < 0.05$).

2013). What is more, research has reported an inverse correlation between autophagy and cell migration (Catalano et al., 2015). Further investigations are required to elucidate whether the ODN 2006-induced decrease in cell migration of FaDu^{TLR9def} cells is due to similar associations between these functions (Fig. 16).

Lastly, the ODN 2006-induced reduction of GAPDH in FaDu, but not in FaDu^{TLR9def} cells, was a curious result. Apart from its role as a metabolic enzyme, GAPDH acts as a nitrosylase, incorporating NO into proteins (Kornberg et al., 2010). Together with other PTMs, including e.g. acetylation and phosphorylation, nitrosylation regulates the stability and/or function of several target proteins and impacts critical functions such as autophagy and cell death (Colell et al., 2007; Jacquin et al., 2013). However, since the data cannot disclose the activity of GAPDH, which is regulated by various PTMs, this result is only indicative at this point.

These results are intriguing as several clinical trials are attempting to activate anti-tumour immunity by engaging TLR9 with agonists like vidutolimod, agatolimod, tilsotolimod and SD-101 as mono- or combination therapy with cetuximab- or cisplatin-based treatments to tackle various cancers (Frank et al., 2018; Goutagny et al., 2012; Ribas et al., 2021; Ruzsa et al., 2014). However, the general efficacy of immunotherapies is not simply ‘one-for-all’ as many clinical studies have identified

patient subgroups who have not benefited from immunotherapies (Kirchhammer et al., 2022). Although a quick overview may suggest that the TLR9-mediated effects in breast cancer and HNSCC are minute in the context of immunotherapy, they cannot be presumed irrelevant. In contrast, the actions of TLR9 in malignant cells likely fine-tune cellular responsiveness, which in turn may reduce resistance to other treatments, thereby contributing to the overall balance between anti- and pro-tumourigenic outcomes. In my view, it is advisable to explore further in this field to gain a broader understanding of how tumour-originating TLR9 could one day be used as a putative functional marker in precision medicine to improve a personalised treatment strategy.

Several sources of uncertainty need to be considered here. As mentioned previously, due to the presence of one functional TLR9 allele in FaDu^{TLR9def}, the assessment of the impact of endogenous TLR9 in MΦ polarisation remained limited. Nonetheless, the observed changes in signalling pathways by ODNs indicated that FaDu^{TLR9def} cells differ markedly from the parental FaDu cells. Therefore, it is plausible that instead of merely being expressed, the tumour-originating TLR9 may become a relevant factor only after a ligand-induced stimulus.

Furthermore, the GpC-containing ODN 2137 is labelled as a negative control as it fails to induce cytokine production in immune cells. Following this, the fact that both ODNs generated signalling responses in certain proteins was somewhat surprising. Although this outcome appears unusual, it is consistent with previous findings which have suggested that TLR9 can recognise and interact with short oligonucleotide-related ligands independent of sequence- or structure-related identity (Kauppila et al., 2013; Landrigan et al., 2011; Li et al., 2011; Noack et al., 2012; Vollmer et al., 2004; Yasuda et al., 2006). This type of recognition enables a broader yet adjustable range of responses depending on the context and the stimulus (Klaschik et al., 2010; Man & Jenkins, 2022).

It is also important to consider the context in which TLR9 is investigated, particularly since the data in this thesis are based solely on in vitro experiments. Therefore, caution is warranted when extrapolating these results into the in vivo context. This is demonstrated for example by Damiano et al., as they assessed the influence of TLR9 on cell proliferation by both in vitro experiments as well as *in vivo* xenografts (Damiano et al., 2009). Although CpG-ODN seemingly had no impact on cell proliferation in colon carcinoma cells in vitro, researchers observed a 50% reduction in the growth of tumour xenografts following the same treatment in vivo. Further supporting this concept, Sommariva et al. have found evidence that treatment of human ovarian tumour xenograft mice with intraperitoneal injections of CpG-ODNs led to a reduction in DNA repair gene expression in tumour cells. In contrast, similar genes in the splenic immune cells were upregulated (Sommariva et al., 2011).

In summary, as many of the signalling networks studied here crosstalk and co-regulate multiple target proteins, the analysis of ODNs' impact on HNSCC cell physiology is at best a refined estimate. Nevertheless, this data suggests that it is

advisable to consider the ODN-induced ramifications in non-immune cells, as these responses can either enforce unintended interactions or attenuate vital crosstalk among the TME-residing cell populations.

7 Summary and conclusions

Opinions on TLR9's contribution to cancer have always varied regarding the how, where and when. However, by exploring beyond the conventional immune compartment, the role of TLR9 in cancer is inevitably being revealed.

This thesis investigated the tumour-originating TLR9 in the context of breast cancer, HNSCC, n-BPs, and MΦ polarisation. The main findings broaden the foundation of knowledge by describing that (1) TLR9 suppression in TNBC enhances the cytotoxicity of n-BPs, possibly by increasing the production of non-hydrolysable ATP-analogues, such as AppplI, (2) HNSCC cells execute immunomodulation on the uncommitted MΦ in the form of soluble mediators independent of hypoxia and endogenous tumour-originating TLR9, and (3) ODNs can alter physiological functions and induce changes in HNSCC cell signalling in both conventional and unpredicted pathways.

Despite these insights, the sixty-four-thousand-dollar question remains: is tumour-originating TLR9 a cause or a consequence? Based on the data, TLR9-associated functional pleiotropy seems synergistic/complementary and possibly contingent on ligand-activation. Since the actions of TLR9 are governed by multiple factors and, depending on the context, transmit either anti- or protumourigenic ramifications, it is fair to claim that TLR9 is not an oncogene. While the overexpression of TLR9 has been shown to correlate with factors such as histopathological grade, inflammatory microenvironment, and patient survival, the contingency of TLR9's functionality on multiple external factors challenges its applicability as a general, straightforward, and reliable prognostic biomarker for breast cancer or HNSCC. Instead, its expression appears to reflect the physiological context it emanates from, and a lot can be learned from its actions in different conditions. This thesis suggests that not only can TLR9 function as an instrument to multiple physiological carry-over ramifications, but it also can become an inadvertent recipient, tweaking the output of multiple cancer hallmarks, including tumour-originating persistent inflammation, alternative energy expenditure, and cell motility.

At present, there is a clear need to increase the knowledge of how malignant cells respond, both independently and as a part of the TME, to both n-BPs and immunomodulatory ODN-related treatments. The current understanding of TLR9 is a combination of what we already know and what outcomes we hypothesise its

expression in cancer could generate. Sometimes, outside-of-the-box thinking is needed so that the applicability of old remedies, such as n-BPs, can be tested for a new indication or to enable a TLR9-ligand-related clinical breakthrough in the future.

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> "Straight roads do not make skilful drivers.
>
> Paulo Coelho "

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> "I have had people give who didn't need to give and people help who didn't have to help."

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> "We are like books. Most people only see our cover, the minority read only the introduction, many people believe the critics. Just few will know our content."

Wiz Khalifa sings, "It's been a long day without you, my friend. And I'll tell you all about it when I see you again…" I owe this thesis to my closest family. Although they are no longer present to witness this day, the love that they left behind is engraved in my heart, and I know that I am always looked after.

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